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The Role of Kynurenine Metabolism in the Development of the Central Nervous System

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Thesis submitted in fulfilment for the degree of Doctor of Philosophy

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Abstract

Prenatal exposure to maternal infection has been thought as a major risk factor for neurodevelopmental brain damage and thus contributes to the pathophysiology of neurodegenerative diseases including schizophrenia and autism. The mechanisms of aberrant neurodevelopmental processes on the offspring, in which primary cerebral insults occur during early brain development, are not fully understood. In the present investigation, maternal infection was modelled in timed-pregnant rats at embryonic day (E) 14, 16 and 18 by administering intraperitoneal injections of polyriboinosinic-polyribocytidilic acid, poly(I:C), a viral mimic double stranded RNA complex which activates Toll-Like-Receptor-3 (TLR-3). The aim was to examine the impact of maternal inflammatory response on the regulation of expression of neurodevelopmental proteins that play important roles in many neurodevelopment aspects, including maintenance of synaptic plasticity, intracellular signalling and neurogenesis which may be relevant in cognitive and behavioural functions. An examination of embryo brains 5 h after maternal poly(I:C) showed significant differences in expression of the NMDA receptor NR2 subunits. The expression of NR2A subunits was reduced, whereas infection induced during pregnancy enhanced NR2B subunit expression. Expression levels of both subunits at postnatal day 21 (P21) were not affected by prenatal poly(I:C) exposure. In utero viral challenge led to significant changes among neurogenesis factor only at P21. In the fetal brain, acute poly(I:C) exposure had no effect on the expression of SHH, PCNA and also SOX2 proteins. However, when poly(I:C) was administered during mid and late gestation in the rodent model, long term effects of prenatal viral challenge on survival and maintenance of cell in the brain as indicated by the expression of SOX2 and SHH was clearly demonstrable. Expression of SOX2 level was increased, while SHH was significantly decreased, suggesting possible increase in the number of cells and changes in the rate of differentiation, respectively. The results demonstrate that poly(I:C) challenge in pregnant dams results in selective molecular changes in the brain, with transient alteration in the levels of NMDA receptor subunit NR2A and NR2B in the foetal brain, and also affecting molecules associated with cell genesis processes at later stages of developmental age of offspring.
On the other hand, recent pharmacological interest in kynurenines with respect to CNS diseases has mainly focussed on two neuroactive molecules: quinolinic acid (QUIN) and kynurenic acid (KYNA). Manipulation of the kynurenine pathway and its neuroactive metabolites has been associated with N-methyl-D-aspartate (NMDA) receptor neurotoxicity and dysfunction which linked to the development of various neurological disorders. An early developmental event has been proposed to precipitate alterations in the NMDA receptor function. In this respect, early development during the gestational period of rats is most suitable for investigating the modulating effect of kynurenine pathway inhibition by compound Ro61-8048 (3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]benzenesulphomide) an inhibitor of kynurenine-3-monooxygenase (KMO) in shifting the balance towards the production of neuroprotective, kynurenic acid. Western blots were generated to indicate the expression of a range of proteins relevant to different aspects of CNS development including neuritogenesis, axon guidance, maintenance of synaptic plasticity, intracellular signalling and cell proliferation and migration. Within 5 h of Ro61-8048, there was a significant decrease in NR2A expression and increased NR2B in the embryo brains, with subsequent changes in SHH and NFκB at 24 h post treatment. The litters were left undisturbed until weaning on P21 and other groups were allowed to develop to P60, at which time they euthanized and the brains removed for analysis. At P21, western blot analysis revealed significantly increased protein expression of the NR2A and NR2B subunits and postsynaptic density protein (PSD95). Among several neurodevelopmental proteins, the expression of NFκB and proliferating cell nuclear antigen (PCNA) was increased, while reduced level of SHH was detected. We demonstrate here persisting changes in NR2A expression, with reduced level in the hippocampus while a raised level was noted in the cortex suggesting prenatal modulation of kynurenine pathway causes long lasting modifications of NMDA receptor composition and function. It is important to note that kynurenine pathway inhibition can generate a consistent set of long term changes in the SHH in which the levels of this protein remained repressed in some regional areas of the brain including hippocampus, cerebellum and cortex. We show that there are some common pathways that are affected by kynurenine pathway inhibition, and this early modulation tends to disrupt critical molecular processes that are known to be actively occurring at each specific developmental time. Overall, given these selective and differing developmental
profile, an early life modulation of the kynurenine pathway might be expected to cause a sufficient disturbance of biological processes that are actively occurring at the time of exposure and also able to leave a series of molecular changes that persist into adulthood. This disruption is likely to influence the resulting physiology of the adolescent and adult brain and subsequently can lead to impairments in social behaviour. It is hoped that this study provides a broad analysis of the long term molecular effects of developmental kynurenine metabolism, and that it allows for a viable opportunity of potential therapeutic targets for disease intervention.
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For any errors or inadequacies that may remain in this work, of course the responsibility is entirely my own.

“And if all the trees on earth were pens and the ocean were ink, with seven oceans behind it, to add to its supply. Yet would not the words of Allah be exhausted. For Allah is exalted in power Full of Wisdom”

Luqman [31:27]
Author’s Declaration

I declare that the work presented in this thesis is my own work, with exception of part of the data, ELISA and HPLC analysis which was performed by Dr Forrest.

This thesis has not been submitted in any previous application for any other degree in the University of Glasgow or any other institutions.

......................................

MAZURA MD PISAR
List of abbreviations

ALS  Amyotrophic lateral sclerosis
AMPA  2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propionic acid
ANOVA  Analysis of variance
ASD  Autistic spectrum disorder
α7NCR  α-7 nicotinic acetylcholine receptor
BDNF  Brain-derived neurotrophic factor
BSA  Bovine serum albumin
CA1  Cornu Ammonis area 1
CA3  Cornu Ammonis area 3
Ca<sup>2+</sup>  Calcium
CBM  Cerebellum
CNS  Central nervous system
COX  Cyclooxygenase
COX2  Cyclooxygenase 2
CX  Cortex
DG  Dentate gyrus
dH<sub>2</sub>O  Distilled water
DNA  Deoxyribonucleic acid
E14/16/18  Embryonic day 14/16/18
ECL  Electrochemiluminescence
Eph  Ephrin receptor
EphA4  Ephrin receptor type A4
EphB2  Ephrin receptor type B2
EPSP  Excitatory postsynaptic potential
ESC  Embryonic stem cell
G1  First gap phase: Cell growth, preparation for DNA replication
G2  Second gap phase: Preparation for mitosis
GABA  γ-aminobutyric acid
GFAP  Glial fibrillary acidic protein
5 h/ 24 h  5 hour / 24 hour
3-HA  3-hydroxyanthranilic acid
3-HK  3-hydroxykynurenine
HIP  Hippocampus
HMG  High mobility group
IDO  Indolamine 2,3-dioxygenase
IL-6  Interleukin-6
i.p.  Intraperitoneal
i.v.  Intravenous
ICC  Immunocytochemistry
IDO  Indolamine 2,3-dioxygenase
ir  Immunoreactivity
kDa  Kilo Dalton
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>KAT2</td>
<td>Kynurenine amino transferase 2</td>
</tr>
<tr>
<td>KMO</td>
<td>Kynurenine 3-monoxygenase</td>
</tr>
<tr>
<td>KP</td>
<td>Kynurenine pathway</td>
</tr>
<tr>
<td>KYNA</td>
<td>Kynurenic acid</td>
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<tr>
<td>LPS</td>
<td>Lipolysaccharide</td>
</tr>
<tr>
<td>LTD</td>
<td>Long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>M</td>
<td>Mitotic phase</td>
</tr>
<tr>
<td>MAGUK</td>
<td>Membrane-associated guanylate kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MIA</td>
<td>Maternal immune activation</td>
</tr>
<tr>
<td>MID</td>
<td>Midbrain</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein-1α</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Magnesium</td>
</tr>
<tr>
<td>mNBA</td>
<td>meta-nitrobenzyolalanine</td>
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<tr>
<td>n</td>
<td>Number</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NAL</td>
<td>Nicotinylalanine</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclear antigen</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa light chain enhancer of activated B Cells</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>-----------</td>
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</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NO*</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NR1</td>
<td>NMDA receptor subunit 1</td>
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<td>NMDA receptor subunit 3</td>
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</tr>
<tr>
<td>NR2D</td>
<td>NMDA receptor subunit 2 D</td>
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<td>NSAIDs</td>
<td>Non steroidal anti inflammatory drugs</td>
</tr>
<tr>
<td>P21/60</td>
<td>Postnatal day 21/60</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95/Dlg/ZO-1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Polyribosinic:polyribocytidilic acid</td>
</tr>
<tr>
<td>PPI</td>
<td>Prepulse inhibition</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>PSD95</td>
<td>Postsynaptic density 95</td>
</tr>
<tr>
<td>Ptc</td>
<td>Patched</td>
</tr>
<tr>
<td>QUIN</td>
<td>Quinolinic acid</td>
</tr>
<tr>
<td>Ro61-8048</td>
<td>3, 4-dimethoxy-N-[4-(3-nitrophenyl) thiazol-2-yl]benzenesulfomide</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis: DNA synthesis and replication</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>SH3</td>
<td>Src Homology 3 domain</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
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<tr>
<td>Smo</td>
<td>Smoothened</td>
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<tr>
<td>SOX2</td>
<td>Sex determining region of Y-chromosome related-HMG box 2</td>
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<tr>
<td>rpm</td>
<td>rotation per minute</td>
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<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TBST</td>
<td>Tris buffered saline tween</td>
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<tr>
<td>TDO</td>
<td>Tryptophan 2, 3-dioxygenase</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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<tr>
<td>TF</td>
<td>Transcription factor</td>
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<tr>
<td>WB</td>
<td>Western blot</td>
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<tr>
<td>Zn²⁺</td>
<td>Zinc</td>
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1 Research background

1.1 Maternal infection

The recent widespread interest in the interaction of mind/body and diseases has undoubtedly stimulated the need to integrate research within this conceptual biological framework. There are many views on the aetiology of neurodevelopmental brain disorders, most notably autistic-spectrum disorder (ASD) and schizophrenia, which are highly heterogeneous with myriad causes. Several factors of infectious, neurological, metabolic, environmental and immunological origin have been thought to be involved in the disease development process of these neuropsychiatric disorders. Although the importance of genetic factors in this neuropsychiatric disease is strongly suggested by existing data, recent research in both animals and humans has discovered a number of gene-environment interactions where the exposure to pre- or post-natal environmental pathogens contributes to behavioural changes resembling essential symptoms of ASD or schizophrenia and produces lasting abnormalities in cognition and immune function including neuroinflammation in the offspring (Meyer et al., 2009, Patterson, 2009).

Maternal infection is among the most studied and best-established nongenetic risk factor for schizophrenia. The connection between schizophrenia and maternal influenza infection was first indicated by the work of (Mednick et al., 1988) which reported that the observed viral effect could be interpreted as being one of many potential perturbations of gestation. Studies examining the impact of influenza epidemics on schizophrenia found a higher incidence in offspring present in utero during these outbreaks compared with offspring in utero in a normal environment. One study featured a large pool of archived maternal serum samples collected in the 1960s that was linked to detailed medical records of both mothers and their children. They found that in cases where they were able to confirm maternal influenza infection by assaying the serum, the offspring were 3-7 times more likely to develop schizophrenia than controls. Because of the high prevalence of influenza, they estimated that up to 21% of all schizophrenia cases may be traced to maternal influenza infection (Brown et al., 2004). Similarly, epidemiological studies involving clinical
examination and serological testing have also reported a higher risk of schizophrenia and other psychosis-related disorders following prenatal exposure to rubella and *Toxoplasma gondii* (Brown et al., 2001, Brown et al., 2005). In addition, studies of other maternal infections demonstrated an association between periconceptional exposure to bacterial, genital and reproductive infections with the risk of schizophrenia spectrum disorders in adult offspring (Babulas et al., 2006, Sorensen et al., 2009).

Epidemiological studies have demonstrated that infection-associated immunological events in the maternal host may be one of the key events leading to an enhanced risk for the offspring to develop severe neuropsychiatric disorders in later life. The precise mechanisms underlying the epidemiological link between maternal infection during pregnancy and increased risk for schizophrenia in the offspring remain largely unknown. Considerable evidence has correlated the indirect effects via changes in maternal-foetal immunological parameters such as cytokines with the reported brain and behavioural dysfunctions emerging after prenatal exposure to infection. Elevation of cytokines in the maternal host and thereafter in the foetal environment has been seen as one of the key events responsible for the interaction between maternal infection during pregnancy, altered neuronal development and development of schizophrenia (Gilmore and Jarskog, 1997). It would now appear that many of the psychological changes associated with infections are directly caused by pro-inflammatory cytokines that are produced by infection or stress-induced activation of immune cells. Just as neurotransmitters and hormones elicit their biological responses by activating specific receptors on cells or by combining with intracellular receptors, so the cytokines activate specific receptors on immune, endocrine or neural cells (Meyer et al., 2009, Smith et al., 2010). Consequences of the infectious process during pregnancy are likely to affect foetal brain development. This effect is said to be mediated through pro-inflammatory cytokines released by the maternal immune system which can cross the placenta and enter the foetal circulation (Gilmore and Jarskog, 1997). These molecules are critically important to the immune response, acting as the systemic mediators of the host response to infection. Cytokine elevations increase vulnerability to developmental brain damage and other reproductive outcomes (Damman and Leviton, 1997). Further studies have found links
between elevated levels of cord blood cytokines and development of schizophrenia, strengthening the association of influenza infection with schizophrenia risk (Brown, 2006).

1.1.1 Infectious agents and models of maternal infection

Animal studies are of great importance in the elucidation of the function of specific molecules and as models of disease. Several species are now widely used as systems for the functional analysis of molecules in vivo, which have led to a rapid development of the technology for processes such as gene mutation or gene expression. Other approaches allow the alteration of expression of a gene in particular windows of time and in a tissue-specific way. In addition, animal model studies indicate the plausibility of an infectious basis for a wide range of neuropsychiatric disorders and are increasingly available for testing potential risk factors. Using animal models to follow the effects of exposures to various exogenous factors may indicate an association between the time points during nervous system development at which they are introduced and the manifestation of the behavioural and neuropathological features (Boksa, 2010). This could be especially relevant for an animal model of schizophrenia, considering the late onset of the disease, and support the plausibility that early life infections can give rise to persistent effects on behaviour. Animal experimentation illuminates the behavioural consequences that discrete lesions and their interconnections can produce. This has been demonstrated in a rat model in which some striking behavioural abnormalities emerging during adolescence could be observed (Vicente and Kennedy, 1997). The pathophysiology for schizophrenia has been thought to result from neurodevelopmental processes that start long before the onset of clinical manifestations and are due to a combination of environmental and genetic factors. In addition, several adverse events like infection or harmful stressors during prenatal and postnatal periods have been associated with the development of schizophrenia (Rapoport et al., 2005).

The most well appreciated cause of inflammation during pregnancy is due to infection by a pathogen. During inflammation there is an increase in the maternal circulation of inflammatory cytokines to which the foetus is then at risk of being exposed. Perturbations to the prenatal environment, in particular
maternal infection play a major role in the development of schizophrenia. Many studies have reported an association between influenza infection during pregnancy and an increased risk to schizophrenia in offspring. This association has also been shown in a variety of other maternal illnesses such as rubella and respiratory tract infections. Less is known about the possible relationship between early-life exposure to infectious agents other than viruses and the risk of schizophrenia. Although the environmental agents responsible for the initial neurodevelopmental insult in schizophrenia has not been discovered, prenatal or neonatal viral infections has received considerable attention based on epidemiological, immunological neuropathological studies. This have contributed to a link between viral infections and the etiopathogenesis of schizophrenia (Pearce, 2001).

Several large epidemiological studies have shown that prenatal exposure to viral infection is among environmental factors that may detrimentally affect neurodevelopment and has been implicated in the risk of schizophrenia in adulthood (Adams et al., 1993, Torrey et al., 1988). However, the mechanisms that cause the late emergence of the central nervous system pathology during adulthood still remain unknown. A number of studies suggested that interference with normal development is caused particularly by the proinflammatory cytokines released by the maternal immune system in response to the infection. It is based from the finding that prenatal exposure to a variety of infections has been associated with an increased incidence of schizophrenia (Gilmore and Jarskog, 1997, Pearce, 2001). To study whether the consequences of prenatal infection are really due to the infection itself or to the maternal immune response following infection, a model of animal study on rodents that utilize systemic administration of the synthetic double-stranded RNA, polyriboinosinic:polyriboctydilic acid [poly(I:C)] has been developed. In this approach, pregnant dams were injected with poly(I:C) solution with set dosages to mimic viral exposure with the aim of inducing a maternal immune response without exposure to a virus itself (Katafuchi et al., 2003, Kimura et al., 1994).

Several experimental models of maternal infection challenge by agents such as the viral pathogen viral mimic polyriboinosinic-polyribocytidilic acid, [poly(I:C)], the bacterial endotoxin lipopolysaccharide (LPS) and the pro-inflammatory
cytokine interleukin (IL-6) have provided considerable support to the causal relationship between prenatal exposure to maternal infection and the disruption of neurodevelopment in the prenatal brain. This predisposes the offspring to long lasting pathological changes in the brain as well as profound disturbances in mental functions, emotions and behavioural changes in adolescence or early adulthood. A body of evidence from animal studies have indicated that prenatal immune activation causes a wide spectrum of structural and functional abnormalities implicated in schizophrenia and other psychosis-related disorders (Smith et al. 2007; Hao et al. 2010). However, little is known about the neurodevelopment mechanisms underlying this epidemiological link and empirical support to clarify whether immune dysfunction during early neurodevelopment leads directly to central nervous system abnormalities has yet to emerge. In addition, the finding of elevated antibody levels in maternal sera suggest that maternal infection with Toxoplasma gondii can be a potential risk for schizophrenia and schizophrenia spectrum disorder in the offspring. An association between the risk of psychosis in adulthood following such exposure, and elevated levels of the antibodies in the newborn blood is known to exist (Brown and Derkits, 2010). Taken together, these studies suggest that infectious agents may be involved in the risk of developing schizophrenia.

1.1.2 Immune response to infection in the central nervous system

The exact mechanisms that are associated with virus related prenatal neurodevelopmental damages are not thoroughly defined. Considering the multiplicity of viruses that are implicated in a multitude of neuropsychiatric conditions, common mediators of maternal immune responses are suspected. Studies have shown that infection to pregnant women has been implicated in the pathophysiology of schizophrenia and several viruses are thought to contribute to the mechanism of disease. This observation has led to propositions that these viruses may be acting through a common pathway, possibly involving increased levels of cytokines, which could impair several aspects of neurodevelopment. Support for this hypothesis include studies showing that cytokine levels are increased in pregnancies complicated by infection and altered level of several cytokines have been found in the neonates from infected mothers. Both protein and mRNA of IL-1β, IL-6, TNF-α and IL-10 are upregulated at various time points
ranging from 2 - 24 h post injection of either LPS or poly(I:C). Some of these responses seem to be dose-dependent, with more robust increases in fetal brain cytokines following higher doses of treatments. Furthermore, maternally produced cytokines (IL-6 and IL-2) can cross the placenta and enter the foetal circulation, and can also cross the blood brain barrier. It has been proposed that the alterations to foetal brain development caused by increased cytokine signalling likely contribute to the abnormal behaviour in the adult offspring (Urakubo et al., 2001, Gilmore and Jarskog, 1997, Ozawa et al., 2006, Meyer et al., 2006, Ashdown et al., 2006). One key mediator that plays a critical role in mediating the behavioural changes in the offspring is IL-6, and a single maternal injection of IL-6 was observed to cause deficits in prepulse inhibition and latent inhibition. Co-injection of an anti-IL-6 antibody prevented the behavioural deficits caused by poly(I:C). Moreover, blocking the effect of IL-6 also normalized maternal poly(I:C)-induced gene expression changes in the adult offspring brain, as measured by RNA microarray. In the same study, IL-6 knockout mice did not show behavioural deficits after MIA (Smith et al., 2007).

Cytokines are low molecular weight proteins that known to be produced during events associated with an increased risk to schizophrenia, like infection. Cytokines are able to modulate the systemic and central nervous system response to infection and inflammation, and it has been demonstrated that they also play important roles in the regulation of many neuronal functions such as neurotransmission, neuronal survival and synaptic plasticity (Rothwell and Hopkins, 1995). Studies have revealed abnormalities of cytokines in schizophrenic patients. It has been known that cytokines can modulate neuronal proliferation, survival, differentiation and function. Other CNS functions influenced by cytokines include regulation of cognition, social interaction and at a neurochemical level, modulation of corticosteroid secretion and turnover of monoamines. Thus, cytokine production by the maternal immune system may be responsible for the interaction between maternal infection during pregnancy and altered neural development towards pathogenesis of neuropsychiatry diseases (Meyer et al., 2009, Watanabe et al., 2010).
1.1.3 Behavioral changes and neurochemical alterations

The mechanisms by which MIA leads to behavioural changes are not fully understood. It is possible that MIA sets into motion an ongoing immune activation that include alteration in the level of proinflammatory cytokines in maternal and foetal compartments, which can potentially affect multiple aspects of neuronal development. This deregulation may be responsible for some of the behavioural abnormalities observed in the adult offspring. It is clear that MIA has the potential to cause ongoing inflammatory processes and alter the balance between pro and anti-inflammatory signalling. Chronic elevation of cytokines and associated cellular inflammation would have an adverse effect on behaviour (Ashdown et al., 2006, Urakubo et al., 2001). For instance, a report showed injection of certain cytokines e.g. IL-6 triggers microglia activation in the brain, elevated IL-6 levels in the adult hippocampus, which indicate ongoing inflammation and increased glial fibriliary acidic protein (GFAP) and GABA<sub>A</sub> receptor levels that could all contribute to produce working memory deficit in the adult offspring (Nyffeler et al., 2006)

Different experimental models provide support for persistent effects of viral infections during early life on host behaviour. Experimental models of maternal infection have been used to model neurodevelopmental damage of relevance to neuropsychiatric disorders. Animal studies of rodents offspring prenatally exposed to influenza virus and viral mimic poly(I:C) display behavioural deficits. A range of behavioural methods has been used to examine various domains of schizophrenia-related behaviours in rodents such as sensorimotor gating, drug-induced locomotion and learning and memory. Sensorimotor gating refers to the process by which a weak sensory stimulus inhibits a motor response elicited by a stronger sensory stimulus, and most commonly assessed by performance on prepulse inhibition (PPI) tasks. This task refers to an attenuation of the startle reflex when the startle eliciting stimulus (the pulse) is preceded by a weaker sensory stimulus (the prepulse). Individuals with schizophrenia display deficits in sensorimotor gating and a failure to attenuate the acoustic startle reflex on PPI tasks, which are the most commonly reported behavioural disturbances in rodents subjected to the MIA model. To this regard, reduced PPI has been observed in rat and mouse offspring born to dams prenatally exposed to LPS or
poly(I:C). Furthermore, the PPI deficits only occur after puberty, mimicking the adult onset of schizophrenia. The use of PPI as an operational measure of sensorimotor gating have made it possible to identify the underlying neuronal brain circuitries and may also be useful in understanding the biology of drug effects in the normal and abnormal human CNS (Borrell et al., 2002, Meyer et al., 2008b, Shi et al., 2003, Fortier et al., 2007). Although the focus of using the Morris water maze has been to investigate memory encoding and hippocampal activity, many have used this task in schizophrenia research and it is regarded as the standard procedure to evaluate the spatial learning and memory abilities of rodents. In addition, impairment in this ability has been noted following systemic maternal inflammation of LPS (Hao et al., 2010). Another commonly reported effect of MIA in offspring, specially related to schizophrenia is altered amphetamine (AMPH)-induced locomotion which is a behavioural measure of mesolimbic dopamine activity. Offspring born to immune-challenged dams display enhanced locomotor responses to a low dose of AMPH, suggesting that these offspring have enhanced susceptibility to dopaminergic stimulation and are highly relevant to the positive symptoms of schizophrenia. MIA also affected the adult offspring’s sensitivity to the locomotor-stimulating effects of systemic administration of non-competitive NMDAR antagonist, dizocilpine (MK-801). It has been shown that maternal poly(I:C) exposure enhanced the locomotor reaction to systemic MK-801 in the adult offspring. Many of these behavioural deficits respond to antipsychotic drugs (Meyer et al., 2008a, Meyer et al., 2008b).

In addition to behavioural abnormalities, MIA also potentially affects multiple aspects of neuronal development. Particularly relevant are the findings that suggests cytokines affect the development of hippocampal and cortical neurons including abnormal hippocampal structure and neuronal loss in mature periods of maternal immune activation models (Hao et al., 2010). Morphometric analysis showed a decreased hippocampal volume in offspring following prenatal viral infection which is consistent with the observed reductions of hippocampus in subjects with schizophrenia (Fatemi et al., 2009b). On the other hand, MIA also impairs synaptic function and synaptic protein expression. It has been demonstrated that the reduction of the presynaptic function-relevant molecule synaptophysin in the hippocampus is responsible for the synaptic dysfunction in
the offspring (Oh-Nishi et al., 2010). In addition to neuronal changes, there is also evidence for neuroanatomical changes in the central dopaminergic, GABAergic and glutamatergic systems following prenatal immune activation (Meyer et al., 2008a, Meyer et al., 2008c). Interactions between inflammatory cytokines and neurotrophins in the nervous system also have been studied following prenatal immune challenge in rodents. The levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are differentially regulated by LPS or poly(I:C) depending on the severity and magnitude of the immune response within the maternal-fetal unit evoked following each agent. Taken together, alterations of these systematically generated neurotrophic factors after maternal infection may contribute to abnormal brain development and increase the risk of neurodevelopmental disorders (Gilmore et al., 2005b, Gilmore et al., 2003). In light of the neurodevelopmental hypothesis for schizophrenia, it is imperative to search for candidate genes related to pathogenetic processes that are induced by interaction of susceptibility genes and also environmental factors. There is now large body of evidence which demonstrates the altered expression of several genes that are involved in various neurodevelopmental aspects including neuronal proliferation and migration, neuronal polarization and axonal guidance, dendrite formation and myelination and synaptic plasticity (Fatemi et al., 2009b, Fatemi et al., 2008, Hayashi-Takaga and Sawa, 2010, Liverman et al., 2006)

Studies have widely demonstrated that MIA and related neuroinflammatory responses characterized by alterations to cytokine expression have detrimental effects on the behavioural and neurophysiological development of offspring, but the exact mechanisms by which maternally, placentally, and/or fetally-generated cytokines ultimately disrupt brain and behavioral development remain unclear. Taken together, this evidence demonstrated that MIA not only alters both behaviour and immunological parameters in the adult offspring, but behaviour and immunological parameters are tightly regulated. It should be emphasized that a comprehensive approach to investigating the pathogenesis of neurodevelopmental disorders must consider the interaction of host and environmental factors in a broader context. Evidence is emerging from epidemiology and animal models suggesting that prenatal infection with a
variety of agents can trigger complex behavioural disorders by impacting the function of specific neural cells and circuits (Zuckerman and Weiner, 2005).

1.2 The Kynurenine Pathway (KP)

On the other hand, neurotoxicity associated with maternal-immune activation may be the result of a series of small perturbations in brain metabolism and this may include the involvement of kynurenine pathway enzymes and a series of metabolites produced along the pathway. It is known that the principal role of the essential amino acid tryptophan is to form a constituent of protein synthesis and serves as a precursor for serotonin. As animals cannot synthesize tryptophan, they are therefore dependent on ingestion of proteins containing tryptophan. Ingested tryptophan is primarily degraded in the liver through several metabolic steps known as the kynurenine pathway (KP). The specific reactions associated with each enzyme of the kynurenine pathway are shown in Figure 1-1. Under physiological conditions, the majority of tryptophan is metabolized in the liver by enzyme tryptophan 2,3-dioxygenase (TDO), which acts as the rate-limiting enzyme for tryptophan in the liver and is up-regulated by corticosteroid. Whereas indolamine 2,3-dioxygenase (IDO) is the first enzyme of the KP in extrahepatic tissues and this enzyme is induced when tissues are invaded by viruses, bacteria or endotoxin. Oxidation of tryptophan, predominantly by the enzyme IDO can be found in numerous cells including macrophages, microglia, neurons and astrocytes. Generally, induction of this enzyme increases tryptophan metabolism leading to an increase in kynurenine and reductions in tryptophan concentrations. Kynurenine, a central compound of the pathway, can then be metabolized by several enzymes, namely kynurenine aminotransferases (KAT1, KAT2, KAT3), which generate kynurenic acid (KYNA), kynureninase which generates anthranilic acid (AA) and finally kynurenine 3-monooxygenase (KMO) which is the enzyme responsible for the production of 3-hydroxykynurenine (3-HK). The latter metabolite can be further metabolized into 3-hydroxyanthranilic acid (3-HAA) which subsequently is metabolized by 3-hydroxyanthranilate 3,4-dioxygenase (HAAO) to 2-amino-3-carboxymuconic semialdehyde and is converted to either picolinic acid or quinolinic acid (QUIN). Finally, the end product nicotinamide adenosine dinucleotide (NAD) is produced via the action of the enzyme quinolinate phosphoribosyltransferase (QPRT) (Stone, 1993, Moroni, 1999). Recent studies on the biochemical and
molecular biological function of the kynurenine pathway have triggered renewed interest in the characteristics of individual kynurenine pathway enzymes.

**1.2.1 Neuroactive metabolites**

**1.2.1.1 Quinolinic acid (QUIN)**

Interestingly, this pathway generates several immunomodulatory and neuroactive metabolites and can be induced by infections. It is up-regulated by inflammatory molecules such as lipopolysaccharide, amyloid peptide, human immunodeficiency virus proteins, and its potent stimulus, interferon-γ (IFN-γ), which is able to induce both the gene expression and enzymatic activity of IDO (Fujigaki et al., 2001; Takikawa, 2005). As tryptophan proceeds along the kynurenine pathway to achieve the final product nicotinamide adenine dinucleotide (NAD), a number of neuroactive intermediates are subsequently generated, most importantly the free-radical generator 3-hydroxyanthranilic acid (3-HA), the excitotoxin and N-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid (QUIN) and a broad spectrum excitatory amino acid receptor antagonist with a particularly high affinity for the glycine recognition site present on the NMDA receptor-ion channel complex kynurenic acid (KYNA) (Stone & Perkins 1981; Perkins & Stone 1982; Stone, 1993; Schwarcz et al., 1983). In contrast to KYNA, 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) are synthesized from kynurenine en route to NAD⁺ by the enzyme kynurenine 3-monooxygenase (KMO). This enzyme is increasingly viewed as a major gatekeeper of the kynurenine pathway. The ability to readily generate damage-promoting free radicals and NMDA receptor activation accounts for QUIN’s unique neurotoxicity and neurotoxic profile (Stone, 1993). In experimental studies, high concentrations of KYNA are frequently employed to inhibit excitatory amino acid receptor function non-specifically and to protect against excitotoxic insults, whilst QUIN is primarily used as a tool to produce excitotoxic brain lesions (Schwarcz & Kohler 1983). Quinolinic acid is probably the most studied kynurenine metabolite because it may cause convulsions and excitotoxicity activity by interacting with glutamate receptors of the NMDA type (Stone and Perkins, 1981). The QUIN concentration is normally low within the brain tissue. However, during an immune response activation, the level of QUIN is highly elevated in the brain and exerts its excitatory effects at the NMDARs. In
this situation, the endogenous formation of kynurenine and its downstream metabolites are greatly enhanced due to the activation of IDO by interferon-γ (IFN-γ), cytokines, viral and bacterial insults. Infiltrating macrophages, microglia and dendritic cells have been shown as major sources of QUIN production under inflammatory conditions in the brain (Schrocksnadel et al., 2006, Chen and Guillemin, 2009). QUIN has been shown to induce neuronal and astrocytic apoptosis, and it has been thought that the over-activation of the NMDAR and subsequent Ca\textsuperscript{2+} influx into neurons activates the downstream enzyme nitric oxide synthase (NOS) leading to the production of nitric oxide (NO\textsuperscript{•}) which promote free radical damage (Braidy et al., 2009, Stone and Perkins, 1981). In addition to NMDAR agonism, it also induces lipid peroxidation and generates free radicals which in part are responsible for the compound neurotoxic profiles (Forrest et al., 2002). In certain pathological conditions, in which microglia activation occurs, elevated QUIN levels were detected in the brain and the accumulation of this compound has been implicated in the etiology of a broad spectrum of neurological diseases, particularly those with inflammatory reactions. The most notable ones are AIDS-dementia, spinal trauma, epilepsy, Parkinson’s disease, Huntington’s disease and Alzheimer’s disease (Schwarcz et al., 2012, Schwarcz and Pellicciari, 2002, Schwarcz, 2004, Schwarcz et al., 2010).

1.2.1.2 Kynurenic acid (KYNA)

Importance is also attached to KYNA by the fact that this is one of the few known endogenous excitatory amino acid receptor blockers with a broad spectrum of antagonistic properties on the glycine modulatory site of the NMDA receptor at low concentrations. At high concentrations, antagonism at the glutamate site of the NMDA receptor and also on the α-amino3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, suggesting its physiological function in glutamatergic neurotransmission (Perkins and Stone, 1982). In addition, it also non-competitively antagonizes α 7-nicotinic acetylcholine receptors (αNCRs). KYNA is present in the mammalian brain at low concentrations (nanomolar) and has been described as an inhibitory component exerting anticonvulsant and anti-excitotoxic actions (Foster et al., 1984). As shown in Figure 1-1, KYNA is generated directly from L-Kynurenine by KAT enzymes. Two distinct enzymes have been described in rat and human brains,
KAT I and KAT II, which have shown immunoreactivity in astrocytes and also seems to be present in a small percentage of neurons. In most brain regions and lesioned brain tissue, KYNA results primarily from KAT II activity and newly synthesized KYNA is rapidly released into the extracellular space (Amori et al., 2009, Alkondon et al., 2011). The levels of extracellular KYNA increase linearly with kynurenine availability, as astrocytes generally lack KMO and therefore favour KYNA synthesis, whereas microglia cells contain only a little enzyme KAT and preferentially produce intermediates of the QUIN branch of the pathway (Schwarcz and Pellicciari, 2002).

Investigation of the KYNA arm of the pathway has provided an insight into their effects, that elevated level of this metabolite may cause cognitive impairment by virtue of its ability to antagonize the α-7 nicotinic acetylcholine presynaptic receptor (α7NCR) and the glycine site of the NMDA receptor. These are both critically involved in physiological processes underlying learning, memory and other manifestations of synaptic plasticity (Schwarcz et al., 2012). It has been proposed that by shifting the kynurenine metabolism toward KYNA formation, it may be possible to reduce glutamate receptor activation and excitotoxic neuronal damage. High concentrations of KYNA are anticonvulsant and neuroprotective, its glutamate antagonist activity is probably responsible for its ability to prevent brain damage following various pathological settings. It was found to be protective against brain NMDA neurotoxicity of ischemia and demonstrated the ability to block QUIN-induced excitation of CNS neurons, thus supporting the role of KYNA as an endogenous modulator of neurodegenerative and seizure phenomena (Foster et al., 1984). It has been shown that a reduction of KYNA level enhances the vulnerability to excitotoxic insults and the ability to decrease inflammatory molecules such as tumour necrosis factor-α (TNFα) and nitric oxide which is likely to be involved in LPS-mediated toxicity (Chen and Guillemin, 2009, Moroni, 1999, Stone, 2001, Vamos et al., 2009).

On the other hand, up regulation of KYNA branch of the pathway has been theoretically thought to mediate the negative effects of kynurenine pathway up regulation on mental status. It has also been demonstrated that a minor elevation of KYNA causes a decrease in the extracellular levels of neurotransmitters associated with cognitive function, i.e. glutamate,
acetylcholine and dopamine. The reduced level of glutamate in cerebrospinal fluid of individuals with schizophrenia was consistent with hypoglutamatergic function (Linderholm et al., 2010). Evidence has also supported the observation that altering the concentration of KYNA during critical times of brain development can affect behaviour later in life. It should also be noted that chemical manipulation by injection of kynurenine induced alterations in working memory function in adulthood, therefore establishing a notion that altered concentration of KYNA is capable of influencing cognitive function that depends on intact NMDAR transmission (Akagbosu et al., 2010, Chess et al., 2007). With regard to schizophrenia risk, exposure to a number of infectious agents during early life or childhood has previously been associated to the later development of symptoms related to schizophrenia. Experimental studies have shown that exposure to various insults during early life can induce long term effects on gene expression in the brain as well as behaviour (Shi et al., 2003, Fatemi et al., 2009a). However, the impact of CNS infections on the KP during early life in the brain is not fully described. Infection of neurotrophic influenza A virus during early postnatal period (P3) in mice triggered an altered expression of several genes in the KP whilst increased levels of IDO and KMO have been observed on P7 of virus-infected brain accompanied with transient increase of KYNA concentration. The increased levels of KYNA appear to be caused by activation of tryptophan degradation in the brain and the inhibition of NMDA receptors by KYNA would be one of the potential consequences which may functionally link infections with distorted glutamatergic signalling in the developing brain (Holtze et al., 2008). Therefore, the elevation of KYNA as observed in schizophrenic patients provided a new insight into the possible effect of KYNA on the glutamatergic and dopaminergic systems and its potential role in the pathogenesis of schizophrenia (Erhardt et al., 2007, Barry et al., 2009). Novel treatment of the disease could rationally be directed towards brain KYNA formation. Thus, blockade of kynurenine aminotransferase (KAT II which catalyzes the conversion of kynurenine to KYNA) causes a decrease in brain KYNA and may have cognition enhancing effects in the treatment of schizophrenia (Wonodi and Schwarcz, 2010, Erhardt et al., 2009).
1.2.2 Modulation of kynurenine pathway metabolism

By combining evidence of QUIN and KYNA action, excitotoxicity and CNS inflammatory related disorders have been related to excessive stimulation of NMDAR by QUIN, while the other metabolite, KYNA affords protection against it. In line with this notion, a substantial body of research have indicated that changes in the endogenous levels of each of KP metabolites have been implicated in the pathophysiology of several brain disorders including Huntington’s disease, Alzheimer’s disease, affective disorders such as schizophrenia and depression and also been correlated with Autistic-spectrum
disorder (ASD). The presence of neuroactive kynurenines in the mammalian brain has led to the notion that it may be possible to affect synaptic transmission of excitatory amino acid receptors by modulating kynurenine pathway metabolism in the brain. Some studies have found that pharmacological manipulations favouring KYNA in the brain extracellular space by intracerebral administration of exogenous KYNA protected against excitotoxins which may be beneficial in attenuating excessive NMDA receptor function and provide effective neuroprotection after an acute excitotoxic insult (Obrenovitch and Urenjak, 2000).

The pharmacological manipulation of the kynurenine pathway is still in its developmental stage and the pathway has been manipulated in several ways with the aim of developing therapies for the treatment of neurodegenerative diseases. The fact that NMDA receptor function is paramount to early brain development and since over activation of this receptor in the brain has been postulated as a cytotoxic mechanism involved in neurodegenerative process, many researchers have been prompted to look into the kynurenine pathway for a potential therapeutic approach. In this aspect, pharmacological challenges directed to enhance the endogenous formation of KYNA in brain tissue are considered as a successful strategy to counteract toxic events. Several ways of increasing the brain levels of endogenous KYNA have been discovered. One example is administration of a combination of kynurenine and probenecid, an organic acid transport blocker. The immediate precursor to KYNA, kynurenine has been shown to cross the brain barrier (BBB) effectively from the periphery and increased KYNA concentration while probenecid inhibits the efflux of KYNA from the brain via a probenecid-sensitive carrier. This association appears to be able to increase the brain extracellular concentration of KYNA and attenuate QUIN neurotoxicity (Santamaria et al., 1994, Santamaria et al., 1996). Neuroprotection of this combination has also been observed in animal models of Parkinson’disease where systemic administration of kynurenine and probenecid attenuates the dopaminergic damage induced by 6-hydroxydopamine, suggesting that the combined treatment constitutes a pharmacological strategy of considerable therapeutic value to mitigate excitotoxic events (Silva-Adaya et al., 2011). Another approach involved the use of synthetic analogues of kynurenic acid or kynurenine that can easily penetrate the blood brain barrier.
(in contrast with KYNA) as antagonists at glutamatergic NMDA receptors (Stone and Addae, 2002, Stone, 2001). A series of kynurenine analogues that have KMO and kynureninase inhibiting properties have also been tested. This includes nicotinylalanine (NAL) and meta-nitrobenzoylalanine (mNBA) which change the balance of the tryptophan metabolites towards the formation of KYNA (Moroni et al., 1991, Russi et al., 1992). The latter has been described as having more potent competitive and selective inhibitory activity than previous compounds in inhibiting KMO and caused an increase in the brain concentration of KYNA, an effect which is associated with sedative and anticonvulsant actions (Carpenedo et al., 1994). Another possible method to decrease the effects of KP disturbances is by using enzyme inhibitors. It appears that in some pathological states, changes in the two KP metabolites are modest and could be balanced by elevating the level of the antiexcitotoxic metabolite, KYNA or blocking the formation of QUIN. Development of pharmacological agents designed to augment or attenuate the effects of specific kynurenine metabolites, such as QUIN and KYNA is underway and several prototype inhibitors of most kynurenine enzymes are now available. Several of these compounds have been used for proof-of-concept in experimental animals (Nemeth et al., 2007, Moroni, 1999).

It has been shown that inhibition of kynurenine 3-monooxygenase (KMO), an enzyme that is responsible for synthesizing quinolinic acid, will shift kynurenine pathway from the production of excitotoxin quinolinic acid towards the production of neuroprotective kynurenic acid. Neuroprotection provided by the chemical inhibition of KMO has been shown in Huntington’s disease model flies, in which decreases in 3-HK relative to KYNA is thought to confer neuroprotection by antagonizing NMDAR and decreasing glutamate-dependent excitotoxicity as well as by scavenging free radicals (Campesan et al., 2011, Stone, 2000). Reductions in spontaneous locomotor activity and increases to the threshold level of electroshock-induced seizure are the effects produced by systemic administration of KMO inhibitors. In comparison, neuroprotective effects of KMO inhibitors in models of brain ischemia are primarily related to their ability to increase KYNA concentration in the brain and consequently antagonise the activation of NMDAR. Thus, it is reasonable to assume that by inhibiting KMO it is possible to reduce NMDAR function thus affording neuroprotection (Chiarugi et al., 1995, Moroni, 1999). However, it should be noted that the neuroprotective
properties of KMO inhibitors by increasing the KYNA concentration are observed under pathological settings such as in immune-stimulated conditions. Administration of inhibitors of KMO under basal conditions, however do not affect the levels of either blood or brain content of QUIN (Chiarugi and Moroni, 1999). Therefore, it is important to further evaluate the physiological and pharmacological implications of increasing KYNA concentrations in the brain.

A range of studies has been conducted to study the inhibition of KMO as a strategy for inducing a more favourable ratio of KYNA to QUIN in vivo. Among related molecules from a series of N-(4-phenylthiazol-2-yl)benzenesulfonamides, one member known as Ro61-8048 with the molecular structure as 3,4-dimethoxy-N[4-(3-nitrophenylthiazol-2-yl)]benzenesulphonamide has been characterized as a highly effective inhibitor, and has been successfully used as a selective agent to probe the physiological and pathological roles of KP metabolites experimentally (Rover et al., 1997). Previous studies have shown that systemic administration of this compound caused a robust decrease in glutamate levels in selected regions of the brain and this intervention is useful as a pathological increase in glutamate neurotransmission is considered detrimental in various neurological and psychiatric disorders (Meldrum and Garthwaite, 1990, Moroni et al., 2005). Ro61-8048 is a competitive inhibitor of cerebral KMO and caused an increase in brain KYNA concentrations. It has been shown that a build up of this antagonistic intermediate could inhibit activation of brain NMDARs and decrease convulsion in hyperbaric oxygen (HBO)-induced seizure activity (Dale et al., 2000). Subsequent studies that provides both behavioural and biochemical evidence in support of the pharmacological interventions of KMO inhibition showed that Ro61-8048 was neuroprotective in rodent models of ischemia and sleeping sickness, and in model of levodopa-induced dyskinesias (movement disorders) in parkinsonian monkeys. Mechanisms other than direct NMDA receptor blockade such as KYNA-induced αNCR-mediated presynaptic inhibition of glutamate release may therefore have contributed to the therapeutic benefits of the compound (Gregoire et al., 2008, Cozzi et al., 1999, Rodgers et al., 2009).

In this study, we evaluated the effects of KMO inhibition by a novel compound Ro61-8048, administered during the late gestation period of a rat model and later examine whether pharmacologically induced prenatal increases in brain
KYNA levels might interfere with developmentally essential neurological and glutamatergic functions.

1.3 Neurodevelopmental Markers

1.3.1 NMDA receptor subunit NR2A and NR2B

In the vertebrate CNS the predominant mode of excitatory transmission is mediated by the neurotransmitter glutamate and the ionotropic glutamate receptors, which include N-methyl-D-aspartate (NMDA), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. The NMDA receptor plays critical roles in excitatory synaptic transmission, plasticity and excitotoxicity in the CNS and several researchers have indicated that activity-dependent changes in excitatory synaptic function such as long term potentiation (LTP) and long term depression (LTD) are likely cellular correlates to learning and memory (Bliss and Collingridge, 1993). Research has shown that glutamate induced excitotoxic injury or overstimulation of the NMDA receptors may disturb normal glutamate neurotransmission in a variety of neurological disorders including Alzheimer’s disease, ischemia, epileptic disorders and Huntington’s disease (Bi and Sze, 2002, Engelhardt et al., 2007, Gascon et al., 2008). On the other hand, NMDA receptor hypofunction is also implicated in other pathologies such as schizophrenia (Schmitt et al., 2007). Its involvement in diverse processes during CNS development and implication in various psychiatric disorders has made research enthralling around its function to govern synaptic trafficking and stabilization, and is crucial for our understanding of the various processes carried out by NMDA receptors (Bard and Groc, 2011). NMDA receptors are heteromeric ligand-gated ion channels assembled from two families of subunits, NR1 and one or more of the four closely related NR2 subunits. The mandatory NR1 subunit is encoded by a single gene that can generate eight splicing variant isoform and can be found ubiquitously throughout the brain. The NR2 subunit family is composed of NR2A, NR2B, NR2C and NR2D, which are encoded by four distinct genes and shows both regional and developmental variations in animals, with NR2B and NR2D subunits predominating in the neonatal brain, but over the course of development these are replaced by NR2A and in some regions NR2C as well as a mixture of NR2A and NR2B subunits.
Another NMDA receptor subunit that has been characterized is known as NR3 and comprises two subtypes (A and B). Some studies showed variations in the NR2A and NR2B ratio which increases with age and this suggested that the composition of NMDA receptor complexes is dependent on the availability of the subunits (Al-Hallaq et al., 2007). The functional and pharmacological properties of the receptors are determined by subunit composition, post-translational modification and its cellular localization in the CNS (Monyer et al., 1992, Monyer et al., 1994, Cull-Candy et al., 2001, Furukawa et al., 2005). Under normal circumstances, NMDA receptor activation occurs upon the binding of glutamate and glycine (co-agonist) to the NR2 and NR1 subunits, respectively (Figure 1). The channel is permeable to calcium ions and blocked by magnesium ions at resting potentials, which requires postsynaptic depolarization to release the magnesium block and to permit activation of NMDA receptor. Thus, the NMDA receptor is unique in that it requires membrane depolarization to remove the Mg$^{2+}$ and also simultaneous co-activation by two ligands, glutamate/aspartate and glycine/D-serine, to be activated. In addition, the function of the receptor can also be modified through the use of extracellular Zn$^{2+}$ and polyamines. The activated NMDA receptor is permeable to both K$^+$ and Na$^+$, but has particularly high permeability for Ca$^{2+}$, which acts as a second messenger in the cell. The increased Ca$^{2+}$, due to high glutamatergic activity, results in a permanent increase in synaptic efficacy. This process is known as long term potentiation (LTP) and is of critical importance for learning and memory. LTP is a form of synaptic plasticity and typically consists of a prolonged increase in synaptic transmission strength requiring NMDA receptor activation. The concept of NR2 subunit-specific signals is also an ongoing area of interest within the synaptic plasticity field. It has been proposed that NR2A-containing NMDA receptors are preferentially involved in potentiation of synapses, while NR2B-containing NMDA receptors play a role principally in depression (Bartlett et al., 2007). However, there are contradicting findings that claimed a different point, in which NR2B-containing NMDA receptors can mediate synaptic potentiation as well. Both excitotoxicity and pro-survival effects of synaptic signalling can be mediated solely by NR2B-containing NMDA receptors (Martel et al., 2009). Growing evidence that indicates development in elucidating the molecular mechanisms underlying activation of NMDARs, driven by the availability of high resolution X-ray structures for the ligand binding domains, is likely to revitalize the search for
more effective and subtype-specific NMDA receptor drugs (Cull-Candy et al. 2001; Hunt and Castillo 2012).

Inappropriate activation of the receptor has been implicated in the pathogenesis of several disease states; in particular it has been related to the excessive calcium influx through the channel that can cause excitotoxicity. Thus, blockade of the NMDA receptors is considered neuroprotective. A number of pharmacological agents have been shown to distinguish the function of the various NMDA receptor subunits. Among competitive antagonists that exhibit moderate selectivity for certain subunit combinations are channel blockers such as MK-801 (dizocilpine), ketamine, phencyclidine and amantadine. Dopaminergic hyperactivity and behavioural characteristics of schizophrenia has been observed following treatment with non-competitive antagonists such as ketamine or phencyclidine. In electrophysiological experiments, administration of this NMDA receptor antagonists PCP, ketamine or MK-801 is associated with an increase in the firing rate and burst firing activity of ventral tegmental area (VTA) dopamine neurons (French et al., 1993). However, findings showed that chronic administration of PCP resulting in a reduced dopamine release in the prefrontal cortex and increased dopamine release in nucleus accumbens, which are conceivably related to negative symptoms and cognitive deficits consistently seen in schizophrenia. Administration of PCP also disrupted the prepulse inhibition, a behavioural model reflecting sensory gating deficits in schizophrenia (Jentsch and Roth, 1999). Ifenprodil and haloperidol are non-competitive antagonists that are effective at suppressing the activation of NMDA receptor. The usefulness of these drugs are however limited to their actions on normal synaptic transmission and sometimes accompanied by additional side effects. In addition, physiological metabolite in the brain known as kynurenic acid also serves as a broad antagonist at ionotropic glutamate receptors particularly those of NMDA type. In addition to its inhibition of AMPA and kainate receptor, it acts on the glycine recognition binding site of the NMDA receptor within low micromolar concentration and shared common characteristics with PCP and ketamine in which it could induce psychomimetic actions in human (Erhardt et al., 2007).
The mechanism linking NMDA receptor hypofunction and psychosis remains to be established. In view of the potential importance of the NMDA receptor in neural development, identifying the temporal regulation and spatial distribution of the various receptor subunits during development is really important in understanding the molecular mechanisms that modulate NMDA receptor signalling. Based on the receptor’s unique properties, it is not surprising that NMDA receptor hypofunction or overstimulation can result in many cognitive defects and brain dysfunction, making these receptors prime therapeutic targets.

Figure 1-2  Schematic diagram of NMDA receptor with its binding sites. Structure of NMDA receptor is tetraheteromeric combinations of NR1, NR2 and NR3 subunits (diagram only for illustrative purposes). The NR1 subunit binds glycine and NR2 subunits bind glutamate. The receptor is permeable to Na⁺, Ca²⁺ and K⁺ and the pore channel is blocked by Mg²⁺ ions in a voltage-dependent manner.
1.3.2 Post synaptic density 95 (PSD95)

Subunit composition determines multiple NMDA receptor function, including regulation of posttranslational modification, channel kinetics and downstream signalling involving intracellular protein association (Lin et al., 2004b). In this regards, the postsynaptic density-95 (PSD95), a scaffolding molecule abundantly enriched at glutamatergic synapses, is found to have important functions for the modulation of NMDA trafficking and stabilization on the cell surface. It belongs to the membrane-associated guanylate kinase (MAGUK) family which contains three PDZ(PSD-95/Dlg/ZO-1) domains, a SH3( Src Homology 3) domain and a carboxy terminal guanylate kinase domain (Kim and Sheng, 2004). Receptor subunit composition especially those of NMDA are dynamically remodelled during development and in response to neuronal activity and sensory experience. Less is known about the remodelling process of the NMDA receptors, their scaffold and signalling proteins. Several studies have shown that this reorganization occurs by subunit insertion into the synaptic membrane or mobility between the synaptic and extrasynaptic membrane. Scaffolding molecules situated at synapse facilitate clustering of receptors by engaging in several potential interactions, thus changes to the content or organization of scaffold modules at the synapse could alter synaptic transmission by changing either receptor number or distribution. It has been shown that the induction of depression or potentiation is associated with scaffold modifications. Both AMPA and NMDA receptors mediate the postsynaptic effects of glutamate at central synapses and PSD95 could regulate synaptic transmission and synaptic plasticity by selectively altering the ratio of synaptic responses mediated by both receptors (MacGillavry et al., 2011). Overexpression of PSD95 in the postnatal cultured brain slices has been found to change synaptic properties by increasing AMPA receptor-mediated synaptic transmission and favoured the emergence of LTD (Beique and Andrade, 2003). In addition, there is debatable evidence to show the cooperative role of protein-protein association between NR2 subunit domains and PSD95 (Delint-ramirez et al., 2010). Alteration in the relationship between NMDA receptor subunit expression and PSD95 might be related to several neurodegenerative diseases. As PSD95 plays a fundamental role in the trafficking, membrane targeting and internalization of NMDA receptor complexes, changes to this molecule have been reported in many cases including depression, bipolar
disorder and schizophrenia (Kristiansen and Meador-Woodruff, 2005, Toro and Deakin, 2005). In a clinical sample of schizophrenic patients, alterations in the expression of PSD95 protein and mRNA has been found in the brain, suggesting that glutamate dysfunction may involve changes in intracellular molecules critical for glutamate receptor-associated signal transduction (Clinton et al., 2003, Clinton et al., 2006). Evidence also indicates that the amount of anchoring protein PSD95 in post-mortem brain of depressed subjects are elevated and highly correlated with an increase in NR2A subunit reactivity (Karolewicz et al., 2009). Many of these diseases may initially involve dysregulation of synaptic transmission resulting from defects in synaptic organization.

1.3.3 Eprin type-A receptor 4 (EphA4)

A signalling process that involves some form of cel-cell communication controls arrangement of cells in the correct locations and establishment of the correct pattern of neural connections. Several families of membrane-bound cell surface proteins have been implicated in the control of cell movement, which is crucial for tissue organization during development. These proteins include ligand-receptor systems such as the Eph receptor tyrosine kinases. The Eph receptors represent the largest family of receptor protein tyrosine kinases and interact with their ligands, ephrins. The Eph receptors and ephrins are divided into two subclasses, A and B, based on their homologies, structures, and binding affinities. In general, EphA receptors (EphA1-EphA8) bind ephrin-A molecules (ephrinA1-ephrinA5) whereas the EphB receptors (EphB1-EphB6) bind ephrin-B molecules (ephrinB1-ephrinB3). However, one member of each class, namely EphA4 and EphB2, can be activated by both types of ephrins. Most of the members of this family are predominantly expressed in the nervous system during development (Gale et al., 1996, Flanagan and Vanderhaegen, 1998).

Ephs and their cell surface-associated ephrin ligands are important regulators of central nervous system (CNS) development. They are involved in the guidance of axonal growth and establishment of neural connectivity (O'Leary and Wilkinson, 1999). Several Eph receptors and ephrins are also widely expressed in the adult CNS, where they appear to regulate synaptic function and plasticity (Yamaguchi and Pasquele, 2004). Many of the Eph receptors may have overlapping functions
with each other. Protein expression pattern by in situ hybridization and immunohistochemistry have indicated the expression pattern and distribution of Eph proteins during development. Eph receptors and ephrins are involved not only in early developmental processes, but also in the function of the adult organism. In the mature brain, Ephas regulate neuron-glial communication and play a role in synaptic plasticity. Among the subtypes, EphA4 shows widespread expression throughout the CNS starting from early development and persisting in the mature brain (Egea and Klein, 2007, Greferath et al., 2002, Pasquele, 2008).

The genes for Eph receptors and ephrins have been recognized to be differentially expressed in various brain regions including the hippocampus. EphA4 is highly expressed in the hippocampus and is involved in hippocampal synaptic plasticity such as long-term potentiation, and therefore EphA4 may assume a role in hippocampus-dependent behaviour. EphA4 is expressed on dendritic spines of pyramidal neurons and axon terminals where it interacts with ephrin-A3 to shape dendritic spine morphology and influence synaptic plasticity. Eph dysfunction might be relevant to neuropsychiatric and neurodegenerative diseases characterized by abnormal dendritic spines in the hippocampus and cortex (Carmona et al., 2009, Glantz and Lewis, 2000, Yamaguchi and Pasquele, 2004). Increasing evidence also implicates Eph family proteins in cancer in which interaction between Eph-ephrin regulate critical steps of tumour growth and metastasis. This signalling mainly affects the cell shape and also influences cell proliferation and cell fate determination, therefore it is speculated that Eph signalling could play some role in tumourigenesis as one of their possible consequences (Kaenal et al., 2012).

1.3.4 Sonic hedgehog (SHH)

Initially discovered in Drosophila, sonic hedgehog (SHH) is a member of the hedgehog family of secreted signalling molecules, which is one of the key organizers of tissue patterning that undergo several post-translational modifications to gain full activity. SHH signalling plays a role in many developmental processes in different tissue types. SHH acts as a morphogen and induces distinct transcriptional programs on certain target cells depending on its concentration. In addition, this molecule also plays a central role in regulating
cell proliferation, differentiation and morphological patterning during embryogenesis. During development, interaction of various cell types is supported by many molecular events and inductive signals, secreted from within the CNS to ensure proper coordination and functional neuronal development (Marti and Bovolenta, 2002, Traiffort et al., 2010). The activation of SHH signalling is initiated by the binding of the SHH ligand to the membrane receptor Patched (Ptc), which relieves the Ptc-mediated inhibition of the transmembrane protein Smoothened (Smo). Activated Smo initiates an intracellular response/signalling cascade that ultimately drive the activation and nuclear translocation of the Gli transcription factor family member resulting in upregulation of hedgehog target genes including Gli and Ptc which respectively trigger the positive and negative feedback loops following pathway activation. This signalling cascade play fundamental roles during development and aberrant SHH signals underlie many disease conditions (Mullor et al., 2002, Ruiz i Altaba et al., 2002).

Given the pathway’s significant role in tissue patterning and cell number maintenance, events causing misregulation of the pathway are associated with certain cancers. Evidence that show the contribution of SHH signalling to tumourigenesis is emerging particularly in a model using in vitro approaches (Wicking et al., 1999). Inappropriate SHH signalling has been related to tumour initiation and maintenance, for instance in basal cell carcinoma (BCC) and medulloblastoma. The development of tumours such as those two above is through activation of the pathway by misregulation or mutation at the level of its receptor Ptc or Smo (Taipale and Beachy, 2001, Stecca and Ruiz i Altaba, 2005). These components are involved in the multi-molecular network which allows transduction of the SHH signal which drives proliferation and differentiation through the activation of specific downstream effectors of the pathway. Misregulation or mutations in the various elements of the SHH signalling pathway can lead to different phenotypes and also diseases associated with growth deregulation. As the pathway is involved in many developmental events, it will also be associated with human syndromes, defects and malformations (Mullor et al., 2002, Ming et al., 1998).
In addition to functioning in the embryo, SHH signalling components are expressed in postnatal and adult tissues, suggesting that they function in the mature organism. This molecule has been studied mainly in the context of development, however increasing evidence emerges to suggest the mitogenic properties of SHH are retained in the adulthood and promotes oncogenesis or tissue repair in different organs. Regenerative roles of SHH in cell proliferation might be useful in a clinical setting, for instance in inflammation-induced events and mechanical brain injury. Activation of the SHH pathway has been shown in response to hypoxia, in which protein levels of SHH and its transcription factor Gli1 were upregulated in the region of hippocampus after ischemic brain injury (Sims et al., 2009).

1.3.5 Proliferating cell nuclear antigen (PCNA)

The cell cycle is a complex and tightly regulated process by which cells duplicate their contents and divide into daughter cells. Among several cell cycle regulators, the most widely employed endogenous proliferation marker is Proliferating Cell Nuclear Antigen (PCNA). PCNA is a 36 kDa protein known as the cofactor of DNA polymerase delta and it is involved in cellular DNA replication and repair. Cell cycle analysis has shown that PCNA concentration increases rapidly in the mid-G1 phase of the cell cycle, immediately preceding the onset of DNA synthesis and remains elevated throughout the S phase, then rapidly decreases from the G2/M phase to G1 (Kurki et al., 1986). Because of its close relation to the cell cycle, PCNA is used as a physiological or pathological marker protein of proliferating cells (Valero et al., 2005, Valero et al., 2011). Due to the fact that PCNA is involved in DNA replication, it also has been used as a proliferation marker for adult hippocampal neurogenesis where immunoreactivity staining was detected in progenitor cells in the subgranular zone (SGZ) and also subventricular zone (SVZ) (Ino and Chiba, 2000). As DNA replication is a critical event in the cell; errors can be made and importantly unrepaired DNA damage upon DNA replication may give rise to mutations. Thus, many DNA repair proteins interact with the replication machinery in order to ensure efficient and reliable DNA duplication. Numerous studies have demonstrated correlations between dysregulation of the cell cycle machinery and neuronal death. Upregulation of cell cycle proteins occurs after various
injuries such as trauma and stroke and in neurodegenerative disease such as Alzheimer’s disease. Failure of cell cycle regulation might be a common pathway of several neurodegenerative disorders and this implies that cell cycle regulation might be one of the promising therapeutic intervention in these disorders (Wang et al., 2009).

1.3.6 Sex determining region of Y-chromosome related-HMG box 2 (SOX2)

The Sex determining region of Y-chromosome (SRY) was the first SOX gene identified in mammals where it was responsible for determination of the male sex. The transcription factor SOX2 is a member of the SOX gene family related to the SRY with the possession of a high-mobility group (HMG) DNA binding domain, thus the acronym SOX stands for ‘SRY-related HMG box’. Through protein sequence comparison analysis, 20 SOX genes have been identified and categorized into eight groups, A-H with two B subgroups, B1 and B2 (SOX14 and SOX21). The SOX-B group underwent further expansion into a B1 subgroup consists of three transcriptional activators SOX1, SOX2, SOX3 and a B2 subgroup of two transcriptional repressors SOX14 and SOX21. The SOX B group is of particular interest in that the five proteins are highly related and have overlapping expressions patterns (Lefebvre et al., 2007, Lovell-Badge, 2010, Wegner, 1999).

Generally, SOX proteins have been characterized as important transcriptional regulators of many developmental processes during early embryogenesis and are essential for the maintenance of a stem/progenitor cell pool. SOX-B genes in particular perform a wide range of functions by a complex interplay with specific partner factors to regulate gene expression (Kamachi et al., 2000). They are best known for their role in pluripotency and self-renewal, but also have been implicated in specification, maturation and terminal differentiation events. The vertebrate group B genes, SOX1, SOX2 and SOX3, have similar effects and are expressed in progenitor cells from the early stages of CNS development. Reduction in the expression of one of these genes usually does not result in a significant loss of function, indicating possible compensation of function and functional redundancy appears to be confined to subgroup members. SOX2 is
probably one of the best characterized SOX proteins and have a dynamic pattern of expression throughout embryogenesis and in a variety of adult tissue types. Expression of SOX2 is generally localized to proliferating and undifferentiated precursors whereby expression is downregulated with differentiation (Pevny and Placzek, 2005, Guth and Wegner, 2008). This is in line with evidence that constitutive expression of SOX2 inhibits neuronal differentiation resulting in the maintenance of progenitor characteristics (Graham et al., 2003). Thus, neural stem cells (NSC) at specific locations and developmental stages are especially vulnerable to SOX2 deficiency. It also should be noted that complex interactions of SOX2 with other regulatory factors explain its specificity of action and variably affect the outcome of SOX2 ablation or mutation (Pevny and Nicolis, 2010). It has been shown that SOX2 and other regulatory transcriptional factors like Oct4 and Nanog collaborate to maintain pluripotency and self-renewal in human embryonic stem cell. Close interaction between this three factors was maintained in the undifferentiated state of the cells (Fong et al., 2008). In view of this, the use of mutant allelic series to decrease/abolish SOX2 function have shown that neural stem cells from SOX2 knockdown mice were unable to differentiate into mature neurons and undergo apoptosis, whereas homozygous SOX2 mutant embryos do not develop. Mutations of SOX2 in humans result in a complex syndrome anophthalmia, defective hippocampal development and cognitive defects and epilepsy, while mouse models reproduced a broad spectrum of severity mirroring those seen in human patients (Fantes et al., 2003, Favaro et al., 2009, Sisodiya et al., 2006, Avilion et al., 2003).

SOX2 represents a well known marker for NSC and progenitor cells in both embryonic and adult brains, the distribution of SOX2 expression in the adult brain can be found in areas where neurogenesis persists, the subventricular zone (SVZ) in the forebrain and in the subgranular zone (SGZ) of the hippocampal dentate gyrus (Komitova and Eriksson, 2004). Apart from their distribution in all stem/progenitor populations throughout CNS, SOX2 also can be found in other non-neural populations such as the stomach, lung, hair follicles and the tongue (Ishii et al., 1998, Okubo et al., 2006, Driskell et al., 2009). Functional redundancy roles within SOX-B1 factors in maintaining broad developmental processes and identity of NSC could be seen during neurogenesis stages. Their inhibition in the embryo results in premature differentiation of neural precursors.
while mutation of SOX2 in the adult lead to a reduction in the proliferation of neural precursors suggesting a role for SOX2 in neural stem cell maintenance or proliferation in adult brains (Episkopou, 2005). Loss of its function induces cell cycle exit and advances neuronal differentiation. In a way, overexpression of SOX2 would inhibit neurogenesis while failing to reach a certain threshold of SOX2 expression by neural progenitors, render neurons unable to properly differentiate (Bylund et al., 2003, Avilion et al., 2003). In this regard, estimation of both precursors and early/immature neurons at an early differentiation stage could provide a basis indication of neurogenesis efficiency and support the notion that these regulatory proteins have important roles in several distinct cellular differentiation events (Suh et al., 2007). Recent evidence using immunofluorescence labelling revealed that SOX2 and nestin/vimentin are abundant in the primitive neuroepithelial tissue of immature teratomas of CNS which is indicative of aggressive cellular populations (Phi et al., 2007). Higher levels of co-expression between SOX2 and nestin (with its expression indicative of pluripotency and regenerative potential) have also been noted in cultured melanoma cells. Nestin expression is enriched in metastatic tumors and support its role in cancer progression and over expression of both markers are anticipated, considering that the tumour-initiating cell is probably a cell of an immature nature in which SOX2 is reported to be expressed (Laga et al., 2011). In this regard, roles and involvement of SOX2 and other SOX family members in tumorigenesis need to be verified, as there are differences between cancerous stem-like cells and normal neural stem cells. To some extent, this marker has been shown to be involved in the regulation of cancer stem-like cells and the presence of this protein along with other regulatory factors seems to important for the self renewal capacity and the cell proliferation of cancer stem-like cells (Phi et al., 2008).

1.3.7 Doublecortin (DCX)

Normal brain development involves generation of new neurons throughout life and this process is known as neurogenesis, which is tightly regulated in the specialized areas of the mammalian brain. It is a continuous physiological process that occurs in the subventricular zone (SVZ) of the lateral ventricle and subgranular zone (SGZ) of the hippocampus dentate gyrus. These brain regions
contain a pool of neural progenitor cells (NPC) which are capable of producing new neurons (Eriksson et al., 1998, Rao et al., 2006). Normally in the adult, neurogenesis in these regions occurs at a low level; however, increased cell proliferation and the presence of newly generated neurons have been observed in various experimental setting including environmental enrichment, physical activity, learning and physiological stimulation (Valero et al., 2011). Increased neurogenesis may occur in response to neurodegenerative disease processes in the adult brain. In regards to this, an increased number of DCX positive cells have been observed following kainic acid-induced seizures in mice and in epileptic and Huntington’s patients, suggestive of a contribution for newborn neurons in the disease process (Liu et al., 2008, Jessberger et al., 2005). Conversely, formation of new neurons is decreased by age, stress and chronic exposure to adrenal steroids. This combined evidence suggest that the adult brain can respond to activity-induced stimulation and that differential effects in neurogenesis were likely as part of compensatory mechanisms or an attempt by the injured brain to replace lost cells.

As part of detection techniques in neurogenesis, doublecortin (DCX) expression can be used as a candidate marker for adult neurogenesis. DCX is a microtubule-associated protein and its expression corresponds to the early stages of neurogenesis. The role of DCX in neuronal development has been associated with microtubule stabilization, nuclear translocation during neuronal migration and also axonal and dendritic maturation (Francis et al., 1999, Gleeson et al., 1999). In the adult brain, prominent DCX expression continues to persist within the two areas of continuous neurogenesis. The expression of DCX is observed in cell division as demonstrated by co-labeling with the cell cycle marker (Ki67) and downregulated once the cells became older and began expressing markers for mature neurons, thus reflecting a function for DCX in cell division apart from migration. Therefore it has been extensively incorporated as a specific marker of newly born or immature neurons in the adult and also to study the relation between neurogenesis, neurological and neurodegenerative disease processes in the search for putative therapeutic strategies (Brown et al., 2003, Verwer et al., 2007).
1.3.8 Neuronal nuclear antigen (NeuN)

It is known that adult neurogenesis consists of tightly regulated steps that include proliferation, cell cycle exit, differentiation and functional maturity. This process that takes place in the SGZ of the hippocampus and the SVZ of the lateral ventricles involves the development of late born neurons from early neural progenitors, committed neuronal precursors, immature migrating neurons to mature neurons. At various stages of this process, different specific molecules are expressed by the newly formed cells that can serve as a guide to monitor the different stages of neurogenesis (Halbach, 2007, Kempermann et al., 2004). The stability of new neurons might seem to be an important prerequisite for function, in which establishment of their synaptic contacts for signalling is functionally integrated. Neuronal nuclear antigen or NeuN is a neuron-specific nuclear protein that is identified by immunoreactivity with a monoclonal antibody, anti-NeuN. Because of its high specificity for most types of neuronal cells, and its cross reactivity with multiple species, anti-NeuN has gained widespread acceptance as a reliable tool to detect most postmitotic neuronal cell types in various research fields, notably neuroscience, developmental biology, stem cell research as well as diagnostic histopathology. Data from immunohistochemical analyses in embryonic and adult murine tissues have demonstrated the expression of NeuN throughout the nervous system, exclusively in postmitotic and differentiating neurons, and in association with terminal neuronal differentiation indicating permanent exit from the mitotic cycle. The staining using the antibody was primarily localized to nuclei, thus the name ‘Neuronal nuclei’ and also in the neuronal cytoplasm of postmitotic neurons. Although most neuronal cell types express the antigen, there are some major cell types that do not, including cerebellar Purkinje cells, photoreceptor and mitral cells which are all devoid of staining (Mullen et al., 1992).

NeuN has been used as a marker of postmitotic cells and labels both normal postmitotic neurons and newly generated postmitotic neurons in the hippocampus. Double labelling with other markers of neurogenesis such as doublecortin can help to distinguish between stages of this process and enable the monitoring the time course and fate of the newly generated cells in detail. Since the availability of markers to identify distinct stages of neurogenesis is diverse, a combination of different markers should allow for elucidation of the
roles and functions of adult neurogenesis under a variety of conditions, particularly in the context of neurological disorders (Halbach, 2011). The high specificity for neurons and the dense labeling of even small interneurons renders NeuN preparations suitable for semiautomated evaluation of neuronal densities by computer-assisted image analysis. In experimental animals, NeuN staining may be helpful for semiquantitative evaluation of neuronal cell loss in ischemic damage, epilepsy and neurodegenerative diseases. It has been proposed that a failure of adult hippocampal neurogenesis might partially underlie the pathogenesis of these conditions (Hartiga et al., 2009).

1.3.9 Nuclear factor kappa-light-chain enhancer of activated B cells (NFκB)

The NFκB proteins are a family of inducible transcription factors that activate a variety of cellular genes involved in control of the inflammatory response and in regulating cellular growth and survival. The NFκB signaling pathway is also implicated in control of neuronal death and survival (Kaltschmidt et al., 2005). The DNA binding, nuclear form of NFκB is composed of one 50 kDa (p50) and one 65 kDa (p65) subunit, which independently bind to DNA and potentially activate transcription. An inhibitory subunit called IκB protein is required for the inducible activation of NFκB. In complex with IκB, NFκB is present in the cytoplasm of non-stimulated cells. Extracellular stimulation causes phosphorylation of IκB and allows translocation of NFκB to the nucleus which then generally leads to an increase in the expression of target genes (Baeuerle and Henkel, 1994, O’Neill and Kaltschmidt, 1997). In most cell types, NFκB mediates an early pathogenic response by coordinating the initiation of transcription of numerous genes encoding cytokines, chemokines and cell adhesion molecules. Stimuli that activate the preexisting factor mostly represent pathogenic conditions, such as viruses, bacteria and the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor. Upon stimulation, posttranslational activation of the TF directs its participation in nuclear signaling and activates multiple target genes which almost entirely encode proteins relevant for pathogen defense (Baeuerle and Henkel, 1994).
Immunocytochemical studies in cryosection from normal mouse tissue showed a wide distribution of NFκB subunits in neurons and glial cells in the CNS, which are predominantly enriched in the nuclei of most cells. In accordance with ubiquitous distribution either detected as an inducible form (residing in the cytoplasm and associated with IκB) or constitutive forms at low amounts (found in the nucleus free of IκB and capable of binding DNA), suggesting NFκB participation during synaptic transmission (Kaltschmidt et al., 1993). Immunostaining evidence indicates the presence of NFκB in synaptosomes of the cerebral cortex and striatum. Besides, the distribution of NFκB in the brain has also been noted in the cytoplasm, which suggest that the factor may also be activated in response to postsynaptic stimulation, and has therefore the potential to participate in fundamental brain processes (Kaltschmidt et al., 1993). While NFκB is widely expressed in most cell types, in the nervous system the constitutively activated NFκB (nuclear) was restricted to a subset of neurons in the cortex and hippocampus, suggesting that in neurons a physiological, endogenous stimulus was controlling the activity of the transcription factor (O’Neill and Kaltschmidt, 1997, Kaltschmidt et al., 2005).

NFκB is also expressed in the cerebellum, mainly concentrated in the cytoplasm areas that may represent postsynaptic regions. On this basis, the effect of neurotransmitters such as glutamate and NMDA has been tested in vitro on the immunoreactivity of NFκB distribution. The data indicated that activation of NFκB binding activity coincided with nuclear localization following glutamate and NMDA treatment in granular neurons, suggesting the involvement of NMDAR activation. Stimulation of glutamate receptors and elevation of intracellular calcium levels, indicative of membrane depolarization, lead to activation of NFκB in hippocampal pyramidal neurons and cerebellar granule neurons (Guerrini et al., 1995). In addition, a robust increase in NFκB p65 mRNA was observed after long term potentiation in vivo, which then leads to increased DNA binding activity. This activation may contribute to alterations in target gene expression that accompany activity-dependent synaptic plasticity (Meberg et al., 1996). NFκB activation in the hippocampus following passive avoidance learning tasks have also been observed which suggest roles for this TF in directing the synaptic reorganization which is required for long term memory formation. As an abundant TF in the brain, inappropriate activation can cause the expression of
genes whose products interfere with normal brain function or cause the immune system to target brain cells. Overall, alongside its well-known roles in immune and inflammatory responses, NFKB family members also occupy an important role in neuronal processes including the regulation of the efficacy of synaptic transmission in the CNS. Further characterization of signaling mechanisms for this TF could shed light on physiological and pathological consequences. In addition it also provides more conclusive information about the relationship of these signaling processes in the CNS (O’Sullivan et al., 2010).

1.3.10 Cyclooxygenase 2 (COX2)

The discovery of isoforms of cyclooxygenase (COX) enzyme with distinct distribution and regulation has created new interest in the function of COX isoforms in the CNS as well as in other areas. The COX enzymes catalyze the conversion of arachidonic acid to prostaglandins (PGs) and other lipid mediators. COX exists in two related isoforms: COX1 and COX2. COX1 is constitutively expressed in most tissues where it catalyzes PG synthesis and its role is to support physiologic functions and maintaining homeostasis. In contrast, the inducible form COX2 is tightly regulated and is predominantly induced by endotoxins, cytokines and growth factors. In addition to proinflammatory cytokines, oxidative stress and lipid peroxidation have also been reported to be inducers of COX2 gene expression (O’Banion, 1999). Remarkably, COX2 is continuously expressed within a distinct population of neurons in the brain, mainly in hippocampal and cortical glutamatergic neurons where it has a pivotal role in synaptic activity and long term synaptic plasticity. The pattern of COX2 immunostaining in brains of normal rats shows that under physiological conditions, COX2 is expressed in a discrete subset of neurons. Intensity of COX2 staining within a population of neurons represents induction of COX2 expression in response to natural excitatory synaptic activity (Breder et al., 1995, Yamagata et al., 1993). In general agreement with an earlier study, localization of COX2 in the adult rat brain was consistently found in neuronal dendrites and spines of excitatory pyramidal neurons in the cerebral cortex and granule cells neurons in the dentate gyrus, CA3 and hilus of the hippocampus. Because of higher levels of COX2 expression in hippocampal and cortical neurons involved in cognitive function, this has led to speculation regarding the involvement of COX2 in neural
plasticity (Yang and Chen, 2008). In view of an active role for dendritic spines in shaping neuronal activity and synaptic signalling, this characteristic localization indicate a role for COX2 in cellular signalling of specific populations of excitatory neurons (Kaufmann et al., 1996). Due to this isoform being induced in response to inflammatory stimuli, COX2 has been linked to anti-inflammatory effects and non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to demonstrate a neuroprotective effect at least in part by an inhibition of the inflammatory reactions including increased COX2 expression. Thus, this makes the COX2 isoform an appropriate pharmacological target for anti-inflammatory therapy (Kaufmann et al., 1997).

One of the most intensely studied aspects of COX2 is its role in inflammation and secondary brain injury. The inflammatory reaction in the brain associated with most acute or chronic neurodegenerative diseases is often termed neuroinflammation, which is characterized by a glial response prior to significant losses of neurons. Neuroinflammation consists mainly of an innate immune response involving activation of glial cells and central production of cytokines, chemokines, prostaglandins (PGs), complement cascade proteins and reactive oxygen species in response to a central or peripheral immune challenge. COX2 is potentially one of the proteins that participate in the progression of many neurodegenerative diseases, possibly by propagation of inflammatory response elements and oxidative stress (Choi and Bosetti, 2009). In an acute phase response murine model, robust expression of mRNA was induced in perivascular cells throughout the brain after systemic administration of bacterial LPS (Breder and Saper, 1996). Previous reports have demonstrated that expression of COX enzymes in a subset of neurons as evidenced by mRNA levels and immunostaining profiles has been associated with glutamatergic neurotransmission. In this regard, COX2 expression in kainate models of status epilepticus has been used as marker for glutamatergic activation in the epileptic brain. COX2 was profoundly induced by seizures and changes in expression within selected brain regions have been reported (Joseph et al., 2006).

Compelling evidence supports the claim that inflammatory mechanisms are likely to contribute to some neurodegenerative conditions, such as in Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease (AD). In
keeping with this suggestion, inflammatory processes associated with increased expression of the enzyme COX2 and elevated levels of prostaglandin E\(_2\) (PGE\(_2\)) have been implicated in the cascade of deleterious events leading to neurodegeneration in various pathological settings. Upregulation of COX enzymes may influence cellular integrity by contributing to toxicity, degenerative processes and cell death culminating in excitotoxic damage (Almer et al., 2001, Teisman et al., 2003, Nogawa et al., 2003, Ho et al., 1999). Several lines of research have noted an inflammatory response in the brain of schizophrenic patients in response to injuries. Neuronal damage and the inflammation response described in schizophrenia have been considered as a consequence from the production of molecules that exclusively mediate immune function (Yokota et al., 2004). In schizophrenia, COX2 inhibition showed beneficial effects mostly in the early stages of the disease. As COX2 is expressed in regions critically involved in cognitive function and memory, an effect on cognition could also have been expected from the animal data of COX2 inhibitors in which COX2 inhibition directly attenuates inflammation-induced inhibition of long-term potentiation (LTP), an animal model of cognition. Animals with genetic over-expression of COX2 showed more prominent deficits in cognition, which were attenuated by a selective COX2 inhibitor (Muller et al., 2005).

1.4 Research aims

Since many infections during pregnancy in humans are caused by viruses, poly(I:C) has been administered during the late phases of gestation to simulate maternal immune activation response in the rat model. In the present study, we first set out to address this issue and undertook western blot analysis of virally exposed brains to gain insight into regulation of several molecules that play key roles in various neurodevelopmental process including neurogenesis, axonal guidance, synapse formation amongst many others. In another set of experiments, we employed a known KMO inhibitor (novel compound Ro61-8048) as a modulator of kynurenine metabolism during gestation to address the question of whether the physiological activity of the kynurenine pathway would have an impact in the developing brain. The general goal of the research project was to evaluate the involvement of the kynurenine pathway during brain
development. To this end, we provoked a selective increase of KYNA content in the brain to better understand its possible role in the modulation of NMDAR mediated signalling and transmission. We compared protein expression in brains from rat pups treated with Ro61-8048 in utero to those from saline-treated controls. Cellular localization and composition (using immunocytochemistry) of several neurogenesis markers in the hippocampus of mature offspring was also included. In addition, to examine changes in neuronal morphology on selected populations of cells in the hippocampus, dendritic architecture and the complexity of granule cells were examined using Golgi-Cox impregnation study.
2 Research materials & methodology

2.1 Prenatal animal models set up

All procedures on animal work was performed in accordance with the guidance established by the Animal (Scientific Procedure) Act 1986 and has been approved by the University of Glasgow Scientific Ethics Committee. Wistar rats were mated at about an age of 3 months and the first day after the occurrence of vaginal plug (an indication of copulation) was defined as day one of pregnancy. The pregnant dams were housed alone under pathogen-free environmental conditions at a constant temperature with food and water ad libitum.

2.1.1 Prenatal immune challenge

In the rat model prenatal poly(I:C) model (Figure 2-1), pregnant dams were exposed to the immunological manipulation at a specific gestational stage and the brain and neurochemical analyses of the prenatal immunological manipulation were then compared in the resulting offspring relative to offspring born to vehicle-treated control mothers. Commercially available potassium salt poly(I:C) (P0913: Sigma (Poole, UK)) was dissolved in sterile isotonic 0.9% w/v NaCl solution and then heated up to 50°C to allow for complete solubility. The mixture was allowed to cool to room temperature to allow re-annealing of the double-stranded RNA. Solution of the drug for the treatment was sterilised and aliquoted in small sterilised tube and kept in -20°C until further use. The desired dosage for the injection was 10mg/kg and it has been established in previous experiments in the lab that the dose could be given to pregnant dams during the last seven days of gestation with no signs of stress, behavioural changes or abnormal behaviour towards the neonatal pups after birth.

2.1.1.1 Experiment 1: Acute study on embryonic day 18 (E18)

For the acute study, gestational day 18 timed-pregnant rats were injected i.p. with 10mg/kg of poly(I:C) and saline (0.9% NaCl). This experiment was set up for the purpose of assessing the acute effects of maternal poly(I:C) administration on the cytokine and certain chemokine levels in maternal blood. After the
injections the animals were left undisturbed for 5 hours and then anesthetized with an overdose of sodium pentobarbital and decapitated after blood was obtained by cardiac puncture. The blood was collected in heparinised vacutainer tube and kept in ice cold. The animals were dissected and the uterine horn containing embryonic day 18 pups were surgically removed. Placenta, fetal’s whole brain and body were dissected and immediately frozen on dry ice. The collected whole brains were used in the western blot analysis. Maternal liver, thymus and brain were also dissected for further analysis i.e. HPLC analysis.

2.1.1.2 Experiment 2: Postnatal day 21 (P21)

For gestational poly(I:C) administration and vehicle treatment, groups of pregnant dams were administered intraperitoneally (i.p.) at the dose indicated on days E14, E16 and E18 in order to extend the temporal impact of maternal immune activation on the embryos, with no apparent ill effects on the dam or her behaviour towards her litter after birth. The selected gestational window in this study corresponds roughly to the middle second to early third trimester of human pregnancy, with respect to developmental biology and comparable percentage of gestation in humans (Clancy et al., 2001). The selection of the time window was based on the intention to widen the time window over which potential interference with brain development could occur, because examination of the brains would not occur until at least the time of weaning at postnatal day 21 (P21). The long term effects of prenatal poly(I:C) exposure would be captured at around this postnatal age because it has been reported that the full spectrum of prenatal poly(I:C)-induced behavioral, cognitive and pharmacological abnormalities emerges only after the postpubertal stage of development (Zuckerman et al., 2003, Zuckerman and Weiner, 2005). Group of pregnant dams were injected on E14, 16 and 18 between 9am and 11am to minimise variation in responses. Our preliminary works showed that the selected dosage at 10mg/kg could be used repeatedly without producing signs of sickness behaviour in the pregnant dam. Control females were injected with the same volume of 0.9% NaCl. Gestation was allowed to proceed normally and the offspring were undisturbed until weaning on P21 when they were taken from the home cage for euthanasia followed by removal of the brain. Each brain was
divided into the two cerebral hemispheres and frozen immediately on dry ice before being transferred to storage at -80ºC until required for analysis.

**Figure 2-1** Schematic diagram of experimental set up for prenatal immune challenge

### 2.1.2 Prenatal modulation of kynurenine pathway

Compound 3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]benzenesulphonamide (Ro61-8048) (Rover et al., 1997) was prepared at the dose of 100mg/kg by solubilising in a small volume of 60mM NaOH-NaCl and adjusted to pH 7.5 by adding 1M NaOH or 1M HCl. Mixture was vortexed and checked for pH every time small volumes of 1M NaOH or 1M HCl were added. After the required pH has been reached, the volume of 1M NaOH or 1M HCl was calculated and 60mM NaOH-NaCl was added to make up the total volume. Solution of the drug for the treatment was sterilised and aliquoted in small sterilised tube and kept in -20ºC until further use (Figure 2-2).

#### 2.1.2.1 Experiment 1: Acute study on embryonic day 18 (E18)

This experiment was set up for the purpose of assessing the immediate effects of gestational modulation of kynurenine pathway on the levels of protein expression in the embryo brains measured 5 h and 24 h post administration. In
addition, the objective was to demonstrate the effect of the selected dosage in producing changes in the levels of several metabolites of kynurenine pathway.

For this purpose, gestational day 18 timed-pregnant rats were assigned to receive i.p. injections of the following treatment regimens: (1) 5 h group: saline (0.9% NaCl) and (2) 5 h group: 100mg/kg Ro61-8048. After the injections the animals were left undisturbed for 5 hours and then anesthetized with an overdose of sodium pentobarbital and decapitated after blood was obtained by cardiac puncture. The blood was collected in heparinised vacutainer tube and kept in ice cold. The animals were dissected and the uterine horn containing embryonic day 18 pups were surgically removed. Placenta, foetal’s whole brain and body were dissected and immediately frozen on dry ice. In another set of experiment, two groups of pregnant rats were injected at gestational day 18 with single injection of vehicle solution and compound Ro61-8048 (100mg/kg), respectively. Twenty four hours later, the animals were then sacrificed and dissected to obtain foetal whole brain and body, placenta along with mother’s liver, brain and thymus. The levels of kynurenine and kynurenic acid from were measured by high performance liquid chromatography (HPLC) with electrochemical detection. HPLC analysis was solely performed by Dr Forrest (Forrest et al., 2013a).

2.1.2.2 Experiment 2: Postnatal day 21 (P21)

For gestational treatment, groups of pregnant dams were administered intraperitoneally (i.p.) with compound Ro61-8048 (100mg/kg) at the dose indicated on days E14, E16 and E18 in order to extend the temporal impact of kynurenine pathway modulation on the embryos, with no apparent ill effects on the dam or her behaviour towards her litter after birth. Group of pregnant dams were injected on E14, 16 and 18 between 9am and 11am to minimise variation in responses. Control females were injected with the same volume of 0.9% NaCl. Gestation was allowed to proceed normally and the offspring were undisturbed until weaning on P21 when they were taken from the home cage for euthanasia followed by removal of the brain. The long term effects of pharmacological manipulation of kynurenine pathway during gestation would be studied at juvenile age as to capture any maturational delay onset of the pharmacological abnormalities. Each brain was divided into the two cerebral hemispheres and
frozen immediately on dry ice before being transferred to storage at -80°C until required for analysis.

2.1.2.3 Experiment 3: Postnatal day 60 (P60)

In a separate experiment to evaluate the extended effect of prenatal exposure of compound Ro61-8048 in more mature animal, the neonates were being allowed to survive until around 60 days of age (young adult) at which time all litters were euthanized and their brains were removed. It is around this time of age that many studies have shown behavioral changes resulting from prenatal infection of mimetic agents. This study intended to explore the long term functional consequences on pharmacological activities of the brain. The brain was then sectioned into four main regions i.e hippocampus, cerebellum, cortex and midbrain and preserved for further western blot (WB) analysis. Hippocampus slices were prepared and examined for baseline excitability and other electrophysiological analysis including excitatory synaptic potentials. Electrophysiological analysis was exclusively performed by Prof Stone. Slices were then kept frozen for examination of the selected panel of neurochemical markers in order to detect differences in the CA1 region correlating with the electrophysiological responses.
Each member of a litter from all set of experiments was treated identically, with whole litters being taken at the same time so that no animal would experience the possible trauma of losing littermates while themselves surviving to a later date. This protocol also ensured that changes of maternal behavior caused by the removal of some pups could not affect the development of survivors. Two pups from three separate litters (n=6) were used for each treatment and control group; there were at least 3 samples per treatment and control groups to minimize any litter effects. For postnatal day 21 experiment and acute study, three male and three female pups from three separate litters were used for the treatment and control groups. Postnatal age of 21 day in developmental rodent model was chosen as to represent pre-pubertal (adolescence) stages of development whereas postnatal day 60 is typically regarded as the post-pubertal (adulthood) life span.
2.2 General materials

2.2.1 Solutions and reagents

Solutions used in the main experiments including protein assay, gel electrophoresis and immunocytochemistry are shown in Table 2-1, Table 2-2 and Table 2-3, respectively. All chemicals listed in this section were supplied from BDH Laboratories, Fischer Scientific or Sigma-Aldrich unless otherwise stated.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Chemical compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA buffer</td>
<td>50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% Triton X-100, 1% IGEPAL</td>
</tr>
<tr>
<td>BSA standard</td>
<td>20mg/100ml in dH2O (Albumin Bovine A2153)</td>
</tr>
<tr>
<td>50% BIORAD reagent</td>
<td>5ml BIORAD Protein Assay (final volume 10ml of dH2O)</td>
</tr>
</tbody>
</table>

Table 2-1  Protein assay solutions and reagents

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Chemical compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix running buffer</td>
<td>50ml NuPAGE MOPS/MEPS Running Buffer (Life Technologies,UK), 950ml dH2O, 0.5 ml antioxidant</td>
</tr>
<tr>
<td>Transfer buffer</td>
<td>50ml 20X NuPAGE Transfer Buffer (Life Technologies, UK), 1ml antioxidant, 100ml Methanol (Fischer Scientific), 850ml dH2O</td>
</tr>
<tr>
<td>10x stock Tris-buffered saline (TBS)</td>
<td>200mM Tris, 1.36M NaCl in 2000ml dH2O</td>
</tr>
<tr>
<td>Tris-buffered saline (TBS)</td>
<td>20mM Tris, 136mM NaCl, 0.05% Tween-20 (adjusted to pH 7.6 with 50% HCl)</td>
</tr>
<tr>
<td>5% Milk-TBST</td>
<td>5g skimmed milk (Marvel) in 100ml TBST ph 7.6</td>
</tr>
</tbody>
</table>

Table 2-2  General solutions used for gel electrophoresis and western blotting
### Solutions

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Chemical compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2M phosphate buffer (PB)</td>
<td>Solution A: 37.44g of NaH₂PO₄ (2H₂O) in 1200ml dH₂O. Solution B: 84.90g of Na₂HPO₄ in 3000ml dH₂O. Mix thoroughly 1120ml solution A with 2880ml solution B and adjust pH to 7.4</td>
</tr>
<tr>
<td>0.3M NaCl buffered saline (PBS)</td>
<td>100ml 0.2M PB, 1900ml dH₂O, 36g NaCl</td>
</tr>
<tr>
<td>Mammalian Ringer solution</td>
<td>45g NaCl, 2g KCl, 1.25g CaCl, 0.025g MgCl, 2.5g NaHCO₃, 0.25g NaH₂PO₄, 5g glucose in 5000ml dH₂O</td>
</tr>
<tr>
<td>4% Paraformaldehyde</td>
<td>40g paraformaldehyde (6005, SIGMA), 400ml dH₂O (preheat to 68°C), 500ml 0.2M PB and a few drop of NaOH</td>
</tr>
</tbody>
</table>

**Table 2-3 General solutions used for immunocytochemistry**

### 2.2.2 Drugs

The drugs used in experiments are listed in Table 2-4.

<table>
<thead>
<tr>
<th>Final concentration drugs</th>
<th>Quantity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9 % Saline</td>
<td>0.9g NaCl dissolved in dH₂O</td>
<td>BDH Laboratories</td>
</tr>
<tr>
<td>10mg/kg poly(I:C)</td>
<td>10mg dissolved in 1ml of sterile 0.9% saline and heated to 50°C</td>
<td>P0913, Sigma-Aldrich</td>
</tr>
<tr>
<td>60mM NaOH-NaCl</td>
<td>240mg NaOH dissolved in 100ml of sterile 0.9% NaCl</td>
<td>BDH Laboratories</td>
</tr>
<tr>
<td>100mg/kg Ro61-8048</td>
<td>300mg dissolved in 3ml of 60mM NaOH-NaCl, adjusted to pH 7.5 using HCl, sterilised and aliquoted</td>
<td>Synthesized by F. Hoffmann-La Roche Ltd.</td>
</tr>
</tbody>
</table>

**Table 2-4 Drugs solutions**
2.3 Western blot (WB) analysis

The western blot (WB) or also known as immunoblot is a semi-quantitative method to detect a specific protein in biological samples, for example tissue homogenate or extract. Generally, it involves multistep processes in which samples are first separated by LDS-PAGE (lithium dodecyl sulphate-polyacrylamide gel electrophoresis) and then the separated protein are transferred to a membrane where they are incubated with an antibody specific which binds to the protein band immobilized on the membrane (target protein). The antibody is then visualized with a detection system that is usually based on a secondary protein binding which is linked to a colour-yielding reaction.

2.3.1 Sample preparation

The preparation of the sample for protein analysis is the first step in the complete workflow of WB. The main principle is to ensure that the sample is in the best possible environment for analysis. Following prenatal administration of compound, the offspring for each treatment group were killed by overdose sodium pentobarbital (Euthatal) followed by decapitation. The brains were then quickly removed and separated into two hemispheres. Each hemisphere from the same animal was wrapped in a foil and marked before being stored at -80°C for later use (for experiment 2). For experiment 3 of postnatal day 60, the brain was removed rapidly from the skull and rinsed with cold artificial cerebro-spinal fluid (ACSF) solution. The brain area under study was then separated into four main region of interest which constitute of cerebellum, cortex, midbrain and hippocampus. Fresh dissection of brain tissue has the advantage that particular brain regions can easily be dissected based on visual information, such as differences in colour of adjacent tissue and on the natural anatomical boundaries of certain regions present in the brain. Examples of these are the cerebellum that can be easily taken off from the medulla and pons and are distinct in colour, and the hippocampus that differs from the occipital cortex by colour and loosely connected to the thalamus by the fornix. For embryonic brain dissection, the impregnated rats (E18) from respective treatment groups were killed with overdose injection of Euthatal and a small incision was made over the belly before a string of embryos were pulled out and transferred to a petri dish.
on ice filled with ice-cold PBS. The embryos were dissected out of the uterus wall and removed all the membranes including visceral yolk sac. The head was separated from the rest of the body before being wrapped and kept frozen at -80°C. Important consideration for successful sample preparation for the subsequent assay analysis includes maintaining the sample at low temperature throughout the processes and minimizing elapsed time in between procedure (time from sacrificing an animal to homogenization of the sample) in which all procedure steps were kept constant and as short as possible.

Careful control of presence of the target protein in the starting material and its preparation and storage under non-degrading conditions is essential in preserving the epitope abundance for antibody recognition. Standard and validated method for the extraction purpose has been employed to provide good yields and consistent results. For this purpose, brain tissues were lysed/homogenized using a mechanical homogenizer in RIPA buffer containing 60mg TRIS, 87.6mg NaCl, 10mg SDS, 50μl TRITON X-100, 100 μl IGEPAL, 9850 μl distilled water to encourage lysis of tissues and cells and to solublize proteins. The process of proteolysis and denaturation of protein in the lysate were slowed down by adding one tablet of protease inhibitor (Protease Inhibitor Cocktail tablets-Complete™, Boehringer Mannheim GmBH, Mannheim, Germany). Protease inhibitors are often added to prevent the digestion of the sample by its own enzyme. Then, the whole lysate was centrifuged at 13 000 rpm for 5 minutes at 4°C and finally the supernatant was aspirated and aliquots kept at -80°C prior to use.

2.3.2 Protein concentration

Prior to western blot analysis, determination of protein concentration in each sample was performed using the BIORAD protein assay system. Protein concentration in the brain homogenates (half brain or regional area of the brain) for western blot analysis was normalised against a standard curve of a known protein content, Bovine Serum Albumin (BSA; Sigma-Aldrich) dissolved in an appropriate volume of dH2O to give a concentration range of 0, 0.25, 0.50, 1.0, 1.5 and 2.0 (mg/ml). Then, brain samples dilution was prepared by diluting it twice with dH2O at the ration of 1:10 and 1:100 (only hippocampal lysate was straight
diluted into 1:100). Bio-Rad protein reagent (Bio-Rad laboratories, UK) was diluted 1:1 ratio before mixed thoroughly with each BSA standard reagents and brain samples. Subsequently, each BSA standard and samples was pipetted in duplicate into 96-well plate. An automated plate reader Opsys MR (Dynex Technologies) was used to measure optical density at 595nm using the BIORAD.assay program in Revelation Quicklink software (Dynex Technologies, version 4.25). A linear curve fit was plotted using the reading from the BSA standard concentration and was used to quantify the protein content of unknown brain lysates. A calibration curve was established each time a protein assay was performed with freshly prepared BSA standard dilutions. Using the standard curve, the concentration of each sample was determined according to its absorbance by interpolation. The measurements for each brain lysate from the program were then multiplied by 10 (hippocampal lysates were multiplied by 100) to compensate for the dilution factor applied during the sample preparation. Protein concentrations were then normalised to load 20-30 μg of protein onto the gels.

2.3.3 Protein separation

To characterize immunodetection of the antibody (refer Table 2-5), western blotting was carried out following procedures already established in the lab. Prior to the electrophoresis process, proteins from the lysate of brain samples (65% protein sample, 25% LDS sample buffer and 10% reducing agent) was denatured by centrifugation and heating at 2000rpm for 2 minutes at 4°C and for 10 minutes at 70°C, respectively. Equal amount of proteins from each sample (20-30 μg) was loaded into wells in the NuPAGE 4-12% (1.0mm) Bis-Tris polyacrylamide gel 15 lanes (Invitrogen: Life Technologies, Paisley, UK). The first lane on each gel usually reserved for a molecular weight marker: a mixture of proteins with defined molecular weight (SeeBlue pre-stained standard (Life Technologies, Paisley, UK). When voltage is applied along the gel, proteins migrate at different speed and separate into bands based upon size and charge (electrophoretic mobilities), characteristically smaller proteins migrate through the gel faster than larger proteins. Proteins in the sample travel only in the dimension along the gel for most blots. The separation of the protein which employs polyacrylamide gels and buffers loaded with lithium dodecyl sulphate
(LDS) and treated with 10% reducing agents to sufficient final concentration was electrophoresed through 4-12% Bis-Tris NuPAGE gel in 20X NuPAGE MOPS/MEPS running buffer (Life Technologies, UK) at 150V for 100 minutes.

2.3.4 Protein transfer and blocking

The separated protein then electrophoretically transferred onto a hydrophobic Invitrolon poly(vinylidene difluoride) (PVDF) membranes (Invitrogen: Life Technologies, Paisley, UK). For this procedure, the stacking gel removed from the separating gel and the back of the gel was placed on a wet filter paper which had been soaked with transfer buffer. Thereafter the PVDF membrane was applied on top of the gel (the membrane was activated by dipping into methanol). Each layer was gently pressed using a small roller before the next was applied to remove any air bubbles. The sandwich of membrane PVDF, gel, filter papers and appropriate number of fiber pads were then stacked to gel holder cassette and put in the Novex tank cell. Finally, the cell was filled up with transfer buffer and an electric current at 220mAmp, 30 volts for 80 minutes was applied to the gel so that the proteins transfer through the gel and onto the membrane in the same pattern as they separated on the gel. To check the uniformity and transfer efficiency of the transfer, membranes were rinsed in dH$_2$O and stained with Ponceau red dye (Sigma-Aldrich). Following transfer, membranes were placed in blocking solution containing 5% (w/v) dried skimmed milk powder (Marvel) diluted in Tris-buffered saline containing 0.05% Tween-20 (TBST) for at least one hour. The step was included to minimize background staining due to non-specific binding of the antibody by saturating the proteins which are not reacting with the antibody. The accessibility of a target protein for primary antibody is achieved by washing the blocked membranes with TBST for 3 times 15 minutes each, before incubation with primary antibody.

2.3.5 Western blotting: antibodies incubation

Evaluation of effect of maternal infection induced by poly(I:C) and also systemic administration of kynurenine 3-monooxygenase inhibitor, Ro61-8048 were studied on selected panel of neurodevelopmental markers that are involved in many aspect of central nervous system (CNS) development. These target markers
are known to participate in various roles spanning from neural development, neurite outgrowth, neurogenesis, synaptogenesis, neurotransmission and intracellular signalling, among many others.

The primary antibody is the major determinant of the specificity of the target recognition (Table 2-5). Trial set up of membrane with range of dilutions was initiated to check reactivity and also specificity of the selected antibodies. The optimal dilution of the primary antibody has to be determined experimentally for the detection system used as different batch or source of the antibody may even result in different recognition pattern when applied to the same sample (commonly used dilution is between 1:500 - 1:10,000). The incubation with primary antibody in blocking solution (5% milk-TBST) occurred at 4°C under continuous shaking condition. Post incubation: the membrane was washed three times with TBST solution for 15 minutes at room temperature (RT) to remove unbound primary antibody. In order to detect the antibody which have bound, the membrane was exposed to a secondary antibody reactive against the respective primary antibody used (for instance: anti-rabbit to detect primary antibody raised in a rabbit) (Table 2-6). This secondary antibody is known as horse-radish peroxidise (HRP) conjugated antibody dissolved in blocking solution and applied to the membrane and incubated for 1 hour at RT. All antibodies used in this study were commercially available and have been used previously in numerous regions of the human or rat brain as well as in vitro studies. Western blot analysis was carried out using the following primary antibodies raised against target proteins as listed in Table 2-5.
<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2A</td>
<td>Rabbit polyclonal</td>
<td>1:1000, 1:5000</td>
<td>PPS012, R&amp;D Systems, Abingdon, UK</td>
</tr>
<tr>
<td>NR2B</td>
<td>Rabbit polyclonal</td>
<td>1:1000, 1:5000</td>
<td>PPS012, R&amp;D Systems, Abingdon, UK</td>
</tr>
<tr>
<td>PSD95</td>
<td>Rabbit monoclonal</td>
<td>1:10000</td>
<td>3450, Cell Signalling, New England Biolabs, Hitchin, UK)</td>
</tr>
<tr>
<td>EphA4</td>
<td>Rabbit polyclonal</td>
<td>1:5000</td>
<td>sc-921, Santa Cruz, Insight Biotechnologies, Wembley, UK</td>
</tr>
<tr>
<td>NFKB</td>
<td>Rabbit polyclonal</td>
<td>1:1000, 1:5000</td>
<td>sc-372, Santa Cruz, Insight Biotechnologies, Wembley, UK</td>
</tr>
<tr>
<td>COX2</td>
<td>Goat polyclonal</td>
<td>1:1000</td>
<td>sc-1745, Santa Cruz, Insight Biotechnologies, Wembley, UK</td>
</tr>
<tr>
<td>PCNA</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
<td>sc-56, Santa Cruz, Insight Biotechnologies, Wembley, UK</td>
</tr>
<tr>
<td>SHH</td>
<td>Goat polyclonal</td>
<td>1:1000</td>
<td>sc-1194, Santa Cruz, Insight Biotechnologies, Wembley, UK</td>
</tr>
<tr>
<td>SOX2</td>
<td>Goat polyclonal</td>
<td>1:500</td>
<td>sc-17320, Santa Cruz, Insight Biotechnologies, Wembley, UK</td>
</tr>
</tbody>
</table>

Table 2-5 Primary antibodies used for western blotting

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-rabbit HRP</td>
<td>1:5000</td>
<td>12-348, Millipore, Watford, UK</td>
</tr>
<tr>
<td>Donkey anti-goat HRP</td>
<td>1:5000</td>
<td>sc-2020, Santa Cruz, Insight Biotechnologies, Wembley, UK</td>
</tr>
<tr>
<td>Goat anti-mouse HRP</td>
<td>1:5000</td>
<td>sc-20050, Santa Cruz, Insight Biotechnologies, Wembley, UK</td>
</tr>
</tbody>
</table>

Table 2-6 Secondary antibodies used for western blotting

2.3.6 Detection method: Chemiluminescence

Subsequently excess of secondary antibody was washed free of the blot by applying three times washes with TBST for 15 minutes each. For reproducibility purposes, volumes and times are constantly kept during the whole washing
procedure. The immunoblots were exposed to a substrate which will precipitate upon reaction with the secondary, resulting in a visible band where the primary antibody bound to the protein. For this step, enhanced chemiluminescence solution from Thermo Scientific (Pierce® ECL2 Western Blotting Substrate) was added onto the membrane and incubated for 5 minutes. Finally membranes were placed into a cassette and developed using x-ray films (Kodak, UK) on a Kodak X-OMAT 2000 processor with the exposure time varied depending on the antibody used. In each case the aim was to achieve an optimal balance between signal-background ratios.

2.3.7 Image analysis and membrane quantification

Protein intensity in each membrane developed on an X-ray film was quantified by measuring their relative optical density (OD) using the analysis of gels options in Image J software (http://rsbweb.nih.gov/ij/). First, the X-ray films developed from chemiluminescence were scanned onto a computer in full colour using HP Scanjet 4400cc Scanner and saved as Jpeg file. On Image J software, the selected image was transformed into 8 bit image. The relative OD measurements for control and treated samples run on the same gel were normalised to relative OD of the β-actin signal within each lane, because treatment with either poly(I:C) or Ro61-8048 at the desired dosage did not affect β-actin content in the brain (data not shown).

2.3.8 Data analysis

The immunoreactive bands were detected using chemiluminescence and quantification involved densitometry measurements where comparisons were made statistically between groups of pups born to mothers treated (either with poly(I:C) or compound Ro61-8048) and groups of pups born to mothers injected with saline vehicle. Statistical analysis was performed using unpaired t tests to examine differences between the two groups. The level for a statistical difference in the data was set at $P<0.05$. Unless otherwise noted, statistical difference is cited in the text when the data for the respective treatment group were different from the data from control group. We have determined earlier that there was no effect of gender on the treatment of Ro61-8048 at P21, the
data of the male and female offspring in each treatment group were combined for the purpose of statistical analysis.

### 2.4 Immunocytochemistry (ICC) procedures

Immunocytochemistry is a technique that uses antibodies which target antigenic receptors on the cell membranes. The reaction site can be identified in the specimen and associated with specimen structure by attaching a microscopically probe to the antigen-antibody complex. Thus, microscopical analysis by this technique can therefore be used to obtain a unique view of macromolecular changes within individual cells. ICC is widespread in use for light and electron microscope studies in almost every area of biological research from routine diagnostic work through to more complex research projects. The feasibility of the technique in living specimens, making it possible to study dynamic processes, such as gene expression, molecular binding interactions and as part to understand the distribution and localization of specific molecular markers in different parts of a tissue (Beesley, 1993).

#### 2.4.1 Animals and operative procedures

Groups of litter from saline and Ro61-8048-treated animals ($n=7-9$) were killed at postnatal day 60s after gestational treatment at E14, 16 and 18. Both control and Ro61-8048-treated animals were deeply anesthetized using Euthatal (sodium pentobarbital) before transcardially perfused via the left ventricle. In general, conventional tissue fixation protocols for microscopy analysis involve the use of fixative solution, which should allow excellent morphological preservation. The fixation procedure needs to be a compromise between good morphological preservation and the conservation of antigenicity; ideally the composition of the fixative should ensure both. Furthermore, an ideal fixative should stabilize and protect tissues and cells from the damaging effects associated with subsequent treatment. There are a number of fixation procedures used, which are based on various fixatives such glutaraldehyde, formaldehyde and also ethanol, methanol, acetone and formalin. In this respect, the best concentration of fixative we used in all animal study for ICC procedure is 4% parformaldehyde (PFA), which is less damaging to many antigens and give a
good compromise between retention of morphology and antigenicity. After the animal has been anesthetized, mammalian Ringer solution was flushed through each animal to dilate blood vessels and prevent clotting and fixation of the tissues was followed by perfusion with approximately (850ml to 1000ml) of freshly made, warm 4% paraformaldehyde (PFA) (Sigma-Aldrich 16005) in 0.2M phosphate buffer (PB), pH 7.2. Right after perfusion, the brains were removed from the skull and postfixed the same fixative solution for another 4 hour at 4°C. After thorough rinsing in 0.1M phosphate buffer, the brains were protected in a 30% sucrose solution where they remained until equilibrium. In our laboratory, samples were fixed, stored in phosphate buffer for up to one month and subsequently processed and successfully immunolabelled. It should be noted that prompt fixation after perfusion is essential to achieve consistent results. Prolonged fixation for the tissue samples were also avoided as many antigens show a progressive reduction in immunoreactivity with increasing fixation times by producing cross-linking with protein that could diminish the epitopes for the applied antibodies. Therefore, 4 hour post fixation with PFA for adult brain tissue is thought to be optimal that can give adequate ultrastructural preservation.

2.4.2 Tissue processing

Vibratome is an excellent basic vibratory tissue sectioning system. It cuts fresh or fixed, animal or plant tissue without freezing or embedding. Brain samples were divided into two hemispheres and cut to an appropriate size, embed in 5% agar and cooled for a few minutes before mounted on the vibratome base plate with the aid of super glue to stabilize the specimen. All the parameters were set accordingly; section thickness, speed and angle of the sectioning blade. Initial sectioning was performed one or two times to obtain a flat specimen surface. As section quality is influenced by several factors, the setting parameters of the vibratome (Leica 1000) were appropriately checked when performing sectioning. In relation to brain tissue, setting parameters of: low bath temperature (a physiological compatible buffer: 0.1M PB), slow sectioning speed (setting 1-2), section thickness (60 µm) and low steep blade angle were adopted. After initial sectioning, sixty-micrometer thick free floating sections were then cut coronally through the entire hippocampus and collected serially in a series of tubs filled
with 0.1M PB. The sections were incubated with 50% ethanol for 30 minutes to permeabilize and improving the penetration of the antibody, before 3 times washed with 0.3M PB and stored at -20°C in glycerol.

2.4.3 Antibody incubation and staining procedure

For analyses of hippocampal neurogenesis, serial free-floating sections were first washed in 0.3M phosphate buffer saline (PBS) before incubated with optimally diluted primary antibodies in 0.1% Triton in PBS (PBST; pH 7.4) for 72 h at 4°C under continuous shaking condition. The major consideration when choosing the antibodies for multiple immunolabelling is the possibility of cross reactions between reagents. In this study we adopted simultaneous multiple immunolabelling using antibodies raised in different species. Immunolabelling was performed by incubating the specimen with mixtures of chosen antibodies followed by mixtures of appropriate secondary probes. The optimal concentration of each antibody used in this study was optimized individually to obtain maximal contrast between specific signal and background staining. The mixture of primary and secondary antibodies were diluted in tris-buffered saline (TBS) containing gentle detergent (Tween 20) with the purpose to help keeping the tissues/cells permeabilized. Primary antibodies used in our study were monoclonal mouse anti-NeuN (1:500; MAB377 Millipore, Temecula CA92590), polyclonal goat anti-Doublecortin (DCX, 1:250; sc-8066 Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal rabbit anti-SHH (1:100; sc-9024 Santa Cruz Biotechnology, Santa Cruz, CA). Rinses were performed between all steps using 0.3M PBS before or after the primary and secondary antibody incubation.

Following primary antibody treatment, sections subsequently incubated overnight at 4°C in species specific Alexa Flour-tagged secondary antibodies: Alexa 488 (1:500), Alexa 647 (1:500) (Molecular Probes) and Rhodamine-conjugated secondary antibody (1:100) (Jackson Immunoresearch Laboratories Europe Ltd.). The uses of conjugated antibody fluorochromes have been successfully used for multiple immunostaining. The fluorescent probes are particularly useful since each probe can be visualized separately without hinderance from the other by use of different wavelengths of light for excitation.
and detection. Sections were mounted and coverslipped with an aqueous mounting medium for fluorescence H-1000: Vectashield (Vector Laboratories Inc. Burlingame, CA 94010). Covering the sections with a glass coverslip preserves and protects the section and improves optics. Special mountant containing fluorescent anti-fades was used to protect against photobleaching effect. Another aspect when choosing mounting medium is to match its refractive index with objective immersion medium, it is to avoid incorrect depth discrimination and spherical aberration which could lead to loss of light at the detector (Smith, 2008) In all experiments designed to compare the immunolabelling in saline-control and Ro61-8048 animals, sections from the two groups of animals were processed identically and in parallel for each step of the immunocytochemical procedures. Considering that there was a possible high risk of cross contamination in all multiple immunolabelling schedules, even if totally different species were used, a control specimen from each animal with omission of primary antibodies is included and tested in parallel with the samples. The specimens can be kept at 4°C if the material is going to be processed within 1-2 weeks as fluorescence may last up to 2-3 weeks, long term storage should preferentially be at -20°C in a covered boxes. However for best results the specimen should be viewed as soon as possible using confocal microscopy.

2.4.4 Confocal microscopy and image processing

Over the years, confocal microscopy has developed to form as essential research tool for assessing immunocytochemical preparations. The most common application of confocal microscopy is to compare the distributions or behavioural characteristic of multiple probes in the same tissue samples and also reveal the three dimensional structure of the specimen reconstructed from a series of optical sections at different depths (Smith, 2008). Flourescent signals from each section were examined and photographed using an inverted confocal laser scanning microscope (Nikon Eclipse TE300) equipped with RADIANCE2100 BIORAD and Lasersharp 2000 software. In principal, laser scanning microscope captures images by scanning the specimen with a focussed beam of light from a laser and collecting the emitted fluorescent signals with a photodetector. This is made possible by the development of the system that are capable of efficiently detects multiple fluorophores either simultaneously or sequentially by its laser
optics with a wide range of wavelengths from ultraviolet through the infrared. The system employs a pair of pinhole apertures to limit the specimen focal plane by blocking the light from out of focus areas thus determines the exact emitted fluorescence of the focal spot in the specimen (Smith, 2008). Relatively thick specimens can be imaged in successive volumes by acquiring a series of sections along the optical (z) axis of the microscope. Moreover, the use of oil-immersion objective that have similar refractive index with mounting medium for specimens would ensure better image quality.

In this experimental set up, stacks of 20 images were taken through the z-axis using a 40X oil objective and 10X magnification and a frame box of 289µm (x) X 289µm (y) X 20µm (z) (1024x1024 pixels). Setting parameters that needs to be controlled and standardized throughout the scanning include photomultiplier gain for the channels, scan line speed, brightness and pinhole aperture size. By properly adjusting the pinhole size, separation of the background out of focus signal can be achieved thus improves image quality. Whereas the scan speeds slider is adjusted to achieve the maximum scan rate with a minimum amount of signal noise. As the scan speed is increased, a corresponding increase in the amount of background noise is also captured by the photomultipliers (detector). By using 20x magnification the regional areas (CA1, CA3 and dentate gyrus) were delimited by morphological criteria and under 40x magnification objective the dimensions of the area in the specimen were scanned. Within this dimension only immunopositive cells showing a stained unit area sufficient to ensure that the majority of their volume was contained within the selected area were scored for counting purposes. At least 3 coronal sections per animal were immunolabelled and in every section, two to four images were taken followed by image processing to optimally visualize the staining area. Immunopositive cells from the pyramidal layer of CA1 and CA3 as well as granule cell layer of the hippocampus were quantified using a number of gridded counting frames that were within the specific subregions of the hippocampus and cerebellum with each individual counting frame measured 50 x 50 µm. The counting of the DCX, SHH and NeuN positive cells was conducted in a total of 15 grids of area for dentate gyrus and CA1 regions, 25 grids for CA3 region and grid sizes of 4 x 4 for cerebellum. For all the selected subregions, we calculated the number of immunopositive cells that were inside the counting frames for each section and
the total number of immunopositive cells across all counting frames was averaged to give one mean value per animal per subregions. Cell quantification was performed with adherence to several characteristics which includes (i) the presence of nucleus, (ii) immunopositive cells were of homogenously shape and (iii) labelling intensities of immunopositive cells were distinguishable from background staining.

2.4.5 Data analysis

All cell counts were performed blindly with respect to treatment, using immunolabeled sections reacted with selected antibodies. The data set obtained by capturing a series of optical sections through the specimen was used to compute views of the specimen from different viewing angles. Each subregion of the hippocampus (CA1, CA3 and DG) and cerebellum was analysed using Nikon Eclipse TE300 microscope. For counting purpose, a two-dimensional display of both z-series projection and xy projection formed by merging multiple image planes were conducted. The most common type of projection is a maximum projection in which each pixel represents the intensity of the brightest pixel in the z-axis. The cell number analysis was done on a total of 3 sections per animal with at least 2-3 observations per section using ImageJ software (Version 1.43M; National Institute of Health). Immunopositive cells count of 3 sections was averaged to obtain a single value for the number of positive- stained cells per animal. A mean ± standard error (SE) was calculated from the date in each group (n= 7-9 animals). We chose to use mean cell counts as representative of immunopositive labelling as this protocol would permit valid comparisons among treatment groups. Sections from animals with faint immunolabelling for respective antibody were excluded from statistical analyses, producing some variability in sample sizes across groups.

2.5 Golgi staining

2.5.1 Animals and operative procedures

Pregnant rats were injected with compound Ro61-8048 (100mg/kg) on embryonic days 14, 16 and 18, and gestation was allowed to proceed normally. Both male
and female pups were included in this study and they remained with their mothers until weaning at postnatal day 21 (P21) and were assessed at postnatal day 60 for the post-pubertal or adult group. Control animals were born from saline-treated mothers and were evaluated at the same age. At postnatal day 60, the pups from respective group of litters (4 pups per litter) were deeply anesthetized with sodium pentobarbital before cervical dislocation.

### 2.5.2 Tissue processing and staining procedure

The brains were then rapidly removed, rinsed briefly in dH₂O and straight away immersed in impregnation solution at room temperature for 2 weeks in the dark. Golgi-Cox staining was performed using the FD rapid GolgiStain kit (FD NeuroTechnologies) as per the manufacturer’s instructions. The impregnation solution was made by mixing equal volumes of solutions A and B at least 24 hours prior to use and left un-stirred at room temperature. The impregnation solution was refreshed after initial 24 hour of immersion. The tissue is then transferred into solution C and stored at 4°C for at least 48 hour in the dark. The solution was replaced after the first 24 hour of immersion.

Coronal sections of 200 μm thickness were obtained using a vibratome (VT1000s, Leica). Each section was then mounted on gelatin-coated microscope slides (3 sections/slide). A tiny drop of solution C was placed onto the mounted tissue before being dried naturally in the dark at room temperature. For the final development process, slides were arranged in a slide rack and washed twice in dH₂O for 2 minutes each. Then the slide rack was placed in a mixture consisting of one part solution D, one part solution E and two parts dH₂O for 10 minutes with gentle agitation every 2 minutes. The sections were rinsed two times with dH₂O, four minutes each and followed with dehydration process with graded concentration of ethanol in successive baths of 70% (4 minutes), 90% (4 minutes) and 100% (3 x 4 minutes). Subsequently, the sections were dipped in Histoclear solution (2 x 4 minutes) before cover slipped with histological mounting medium (Histomount, ThermoFisher Scientific).
2.5.3 Morphological and data analysis

The ability to obtain good images of neuronal processes allowed us to use this technique for morphological and quantitative studies using appropriate image analysis software. From each group, three litters with four pups in each litter were used for dendritic morphology study in the hippocampus. Brain region identification was aided by a standard atlas of the rat brain. Golgi-stained principal cells in the dentate gyrus, the granule cells were then visualized by light microscopy (Nikon Eclipse E400, Nikon Instruments, Inc., NY, USA). Under a 40x objective lens, Golgi-impregnated dentate granule neurons were identified by their location within granule cell layers and by the shape of their soma and dendritic arbor. Dendrites were visualized under a 40x objective blindly to the pretreatment group for manual tracing and dentate granule cells quantification.

A minimum of one neuron in each hippocampus section were drawn individually. Based on those criterion mentioned above, as a whole for each group of treatments, a total of 68 neurons from control and 69 neurons from Ro61-8048-treated animals were traced in their entirety. Fully stained cells within the panel were first visualized under minimal magnification (4 x objectives). Thus, each examined granule cell body was carefully viewed at various magnifications (4x, 20x and 40x) to determine the continuity of processes in its vicinity. By carefully adjusting the fine focus, it was possible to determine whether basal dendrite was present and to distinguish a passing dendrite from one that was attached to the examined cell body. To ensure consistency of sampling the following criteria were applied: (1) granule cells exhibited vertically orientated dendrites that extended into the dentate molecular layer; (2) granule cells did not have cut off dendrites in close proximity to the cell body and (3) dendrites had negligible overlap with the dendrites of neighbouring cells so that all dendritic branches could be traced explicitly. In addition to those parameters, we also generally observe other morphological criteria prior to the selection of the neurons such as integrity of the selected neuron, homogenous impregnation (dark and consistent impregnation seen throughout the extent of the dendrites) and relative isolation from the blood vessels and deposits of other impregnated cells located nearby (Zhao et al., 2006). Morphometric parameters included were the total number of branches and total dendrite length and used to assess
the overall magnitude of morphological change following prenatal modulation of kynurenine pathway. The number of dendritic branches was calculated manually from the camera drawings. The dendritic branches originating directly from cell bodies were designated as first order or primary segments and until they branched off into second order segments (secondary). To measure the extent of dendritic growth away from the soma and the branching of dendrites at different distances from the soma, the concentric circle analysis of was performed (Sholl, 1953, Gibb and Kolb, 1998). A transparent grid with equidistant 20µm was centred over the cell soma and the number of ring intersections was used to estimate the total dendritic length and dendritic arborisation. The branching complexity of dendritic trees was evaluated by applying the method of concentric rings. Rings were calculated at 20µm intervals (0-20, 20-40, 40-60, 60-80, 80-100, 100-120, 120-140). Sholl analyses were conducted on the mean values per animal for the above morphometric parameters. In Sholl analysis, the number of concentric ring intersections was used as an estimate of total dendritic length. While dendritic branching was also measured by counting the total number of dendritic branches at each order away from the cell body. Graphically, this generates a profile which can be statistically compared with other profiles from other groups. Morphological data were analysed using an unpaired t-test to assess differences in the number of dendritic branches and their length between control and Ro61-8048 treated groups of animals. The analysis of dendritic complexity (Sholl, 1953) was subjected to a two-way analysis of variance (ANOVA) followed by Tukey’s post hoc analysis to examine the contributions of drug treatment, sample number and distance from the cell soma. A probability value of 0.05 was taken as the criterion for significance.
3 Effect of maternal immune activation on neurodevelopmental markers expression in foetal and postnatal day 21 (P21) brain

3.1 Introduction

A body of evidence has indicated that prenatal immune activation causes a wide spectrum of structural and functional abnormalities implicated in a diverse range of disease process including AIDS-dementia complex, schizophrenia, autistic spectrum disorder, among many others. However the neurodevelopment mechanisms and biochemical changes underlying these abnormalities are not definitively established. The most widely debated possibility is that a maternal antiviral antibody in some genetically predisposed mothers may cause an immunological reaction that is harmful to the foetal brain (Gilmore and Jarskog, 1997). Maternal inflammation that caused a disruption to the maternal environment has been linked to the above disease states in adult life. Several reports showed that activation of the immune system and increased levels of cytokines during gestation affects the development of brain areas involved in social interaction and memory which later results in a schizophrenia-like phenotype (Ashdown et al., 2006, Hsiao and Patterson, 2012). This finding is also supported by epidemiological studies that demonstrate an association between maternal infection and the development of schizophrenia (Brown et al., 2004).

The use of animal models to model maternal immune activation has provided insights into the host’s response to an immunological stimulus, as well as the acute and lasting effects of immune activation on offspring development. Precise manipulation of a number of variables of interest, such as the immune stimulus, route of administration, dosage and timing of exposure, allows for the testing of various hypotheses regarding the mechanisms of action on the developing foetal brain. The most well known cause of inflammation during pregnancy is due to infection by a pathogen. As previously discussed in the introduction, the two major maternal immune activation models use either LPS or poly(I:C) administered to pregnant dams, although there are a number of other additional models which have used the human influenza virus and
individual cytokines such as TNF-α or IL-6 (Boksa and Luheishi, 2003, Boksa, 2010).

Despite the large number of studies describing abnormalities in rodent brains following exposure to prenatal immune stimulus, the precise molecular mechanisms by which infection during gestation and the effect of maternal immune response on the developing central nervous system remain unclear. The fact that a wide variety of pathogens are able to increase schizophrenia risk in the offspring suggests that these pathogens alter foetal development by one or more common mechanisms. A major causal link underpinning this relationship is thought to be the cytokines released following a maternal immune response to infection (Ashdown et al., 2006, Brown, 2006). Maternal immune activation is thought as a plausible mechanism due to all of the implicated pathogens triggering a maternal immune response, which will alter the biochemical environment of the foetus in a way that increases the risk for neurodevelopmental disorders. Despite the postnatal onset of clinical symptoms, evidence suggests that schizophrenia have their origins in early brain development. In line with the neurodevelopmental hypothesis of schizophrenia, which postulates that neuronal development is affected by events that modify physiological course of maturation in neuronal system. Therefore, maternal immune activated rodent models have been particularly helpful for examining in vivo effects of the maternal immune system on the developing foetal and postnatal brain (Patterson, 2009). These models predominantly involve administration of either bacterial lipopolysaccharide (LPS) or double-stranded viral RNA (polyI:C) to pregnant rats or mice. This induces an immune response, which increases circulating levels of cytokines without the confounding effects of the pathogen itself. The offspring of mothers with infections display diverse neuropathology, depending in part on the nature and timing of the infection. Offspring of these immune activated have been reported to show a ‘schizophrenia like’ phenotype including deficits in prepulse inhibition (PPI), latent inhibition (LI), anxiety, locomotion and social interaction (Meyer et al., 2008c, Meyer et al., 2008a, Ito et al., 2010, Shi et al., 2003).
3.2 Research aims

Based on the in vitro evidence of pro-inflammatory cytokine action in the brain, it is hypothesized that the presence of cytokines in the foetal brain as a result of maternal infection could result in altered brain neurochemical development. The main aim was to investigate if exposure to viral mimetic poly(I:C) during early life can affect the neurochemical balance of a selected panel of neurodevelopmental markers in the young offspring that could undermine normal brain development and predispose the offspring to developing psychiatric illness.

3.3 Experimental approaches

Exposure to a wide range of viruses pre-or perinatally has been implicated in the pathogenesis of neuropsychiatric disorders. To this aim, a synthetic analog of double stranded RNA (polyribonosinic-polyribocytidilic acid, [poly(I:C)] was administered intraperitoneally in mouse dams on embryonic days (E) 14, 16 and 18 in order to extend the temporal impact of maternal immune activation with no apparent ill effects on the dam or her behaviour towards her litter after birth. Administration of this agent to rat dams at three different time points during pregnancy (E14, E16 and E18) correspond roughly to the later periods during the second trimester of human pregnancy. In another set of experiments, poly(I:C) was administered to pregnant dams on embryonic day 18 (E18) as to demonstrate the effect of the immune activation in terms of changes in the levels of cytokine in the maternal plasma as well as protein expression in the embryo. Poly(I:C) is used experimentally to model viral infections because it stimulates the antiviral activities of the innate immune system including cytokine production, without the confounding effects of viral infection. We have decided to focus on poly(I:C) as we find it as a more reliable and consistent activator of the immune system and also because viral rather than bacterial infections predominate in human pregnancy. Injection at a dose of 10mg/kg of poly(I:C) is the highest dose which can be given to a pregnant mouse with no signs of acute stress, behavioural changes or abnormal behaviour towards the neonatal pups. Preliminary work on the neurochemical analyses showed little changes in the selected molecular markers at postnatal day 2 (P2) with a
significant increase of EphA4 at P7 and a reduction of both EphA4 and VAMP-1 expression at P21. In this study, we therefore concentrated on the molecular analyses of brain samples collected from E18 and P21. These two developmental time-points were aimed at capturing early and later/persisted changes in protein expression levels. It is therefore of considerable interest to evaluate biological markers that are involved in the early development of the brain. The goal of the study is to assess differences in the expression profiles of selected neurochemical markers after poly(I:C) induced-maternal immune activation and from unchallenged normal mice by analyzing the activated expression signalling pathways following infection.

### 3.3.1 Animals and drug preparation

All procedures on animal work was performed in accordance with the guidance established by the Animal (Scientific Procedure) Act 1986 and has been approved by the University of Glasgow Scientific Ethics Committee. Wistar rats were mated at an age of around 3 months and the first day after the occurrence of vaginal plug (an indication of copulation) was defined as day 1 of pregnancy. The pregnant dams were housed alone under pathogen-free environmental conditions at a constant temperature with food and water ad libitum. Potassium salt poly(I:C) (P0913: Sigma (Poole, UK)) was dissolved in sterile isotonic 0.9% w/v NaCl solution and then heated to 50°C to allow for complete solubility. The mixture was allowed to cool to room temperature to allow re-annealing of the double-stranded RNA. The desired dosage for the injection was 10mg/kg and it has been established in previous experiments in the lab that the dose could be given to pregnant dams during the last seven days of gestation with no signs of stress, behavioural changes or abnormal behaviour towards the neonatal pups after birth (Forrest et al. 2012). The solution for the drug treatments was sterilised and aliquoted in small sterilised tube and kept in -20°C until further use.

#### 3.3.1.1 Experiment 1: Acute study on embryo

For the acute study, gestational day 18 timed-pregnant rats were injected i.p. with 10mg/kg of poly(I:C). Age-matched pregnant rats were injected with the same volume of saline (0.9% NaCl). This experiment was set up for the purpose
of assessing the acute effects of maternal poly(I:C) administration on several cytokine and chemokine levels in maternal blood. After the injections the animals were left undisturbed for 5 hours and then anesthetized with an overdose of sodium pentobarbital and decapitated after blood was obtained by cardiac puncture. The blood was collected in a heparinised vacutainer tube and kept ice cold. The animals were dissected and the uterine horn containing embryonic day 18 pups were surgically removed. The placenta, foetal whole brains and bodies were dissected and immediately frozen on dry ice. The collected whole brains were used in the western blot analysis. Maternal liver, thymus and brain were also dissected for further analysis for example HPLC analysis. ELISAs for detecting cytokines and HPLC analysis was performed by respected colleague Dr Forrest and some findings are mentioned in the text.

3.3.1.2 Experiment 2: Postnatal day 21(P21)

For gestational poly(I:C) administration and vehicle treatment, groups of pregnant dams were administered intraperitoneally (i.p.) at the dose 10 mg/kg on days E14, E16 and E18 in order to extend the temporal impact of maternal immune activation on the embryos, with no apparent ill effects on the dam or her behaviour towards her litter after birth. Repeated systemic challenges during this time point was meant to examine the effect or repeated viral stimulation on the progression of neurological changes at postnatal day 21 since this represents pre-pubertal stage of development. Group of pregnant dams were injected on E14, 16 and 18 between 9am and 11am to minimise variation in responses. We have shown that treatment of pregnant dams with poly(I:C) at the dose indicated did not affect litter size and pup body weight and could be given during the last seven days of gestation with no sign of stress, behavioural changes of abnormal behaviour towards the neonatal pups after birth. The dosage regime at 10mg/kg was well tolerated in our experimental approach and could provide the maximum advantage for observing any changes in the offspring. In the control group, pregnant dams were injected with the same volume of 0.9% NaCl. Gestation was allowed to proceed normally and the offspring were undisturbed until weaning on P21 when they were taken from the home cage for euthanasia followed by removal of the brain. Each brain was
divided into the two cerebral hemispheres and frozen immediately on dry ice before being transferred to storage at -80ºC until required for analysis.

Each member of a litter from all set of experiments was treated identically; with whole litters being taken at the same time so that no animal would experience the possible trauma of losing littermates while themselves surviving to a later date. This protocol also ensured that changes of maternal behavior caused by the removal of some pups could not affect the development of survivors. To avoid possible litter-specific effects, groups of at least three independent litters were used in which two pups from three separate litters (n=6) were used for each treatment and control group; there were at least 3 samples per treatment and control group. For postnatal day 21 experiments and acute studies, three male and three female pups from three separate litters were used for the treatment and control groups.

Figure 3-1   Summary of diagrammatic representative of the experimental approach
3.3.2 Western blot analysis

The preparation of the sample is the first step in the complete protocol for western blotting. The main principle is to ensure that the sample is in the best possible environment for analysis. For this purpose, brain tissues were lysed/homogenized in RIPA buffer containing 60mg TRIS, 87.6mg NaCl, 10mg SDS, 50μl TRITON X-100, 100 μl IGEPAL, 9850 μl distilled water, with the process of proteolysis and denaturation of protein in the lysate slowed down by adding one tablet of protease inhibitor (Protease Inhibitor Cocktail tablets- Complete™, Boehringer Mannheim GmBH, Mannheim, Germany). The whole lysate was then centrifuged at 13 000 rpm for 5 minutes at 4°C and finally the supernatant was aspirated and aliquots kept at -80°C prior to use. Total protein concentrations for all samples were determined using the BIORAD protein assay system. This was achieved by comparing each brain lysate against a standard curve of known protein content - Bovine Serum Albumin (BSA: Sigma-Aldrich) dissolved in dH₂O to obtain a concentration range 0, 0.25, 0.5, 0.5, 1.0, 1.5 and 2.0 (in mg/ml). The brain lysate (diluted 1/10) and BSA standard was mixed with diluted BIO-RAD protein assay dye reagent (1:1 in dH₂O) (BIO-RAD Laboratories, UK). Subsequently each standard and brain lysate was pipetted twice into a 96-well plate and the absorbance measured with an Opyss MR plate reader (Dynex Technologies, UK) using the BIORAD.assay program of Revelation Quicklink software (Dynex Technologies, version 4.25) at a wavelength of 595nm. Protein concentrations were then normalised to load 20-30 μg of protein onto the gels.

To characterize immunodetection of the antibody for the selected markers mentioned above, western blotting was carried out following procedures already established in the lab. Prior to the electrophoresis process, proteins from the lysate of brain samples (65% protein sample, 25% sample buffer and 10% reducing agent) was denatured by centrifugation at 2000rpm for 2 minutes at 4°C and heated for 10 minutes at 70°C. Equal amount of proteins from each sample (20-30μg) was separated according to molecular weight on NuPAGE 4-12% (1.0mm) Bis-Tris polyacrylamide gels 15 lane gels (Invitrogen: Life Technologies, Paisley, UK) for 80 min at 150 volts. SeeBlue pre-stained standard (Life Technologies, Paisley, UK) was included on each gel as a molecular weight marker. The separated proteins were then electrophoretically transferred onto hydrophobic
Invitrolon poly(vinylidene difluoride) (PVDF) membranes (Invitrogen: Life Technologies, Paisley, UK) for 60 min at 30 volts. The membranes were incubated for 1 hour at room temperature with 5% non-fat dried milk in Tris-buffered saline (TBS: 20mM Tris Base and 0.14M NaCl, pH 7.6) containing 0.05% Tween-20 to block nonspecific binding sites and then incubated with the selected panel of primary antibody (see Antibodies section below) overnight on a shaker at 4 °C. After washing 3 times for 15 minutes each with TBS containing 0.05% Tween 20 (TBST), membranes were incubated for 1 hour at room temperature on a shaker with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody prepared in 5% milk-TBST. Following secondary antibody incubation, blots were rinsed with distilled water followed by 3 times wash with TBST (15 minutes each). Protein bands were developed using Enhanced Chemiluminescence Plus western blotting detection reagents (GE Healthcare, Chalfont St Giles, UK) and immediately exposed to film (KODAK Medical X-ray) and visualized using XOMAT.

For ELISA analysis, this procedure was performed by Dr Forrest as part of the aim to evaluate the maternal immune response following poly(I:C) administration during pregnancy. For these experiments, maternal plasma was obtained after centrifuging heparin-treated blood samples for 15 min at 2000 rpm. Specific rat inflammation profiling strips (SIGNOSIS, EA-1201) which simultaneously detects 4 inflammatory cytokines was used i.e. macrophage inflammatory protein-1α (MIP-1α), monocyte chemotactic protein-1 (MCP-1), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α). The assay was performed in 96 well plates according to the manufacturer’s instruction. Quantification of the data obtained in ELISA studies was performed by Dr Forrest.

### 3.3.3 Antibodies

All antibodies used in this study were commercially available and have been used previously to detect NR2A, NR2B, EphA4, PSD95, NFkB, COX2, SHH, SOX2 and PCNA in numerous regions of the human or rat brain as well as in vitro studies. Rabbit polyclonal antibodies were used to label NMDA receptor subunits NR2A and NR2B (1:5000; PPS 012 and PPS 013, R&D System, Abingdon UK), Ephrin type-A receptor (EphA4) (1 : 5000; sc-921, Santa Cruz, Insight
Biotechnology, Wembley, UK), Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (1:5000; sc-372, Santa Cruz, Insight Biotechnology, Wembley, UK) and rabbit monoclonal antibody for Post synaptic density protein 95 (PSD95) (1:10,000; 3450, Cell Signalling, New England Biolabs, Hitchin, UK). Goat polyclonal antibodies were used to label Cyclooxygenase 2 (COX2) (1:1000; sc-1745, Santa Cruz, Insight Biotechnology, Wembley, UK), Sonic hedgehog (SHH) (1:1000; sc-1194, Santa Cruz, Insight Biotechnology, Wembley, UK) and [Sex-determining region of Y-chromosome (SRY) related high mobility group (HMG) box]SOX2 (1:250 or 1:1000; sc-17320, Santa Cruz, Insight Biotechnology, Wembley, UK). Proliferating cell nuclear antigen (PCNA) was labelled using mouse monoclonal antibodies diluted at 1:1000 (sc-56, Santa Cruz, Insight Biotechnology, Wembley, UK). As a control for transfer and loading, actin was detected on blots using anti-actin (goat polyclonal, 1:10,000; sc-1615 Santa Cruz, Insight Biotechnology, Wembley, UK).

The following secondary HRP-conjugated antibodies were used at a 1:5000 dilution: goat anti-rabbit HRP (12-348) (Millipore, Watford, UK), donkey anti-goat HRP (sc-2020) and goat anti-mouse (sc-2005) (Santa Cruz, Insight Biotechnology, Wembley, UK). The concentrations of primary and secondary antibodies, along with the exposure time to the X-ray film were optimized to provide optical density values that fell within the linear range.

3.3.4 Data analysis and statistics

Quantification of all neurochemical markers was achieved by means of optical densitometry using ImageJ software (ImageJ, NIH, MD, USA). To control for variations and differences in protein loading, each membrane was stained with Ponceau S solution (Sigma, Poole, UK) after proteins were electrophoretically transferred onto a hydrophobic Invitrolon poly(vinylidene difluoride) (PVDF) membrane. Each membrane was reprobed and examined for actin expression and the relative optical density measurements for the respective target proteins were normalized to the relative optical density of the actin signal intensity within each lane. Actin served as an internal standard control and was not affected by treatments in this study. For all experiments, three pregnant rats were injected in each of the treatment groups and two pups from each litter
were randomly selected for western blot analysis \((n=6)\). To assess the long term effects of prenatal poly(I:C) exposure on the P21 offspring, comparisons of each treatment group with their respective control group was analysed using unpaired t-tests. In the second set of experiments, the acute effects of a single injection of saline and poly(I:C) on neurochemical alterations in foetal brain were determined by performing unpaired t-tests. Analyses were conducted using statistical software GraphPad Instat (version 3.0). Statistical significance was set at 0.05 for all analyses.

### 3.4 Results

#### 3.4.1 Cytokine expression

Measurement of cytokine responses were also assessed in maternal plasma and the amount of pro-inflammatory cytokines interleukin-1\(\beta\) (IL-1\(\beta\)), tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), the chemokines monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1\(\alpha\) (MIP-1\(\alpha\)) were analysed using ELISA. Poly(I:C) is recognized primarily by toll-like receptor-3 (TLR-3), in which upon recognition of dsRNA, induces an inflammatory cascade that results in the production of antiviral cytokines and chemokines (Alexopolou et al., 2001, Matsumoto and Seya, 2008). We examined the levels of several cytokines/chemokines in the maternal serum after 5 hours post administration of poly(I:C) so as to capture early changes of inflammatory responses. Activation of the maternal immune response by poly(I:C) caused a significant increase of chemokines MCP-1 5 hours after treatment compared with saline-treated animals, whilst the proinflammatory cytokines IL-1\(\beta\) and TNF-\(\alpha\) were not substantially affected 5 hours after poly (I:C) injection (data for IL-1\(\beta\) and TNF-\(\alpha\) not shown). Analysis of these results was performed by one of our colleagues and data for MCP-1 was indicated to highlight the effectiveness of the drug dosage used in this model (Forrest et al. 2012). The levels of this compound in the maternal blood is shown in Figure 3-2 which indicate a significant increase in concentration.
Figure 3-2 Effect of prenatal administration of poly(I:C) on maternal plasma levels of MCP-1

Bar chart showing the maternal blood levels of monocyte chemoattractant protein-1 (MCP-1) measured 5 h following the administration of poly(I:C) 10mg/kg. Values are shown as the mean ± SEM (n=3) in arbitrary units of optical density (OD).

3.4.2 Protein expression

3.4.2.1 Maternal infection induced by poly(I:C) alters protein expression in the embryo brain after 5 h administration.

In a set of an experiment to assess protein expression following poly(I:C)-induced immune stimulation, pregnant dams were administered with poly(I:C) 10 mg/kg or vehicle treatment (sterile 0.9% NaCl) on embryonic day (E18) and sacrificed 5 h post treatment. Expression levels of several neurodevelopmental proteins in the exposed embryo were examined using western blotting. These neuroregulatory molecules are known to be critically involved in several aspects of neuron development such as synaptic formation and plasticity, axonal guidance, and neurogenesis processes amongst many others. This included analysis of components of the NMDA receptor subunits complex (NR2A, NR2B and postsynaptic molecule PSD95), tyrosine kinase receptor EphA4 that are associated with axogenesis and formation of synaptic contact, and also included inductive signalling factor sonic hedgehog (SHH). In addition we examined the inflammatory regulated molecules (COX2 and NFKB) as well as general marker for neurogenesis in the brain, PCNA together with SOX2 in the exposed progeny.
As indicated in the previous section, acute poly(I:C) treatment in pregnant dams induced an elevation in one of the most important chemokines, MCP-1 in the maternal blood, thus confirming the efficacy of the dose used in the animal set up. Western blot analysis of the level of β-actin, a housekeeping protein, showed no difference between Ro61-treated rat and control. Both NMDA receptor subunits, its postsynaptic membrane proteins (PSD95) and axonal guidance molecule EphA4 from immunoblot analyses resulted in a strong signal at 180 kDa, 95 kDa and 120 kDa, respectively. In the foetal brain significant changes in the levels of NMDA receptor subunits were observed 5 h after maternal poly(I:C) injection, which showed a highly significant decrease of NR2A (expression level as compared with maternal control treatment Figure 3-3 (A), p=0.04). In contrast, expression of the NR2B subunit was increased in the brains of foetuses derived from poly(I:C)-treated mothers at 5 h post treatment (Figure 3-3(B), p=0.03). The expression of the NMDA receptor associated protein, PSD95 and one member of tyrosine kinase receptor, EphA4 were not significantly affected by maternal immunological manipulation at E18 (for PSD95: Figure 3-4 (A), p=0.06; for EphA4: Figure 3-4 Figure 3-4 (B), p=0.30).

In addition, we also examined several molecules with generic roles in neuronal proliferation, migration and maturation. Immunoblot analysis of the inductive signaling protein (SHH), neuronal proliferation marker (PCNA) and transcription factor in early stages of cell maintenance and survival (SOX2) resulted in a signal with a molecular weight of 45 kDa, 36 kDa and 34 kDa, respectively. In the foetal brain, acute poly(I:C) exposure had no effect on the expression of SHH (Figure 3-5 (A), p=0.19), PCNA (Figure 3-5 (B), p=0.37) and also SOX2 proteins (Figure 3-5 (C), p=0.35). Finally, we evaluated the effects of prenatal exposure to viral challenge on the expression of molecules involved in the inflammatory pathway which include transcription factor (TF) NFkB and the inducible type of rate-limiting enzyme of PGs synthesis, COX2. In WB analysis, immunoreactivity for polyclonal p65 anti-NFKB was found as a 65 kDa protein whereas anti-COX2 showed at 70 kDa. Single administration of poly(I:C) during late gestation at E18 revealed no significant difference in the level of relative amount of NFKB p65 subunit (Figure 3-6 (A), p=0.90) and COX2 protein levels (Figure 3-6 (B), p=0.25) in the foetal brain from poly(I:C) exposed versus control dams.
The graphs show immunoreactivity optical density values of NR2A (M.W. 180 kDa) and NR2B (M.W. 180 kDa) after normalization with corresponding actin on the same gel. Intraperitoneal injection of poly(I:C) 10mg/kg was administered on E18 and samples were collected 5 hour later. Prenatal poly(I:C) administration decreased the levels of NR2A protein expression while displayed an increase in NR2B immunoreactivity relative to prenatal control animals. Actin was served as an internal standard control and was not significantly different across lanes. All data were expressed as mean ± standard error of the mean (SEM). Representative western blots illustrate the data obtained from animals exposed to the saline (S) [n=6] or poly(I:C) (P) [n=6] and show the relevant protein and the corresponding actin (M.W. 42 kDa). *P<0.05 unpaired t-test.
Figure 3-4  Expression of PSD95 and EphA4 proteins in the embryo brains at 5h post administration. The graphs show immunoreactivity optical density values of PSD95 (M.W. 95 kDa) and EphA4 (M.W. 120 kDa) after normalization with corresponding actin on the same gel. Intraperitoneal injection of poly(I:C) 10mg/kg was administered on E18 and samples were collected 5 hour later. Actin was served as an internal standard control and was not significantly different across lanes. All data were expressed as mean ± standard error of the mean (SEM). Representative western blots illustrate the data obtained from animals exposed to the saline (S) \([n=6]\) or poly(I:C) (P) \([n=6]\) and show the relevant protein and the corresponding actin (M.W.42kDa).
Figure 3-5  Expression of neurodevelopmental proteins in the embryo brains at 5h post-administration.
All the graphs show immunoreactivity optical density values of SHH (M.W. 45 kDa), PCNA (M.W. 36 kDa) and SOX2 (M.W. 34 kDa) with corresponding actin on the same gel. Intraperitoneal injection of poly(I:C) 10mg/kg was administered on E18 and samples were collected 5 hour later. Actin was served as an internal standard control and was not significantly different across lanes. All data were expressed as mean ± standard error of the mean (SEM). Representative western blots illustrate the data obtained from animals exposed to the saline (S) [n=6] or poly(I:C) (P) [n=6] and show the relevant protein and the corresponding actin (M.W. 42 kDa).
Figure 3-6  Expression of inflammatory proteins in the embryo brain at 5h post-administration.
The graphs show immunoreactivity optical density values of NFκB (M.W. 65 kDa) and COX2 (M.W. 72 kDa) after normalization with corresponding actin on the same gel. Intraperitoneal injection of poly(I:C) 10mg/kg was administered on E18 and samples were collected 5 hour later. Actin was served as an internal standard control and was not significantly different across lanes. All data were expressed as mean ± standard error of the mean (SEM). Representative western blots illustrate the data obtained from animals exposed to the saline (S) \([n=6]\) or poly(I:C) (P) \([n=6]\) and show the relevant protein and the corresponding actin (M.W. 42 kDa).
3.4.2.2 Maternal infection induced by poly(I:C) alters protein expression in the postnatal day (P) 21 offsprings.

Maternal viral mimicked infection was modelled in timed-pregnant rats at embryonic days (E) 14, 16 and 18 by administering injections of poly(I:C) at (10mg/kg, i.p.) to study the impact of the maternal inflammatory response on the brain development of the resulting offspring at post natal day 21. Although not significantly altered in comparison to the 5 h treatment, the level of NR2A remained low, when observed in pups exposed to poly(I:C) at the later age of postnatal day 21 (Figure 3-7 (A), p=0.12). Similarly with NR2A, expression of NR2B subunit was not altered in poly(I:C)-pups at postnatal day 21 (Figure 3-7 (B), p=0.64). The expression of neither PSD95 (Figure 3-8 (A), p= 0.51) nor EphA4 (Figure 3-8 (B), p= 0.12) showed any significant difference from control pups from saline-treated dams.

As indicated in the section 3.4.2.1, acute poly(I:C) exposure had no effect on the expression of SHH, however repeated in utero viral challenges led to significant changes among this neurogenesis factor only at P21. As shown in Figure 3-9 (A) and Figure 3-5 (A), antibodies used against the SHH precursor protein (45 kDa) was clearly detected in the embryonic and postnatal brain tissues. Although there was no change in the level of expression of the SHH precursor protein at 5 h after poly(I:C) injection compared with that in age-matched uninfected controls, a statistically significant reduction was shown in the brain of postnatal day 21 (p= 0.03). Similar situation was observed when poly(I:C) was administered during the mid and late gestation in the rodent model, long term effects of prenatal viral challenge on survival and maintenance of cells in the brain as indicated by the expression of SOX2 was clearly demonstrated. Prenatal poly(I:C) exposure significantly increased the relative amount of SOX2 protein in the P21 pups compared with control pups (Figure 3-9 (C), p= 0.009). Although strong signals were seen in the postnatal brain, PCNA protein levels of P21 offspring were not statistically different as compared with control rats (Figure 3-9 (B), p= 0.64). Results of western blot analysis with this developmental markers showed strong signals suggesting conserved levels of endogenous expression during relatively early developmental period. Bands were also observed at moderate
intensities in the postnatal brain at the same position with molecular weight of 36 kDa.

Finally, we examined the levels of the inflammation transcription factor (TF) NFκB and the inducible type of rate-limiting enzyme of PGs synthesis, COX2, whose expression is linked to inflammation-induced oxidative stress. In the brains from P21 pups exposed to poly(I:C), the expression of neither NFκB (Figure 3-10 (A), \( p = 0.97 \)) nor COX2 (Figure 3-10 (B), \( p = 0.35 \)) showed any significant difference from control treatment. Thus, prenatal viral challenge at either E18 or three time points during mid-late gestation showed no apparent difference between treatments on the levels of protein expression of selected inflammatory markers.
Figure 3-7  NMDA receptor protein levels in the P21 offsprings after the maternal injection of poly(I:C).
Quantified protein expression of (A) NR2A and (B) NR2B in the brains of P21 rat offspring. Poly(I:C)-exposed groups consisted of offspring rats whose mothers were subjected to administration of a dose 10 mg/kg (i.p.) of poly(I:C) (P) on days E14, 16 and 18 of gestation. Control groups consisted of offspring rats whose mothers were submitted to the same treatment schedule with saline (S). Exposed and control rats display no difference in the levels of protein. Results are expressed as mean ± SEM. Representative western blots illustrate the data obtained from both groups and show the relevant protein and the corresponding actin. There was no significant difference between control (n=6) and poly(I:C)-treated groups (n=6).
Figure 3-8  PSD95 and EphA4 protein levels in the P21 offsprings after the maternal injection of poly(I:C).
Quantified protein expression of (A) PSD95 and (B) EphA4 in the brains of P21 rat offspring. Poly(I:C)-exposed groups consisted of offspring rats whose mothers were subjected to administration of a dose 10 mg/kg (i.p.) of poly(I:C) (P) on days E14,16 and 18 of gestation. Control groups consisted of offspring rats whose mothers were submitted to the same treatment schedule with saline (S). Exposed and control rats display no difference in the levels of protein. Results are expressed as mean ± SEM. Representative western blots illustrate the data obtained from both groups and show the relevant protein and the corresponding actin. There was no significant difference between control (n=6) and poly(I:C)-treated groups (n=6).
Figure 3-9  Neurodevelopmental protein levels in the P21 offsprings after the maternal injection of poly(I:C).
Quantified protein expression of (A) SHH, (B) PCNA and (C) SOX2 in the brains of P21 rat offspring. Poly(I:C)-exposed groups consisted of offspring rats whose mothers were subjected to administration of a dose 10 mg/kg (i.p.) of poly(I:C)(P) on days E14,16 and 18 of gestation. Control groups consisted of offspring rats whose mothers were submitted to the same treatment schedule with saline (S). SHH level was decreased in the poly(I:C)-exposed group compared to control rats. SOX2 was upregulated when observed at P21. Results are expressed as mean ± SEM. Representative western blots illustrate the data obtained from both groups and show the relevant protein and the corresponding actin. *p<0.05, **p<0.01 by unpaired student t-test.
Figure 3-10  Expression of inflammatory proteins in the P21 offsprings after the maternal injection of poly(I:C).
Quantified protein expression of (A) NFκB and (B) COX2 in the brains of P21 rat offspring. Poly(I:C)-exposed groups consisted of offspring rats whose mothers were subjected to administration of a dose 10 mg/kg (i.p.) of poly(I:C) (P) on days E14,16 and 18 of gestation. Control groups consisted of offspring rats whose mothers were submitted to the same treatment schedule with saline (S). Exposed and control rats display no difference in the levels of protein. Results are expressed as mean ± SEM. There was no significant difference between control (n=6) and poly(I:C)-treated groups (n=6).
3.5 Discussion

3.5.1 Maternal immune activation

In line with cases of infection during pregnancy, several lines of evidence indicate that the maternal immune response rather than direct infection of the foetus is responsible for the increased incidence of schizophrenia and also autism in the offspring of mothers who suffer infections during pregnancy (Patterson, 2002, Gilmore and Jarskog, 1997). This evidence is supported by studies that show behavioural and histological abnormalities in the adult offspring following exposure to either bacterial lipopolysaccharide (LPS) or doublestranded RNA, poly(I:C) similar to those seen in the offspring of infected mothers. The variability of immunological and neurological responses from using active viral agents help rationalize the use of non-replicating viral mimetics like poly(I:C) that act through the same toll-like receptor 3 (TLR3) as all other viral infections. Intravenous (i.v.) and i.p. administration of poly(I:C) are widely used as inducers of the innate immune response, which mimics the first phase of defensive mechanisms against viral infections. Systemic administration of viral mimetic is a widely accepted model of immune activation, resulting in TLR3 stimulation which then induces a robust anti-viral response including type 1 interferons α and β and also other inflammatory cytokines (Matsumoto and Seya, 2008, Alexopolou et al., 2001). For the purpose of prenatal immune activation models, the use of poly(I:C) instead of live pathogens has the advantages of avoiding the use of infectious agents within the working environment and treatments can be standardized which provides a predictable intensity and duration by appropriate dosage. This aspect facilitates an evaluation of the influence of the timing of maternal poly(I:C)-induced immune challenge on the emergence of brain and behavioural dysfunctions in the resulting offspring (Meyer et al., 2006, Meyer and Feldon, 2011).

To shed more light on the potential involvement of viruses in the etiological role during certain stages of brain development, models of early life exposure to the viral mimetic poly(I:C) was studied with regard to the long and short term effects on protein expression in the brain. We choose to mimic a viral infection instead of the most frequently used bacterial mimic agent, because viral
infections during pregnancy are common during the influenza season and appear to predispose the offspring to develop psychiatric illness (Brown et al., 2004). By using the synthetic double-stranded RNA poly(I:C) to induce maternal immune activation (MIA), we are able to study the effects of MIA on the embryo, and explore the impact and association between prenatal immune challenge and postnatal brain dysfunction. We noted no obvious malformations in the pups of poly(I:C) treated dams and no differences in body weight between the poly(I:C)-treated rats and control rats. Early reports from our laboratory has confirmed the efficiency of the poly(I:C) used in the study to activate maternal immune system since the expression of MCP-1 is increased 5 h after its injection, indicating that the maternal is eliciting an inflammatory chemokine response which may then enhance the recruitment of mast cells. Mast cells express TLR-3 and are known to be strongly activated upon treatment with poly(I:C) (Kulka et al., 2004). The chemokine MCP-1 is reported to function in CNS inflammation and injury as it has been found expressed rapidly following brain injury by both microglia and astrocytes. Apart from that, chemokines contribute to normal brain development by providing cues for the migration of newly generated neurons and glial cells and also modulate axon path-finding. In addition, MCP-1 enhances neuronal excitability and synaptic transmission in hippocampal neurons (Zhou et al., 2011).

A correlation between higher expression of MCP-1 and colonization of activated mast cells remains to be determined. Mast cells form close interactions with neurons and transfer intracellular content by transgranulation, which may modulate neuronal functions and affect CNS development (Wilhelm et al., 2005, Conti et al., 1997). However, we did not observe any changes in the level of other cytokines such as IL-1β and TNF-α in the maternal plasma (Forrest et al., 2012). The basis for the observed changes and lack of changes in the other cytokines at 5 h following the administration of poly(I:C) is in agreement with the other studies that showed a robust increase of TNF-α protein level in the maternal plasma at 2 h after exposure, decreased thereafter and below the level of detection by 6 h post injection, while plasma concentration of IL-1β did not change after poly(I:C) injection. It also has been observed at a protein and mRNA level that the concentration of IL-1β may vary during development (Fortier et al., 2004). Analysis of i.p. administration of poly(I:C) indicates the
triggering of a broad antiviral maternal innate immune activation and represents a unique response of the maternal environment and would have a variety of effects on the developing brain (Gilmore et al., 2005a). The mechanisms whereby viral infection can lead to neurodevelopmental disorders remain poorly understood. Currently it is believed that the virus can act either directly or indirectly to affect neurodevelopment. Following viral infection, the immune system becomes activated and cytokines are released, which can in turn interfere with proper neurodevelopment as already mentioned. It should be noted that although the physiological function of cytokines might be to preserve or restore homoeostasis, sustained or excessive production of cytokines could cause damage. Expression levels of a subset of cytokines and chemokines in the foetal brains somehow do not reflect the changes in concentration levels observed in maternal serum. Since only maternal serum was assessed in this study and we have not assessed the cytokine levels in the foetal and placenta, measurement of foetal brain cytokines following a similar set up of prenatal infection would provide additional information with respect to the association between their role in inflammatory responses and effects on CNS development, including effects on neuronal survival, differentiation and also excitotoxicity in developing brain (Rothwell and Hopkins, 1995, Boksa, 2010).

It should be emphasized that the effect of maternal infection on the measurement of foetal brain cytokines was complex, and is dependent on the gestational age at which poly(I:C) was administered and the time of the evaluation after administration. Findings with regard to the changes in protein levels of these cytokines in foetal brain have shown inconsistency and do not provide compelling evidence as to suggest the exact mechanism of how cytokines changes in fetal brain could have altered the function of foetal neurodevelopment (Meyer et al., 2006). However, causal links between maternal infection and the emergence of a wide spectrum of behavioural and pharmacological abnormalities in the offspring have been observed in rodent models (Boksa and Luheshi, 2003). This has led to speculation that the idea that the perturbations caused by the prenatal immunological manipulation are widespread, indicative of multiple structural brain abnormalities and also dependent on a range of normal neuropsychological functions. Therefore, it remains to be experimentally confirmed whether cytokines are the mediators
responsible for long term effects on CNS function in offspring (Shi et al., 2003, Meyer and Feldon, 2009).

3.5.2 Protein expression

Results from this study also indicate the ability of inflammatory challenges by poly(I:C) to produce neuroadaptive changes that might alter neuronal properties and function including changes in the magnitude of NMDA receptor subunit expression. This receptor is known to be an important regulator in neuronal development and function, therefore alterations in the levels of its neuronal properties could have important consequences for synaptic responses (Hunt and Castillo, 2012). To further address this issue, we have examined the effect of poly(I:C) exposure during gestation on the level of two major subunits of the NMDA receptor in the brain obtained from the embryo and also from postnatal age 21 day. The NR2A and NR2B subunits were chosen for examination in this study because they are the most predominant NMDA receptor subtypes in their respective subunit families and underlie functional excitatory synaptic transmission and activity-dependent synaptic plasticity (Cull-Candy et al., 2001, Cull-Candy and Klein, 2007). Both subunits show greater excitotoxicity than the other subunits and brain regions enriched in NMDA receptor composed of NR2A and NR2B subunits (such as hippocampus and forebrain) are commonly injured in excitotoxic insults such as ischemia, brain injury and HIV-1 infection (Waxman and Lynch, 2005, O'Donnel et al., 2006, Schumann et al., 2008). The expression of NMDA receptor subunit subtypes were significantly altered following 5 h post-exposure with poly(I:C). However, the differential changes of the subunit levels induced by poly(I:C) then reached expression levels comparable to those observed in control offspring at postnatal day 21. Our findings suggest that both NR2A- and NR2B-containing receptors would be particularly susceptible to poly(I:C)-induced damage in the embryo brains. The demonstrable low level of NR2A and robust expression of NR2B subunit suggests the likely expression of functional NR2B, since it has been shown that this subunit predominates in early development (Waxman and Lynch, 2005, Babb et al., 2005). The resulting increase in the amount of this subunit may be due to new protein synthesis in the total brain homogenates. It thus seems that the early changes to NMDA receptor subunit expression in the foetus derived from immune-challenged mothers may not have been sufficient to impair the subsequent expression of
NMDA receptor associated molecules in the brain at a later stage of postnatal development (i.e on P21). However, it should be pointed out that infection-induced disruption of early prenatal brain development may lead to long-lasting structural and functional brain abnormalities leading to the emergence of behavioural dysfunctions in later life (Fatemi et al., 2002, Meyer et al., 2008b). Although questions remain to be answered regarding the specific mechanisms of action for synaptic protein, their involvement in the regulation of patterns of synaptic connectivity has been established and suggestive evidence implicates them in schizophrenia (Cull-Candy et al., 2001, Gladding and Raymond, 2011).

Another interesting finding emerging from the present results was the expression of SHH and SOX2. The relative protein level of inductive signal SHH was initially unchanged in the brains of foetuses derived from poly(I:C)-treated mothers 5 h post-treatment, but then decreased significantly at 21 days. This effect was found in the young offspring 21 day after they were born, demonstrating that prenatal exposure to poly(I:C) can produce long-term neurochemical alterations. A related study of maternal poly(I:C) exposure on a different time point, during early/middle pregnancy (E9) found reduced foetal brain gene expression of SHH at 2, 4 and 8 days after maternal immune activation. This study provides evidence on the involvement of SHH and other related genes in the foetal dopaminergic development(Meyer et al., 2008a). SHH signalling is required during embryonic development in the regulation of cell proliferation, migration and survival and in the maintenance of cell in the adult (Bale, 2002). Evidence that strengthen the pivotal role of SHH signalling during early developmental period demonstrate the regulation of cellular proliferation for precise morphogenesis of embryonic tissue and also in the early expansion of developing midbrain and proliferation of granular cell precursors in cerebellum(Wechsler-Reya and Scott, 1999, Britto et al., 2002, Ruiz i Altaba et al., 2002). The influence of SHH signalling activation in response to other types of infection also has been noted. Expression of SHH and its downstream signalling molecules, GLi1 and its receptor Patched (Ptc) were upregulated in *Helicobacter pylori* infection and the level of SHH is elevated in gastric cancer cells. This suggests that SHH signalling pathway is one of the host signals related to cancer growth and metastasis and changes in the level of SHH expression may provide a marker for chemo preventive measures(Kim et al., 2010). In vivo studies showed that
prolonging infection with \textit{H. pylori} resulted in a different pattern of SHH protein expression in the gastric mucosa of the gerbils 51 weeks after bacterial inoculation (Suzuki et al., 2005). Both studies demonstrate that \textit{H. pylori} infection modified the expression of morphogens involved in the regulation of gastric mucosal homoeostasis. In addition, a number of researchers have shown that dysregulated activation of SHH signalling predispose cells to the development of tumours, in which overexpression of SHH have been found in many types of cancers including medullolastoma, basal cell carcinoma, lung carcinoma, adenocarcinoma of digestive tract and oral squamus cancer cell (Dahmane et al., 2001, Wechsler-Reya and Scott, 1999, Stecca and Ruiz i Altaba, 2005, Lupi, 2007, Watkins et al., 2003, Nishimaki et al., 2004). Apart from its role in patterning and the proliferation of embryonic tissue, SHH has been shown to influence the differentiation of various cell types, including dopaminergic neurons and lymphocytes (Hynes and Rosenthal, 1999). The present finding showed that maternal poly(I:C) during late gestation decreased the level of the inductive signal SHH in the P21 offspring as compared with maternal control treatment. Since the emergence of changes was demonstrated only at a postnatal age, this leads to the possibility that SHH signalling pathway abnormalities emerging after prenatal immune challenge are likely to be of developmental origin starting early in foetal life (at the time of injections) and that the pathway is influenced following maternal immune activation.

The high-mobility group transcription factor SOX2 is expressed in embryonic neural epithelial stem cells and represent the unique expression of multipotent stem and progenitor cells in neurogenic regions of the brain of the central nervous system (CNS) throughout adulthood (Brazel et al., 2005, Lefebvre et al., 2007). In this study, we showed that SOX2 protein levels was not affected by maternal poly(I:C) exposure during embryonic development, however an upregulation of SOX2 protein product in the brain of day 21 progeny of virally exposed rat was observed. SOX2 expression has been most commonly associated with non-committed stem and precursor cells in the developing nervous system. It was also being expressed in multipotent cell types which suggest a role for SOX2 in preserving developmental potential. Many SOX genes are expressed in the developing and adult brain, their expression patterns overlapping with each other since a high degree of identity are shared within the same group (Guth and
Wegner, 2008, Brazel et al., 2005). In vitro functional studies showed that the collaboration of SOX2 with the other transcriptional factors, particularly Oct4 and Nanog is required to maintain pluripotency and self-renewal in human embryonic stem cell (ESC), thus preserving developmental potential (Fong et al., 2008). Even though the SOX-B1 members can compensate for each other's loss during early development, they have distinct roles later in development (Graham et al., 2003). Thus proper levels of SOX2 are required for stem cell maintenance as either increased or decreased levels of SOX2 are used as cues for differentiation. Continuous expression of SOX2 led to an increased number of proliferating progenitors and reduction of SOX2 is associated with loss of the pluripotent state and a propensity for differentiation and regulation of target genes requiring cooperation between other critical transcriptional regulators like Oct4 and Nanog (Lefebvre et al., 2007).

As a cofactor for DNA polymerase delta and DNA replication, PCNA expression is a useful marker of cell proliferation under both normal and pathological influences. In this study, following the poly(I:C) exposure, PCNA protein levels were not significantly different than control levels as observed at E18 and P21. As PCNA expression was used to identify the population of proliferating cells and involved in the regulation of cell cycle and neurogenesis, any insult such as ischemia or infectious agents may serve as triggers to activate the cell cycle. Increased density of proliferating cells has been observed in several models of experimental CNS injury such stroke and bacterial meningitis, which postulate the idea of increased neurogenesis as a general reaction after neuronal injury of various etiology (Gerber et al., 2009). Apart from that, it has been regarded as one of either diagnostic or prognostic markers in many disease states, which include predicting the biological behaviour of different types of tumours. The higher content of PCNA positive cells in ameloblastic carcinoma samples perhaps could explain the infiltrative growth of ameloblastoma in which PCNA positivity reflects cell proliferation (Piattelli et al., 1998). However, conclusions drawn from PCNA immunohistochemical studies vary considerably, reflecting differences in case selection, sample selection, numbers of cells counted, evidence of reproducibility and methods of statistical analysis (Kurki et al., 1986, Valero et al., 2005).
We also investigated the impact of maternal immune challenge during pregnancy on the foetal and postnatal expression of several genes known to be critically involved in inflammatory related process. This included analysing the protein expression of COX2 and the transcription factor, NFκB. This served to ascertain whether putative changes in inflammatory markers might be causally related, in part to the known consequences of infection exposure in the human. It should be noted that the nature of the predominant cellular response and the inflammatory factors involved in immune and inflammatory process is extensive, thus the presence of enhanced expression of MCP-1 reflects a general pro-inflammatory status in the maternal environment following poly(I:C). However, poly(I:C)-induced immunological challenges in rodents are short lived, ranging from 24 to 48 h depending on the dose (Meyer and Feldon, 2011). As the peak of detection of protein and mRNA levels of cytokines may vary following stimulation and our results showed no changes to other inflammatory cytokines such as IL-1β and TNF-α in maternal compartment. We suggest that there was no ongoing inflammatory activity that could exaggerate inflammatory responses in which we have noted no evidence of changes in the levels of expression for NFκB and COX-2 in the embryo brain or young adult offspring P21. Although there was no demonstrable difference in immunoreactive of both markers in the fetal and postnatal brain, low steady state levels of COX2 and NFκB are observed throughout the brain. Steady state of COX2 appears to be regulated by normal glutamatergic synaptic activity in the adult brain (Kaufmann et al., 1997). In this regard, endogenous synaptic transmission also might be capable of regulating NFκB activity (Meffert and Baltimore, 2005).

The widespread distribution in the CNS indicates that NFκB may be sensitive to synaptic activation as expression of this gene is increased in hippocampal granule cells after LTP (Meberg et al., 1996). The presence of extracellular signals such cytokines, bacteria, viral proteins or mitogen causes the activation of NFκB with rapid translocation to the nucleus and the transcriptional activation of target genes. A common sign of NFκB-activating conditions is that they either represent a direct threat to the cells or that they are endogenous signals produced in response to pathogens (Baeuerle and Henkel, 1994). The activation of inflammatory transcription factors within the brain is a step that occurs before the expression of target genes, which trigger brain-intrinsic
responses. This phenomenon can be assessed by measuring the expression of markers and their activation. It is well known that NFκB is one of the early markers of inflammatory activation in the brain, and their nuclear translocation can be induced by TNF/IL-1 and IL-6 as shown in previous studies (Dharane et al., 2010). Corresponding to the rather negligible levels of this pro-inflammatory cytokine in the maternal plasma, no clear changes in the expression levels of NFκB was observed either at 5 h post treatment (embryo brain) or at later developmental age of P21.

Although COX2 has limited constitutive neuronal distribution in the CNS, it can be induced in the mammalian brain under a variety of physiological and pathological conditions (Yamagata et al., 1993). For example, a marked elevation of COX2 mRNA levels by kainic acid-induced seizure was detected in the hippocampal CA1-1 pyramidal cell layers, granule layers of DG, cortex and amygdale. Pronounced increases in brain derived neurotrophic factor (BDNF) mRNA has also been noted in the dentate granule cell layer and pyramidal layers. It has been suggested that marked expression of BDNF in rat brain may occur as a trophic response to the neuronal injury caused by kainic acid-induced seizures (Hashimoto et al., 1998). Injuries to the brain activate multiple cellular pathways, some of which are adaptive, while others may persist and become harmful. The severity of neural injury appears to correlate to the degree and duration of some of these inflammatory markers. In agreement with this, increased COX2 expression has been observed with several neurodegenerative disorders such as AD, ALS and PD and also in traumatic brain injury and seizures (Nogawa et al., 2003, Almer et al., 2001, Teisman et al., 2003, Joseph et al., 2006). The present results show no marked changes in the expression of pro-inflammatory cytokines (TNF-α and IL-1β), the inducible form of enzymes for PGE2 (COX2) and the transcription factor (NFκB) following maternal poly(I:C) treatment. Thus, our study showed that repeated challenges do not appear to induce an inflammatory response distinct from that induced by a single challenge and are insufficient to produce any lasting impairment in the inflammatory pathway.
3.6 Conclusion

Changes in behaviours relevant to psychiatric disorders such as schizophrenia and autism are consistently reported following maternal challenge in the mouse or rat, and the MIA model to study prenatal risk factors for these disorders is the focus of much scientific research (Boksa, 2010, Smith et al., 2010). The mechanisms by which MIA disrupts foetal development and sets into motion an atypical trajectory of brain and behavioral development and maturation are largely unknown. It is therefore possible that the induction of cytokines and downstream effects of such activation in the foetal brain following MIA interferes with neurodevelopmental processes or functioning within the brain regions essential to social behaviours (Meyer, 2013, Meyer et al., 2009). It should be pointed out that the current study did not examine the anatomical and behavioural consequences of maternal poly(I:C). Based on the current findings, it is not possible to draw any conclusions as to relate the mechanisms by which poly(I:C)-induced MIA leads to subsequent behavioral changes. In this aspect, a more complete behavioural phenotype of offspring born to poly(I:C)-exposed dams is required to determine the specific behavioural effects of MIA. Additionally, the mechanism by which MIA results in atypical performance on behavioural tasks is unclear and may be addressed by the inclusion of neuroanatomical studies. It is important to note that regulation of expression of molecules used both during early development and in other processes later in life is likely to play a major role in the correct processing of the molecule’s functions. Differential regulation, possibly involving different regulatory molecules or mechanisms, occurs at different stages of life and could be responsible for different phenotypes in different developmental periods. The present study was conducted with the aim to characterize the effects of the infection on CNS function in the offspring at two points of developmental stages (embryo and P21). Assessing acute neurochemical changes in foetal brain are of importance in understanding mechanisms that underlie persistent or longer term CNS changes in the offspring following maternal infection.

In summary, the study carried out showed some evidence that disturbances caused by viral infection during prenatal period of life can produce some neuroadaptive changes that alter neuronal function and development. Some of
the developmental markers showed changes during the period of the insult and disappeared or diminished as animal grew up. This may be an outcome of the compensatory capacity of the CNS to alleviate such changes. In relation to this, changes seen in the expression of the NMDA receptor subunits, NR2A and NR2B could imply that alterations in the receptor number and function may impinge on the formation and establishment of neuronal circuits and connections. The present study demonstrates that poly(I:C) challenge in pregnant dams results in transient alterations in the levels of NMDA receptor subunit NR2A and NR2B in the fetal brain, representing a potential mechanism for altering synaptic development and may contribute to abnormal brain development and risk for neurodevelopmental disorders. On the other hand it is also possible that prenatal poly(I:C) treatment manifested its effects on the postnatal brain independently from its effect at the time of exposure. The acute inflammatory response induced by prenatal poly(I:C) administration somehow induced further long term changes in factors regulating cell proliferation and migration, in order to produce altered neurogenesis in offspring later in development at P21. We show that changes in markers that are involved in neurogenesis (SOX2 and SHH) were observed at a later stage of postnatal development (P21). This could imply that prenatal immune activation elicited by poly(I:C) in rodents may have long term effects on neurogenesis in young postnatal offspring. Although we did not include behavioural assessment and immunolabelling studies, previous data together with current findings suggest such deficits or changes in neurogenesis could conceivably contribute to the alterations in hippocampal-mediated function that have been observed in juvenile or adult rodents following prenatal exposure to poly(I:C) (Meyer et al., 2008b). However the results presented here support the association between immunological manipulation at a specific gestational age and postnatal brain dysfunctions. Unravelling the mechanisms that mediate immunologically sensitive developmental periods that allow for programming of adults’ responses deserves further attention in which it permits possibility for intervention. Taken together, our animal models indicate that distinct neurochemical abnormalities are manifested at different stages of postnatal development after prenatal exposure to viral-like immune challenge during late gestation in rats.
4 The effect of prenatal inhibition of the kynurenine metabolism pathway on the expression of proteins in the rat brains

4.1 Introduction

It has been suggested that changes in the endogenous levels of neuroactive metabolites of the kynurenine pathway are implicated in the pathogenesis of several brain disorders, for instance Parkinson’s disease and schizophrenia. These neuroactive metabolites include KYNA, 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN). The basis for kynurenine pathway up-regulation as seen in psychotic disorders may not reside within one particular enzyme but rather with one of many factors that control the expression of the initiating enzymes involved in the pathway. There is evidence that emphasize the unique antagonistic properties of KYNA as an endogenous factor which is capable of blocking the neurotoxic and seizures caused by QUIN which support the fact that manipulation favouring its accumulation in the brain extracellular space may be beneficial against endogenous excitotoxic abnormalities (Foster et al., 1984). Pharmacological blockade of the enzyme kynurenine-3-monoxygenase (KMO) which is responsible for the formation of 3-hydroxykynurenine is one of the ways of influencing the level of kynurenine in the brain (Stone and Addae, 2002).

For the purpose of this study, a novel compound 3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]benzenesulfonamide or better known as Ro61-8048 which exerts high activity as a selective inhibitor of enzyme KMO was used. The aim was to provide a better understanding of the actions of this inhibitor in the central nervous system and to assess the possible mechanism of action. We examined protein expression of selected markers in brain homogenates of offspring whose mother had been administered at 3 time points (E14, 16 and 18) with compound Ro61-8048 at 100 mg/kg. These markers included the NMDA receptor subunits NR2A and NR2B, NMDA receptor post synaptic density protein PSD95, type A ephrin receptor EphA4, inflammatory markers NFkB and COX2, sonic hedgehog (SHH), stem cell marker SOX2 and proliferation marker PCNA. The protein level of these markers was examined by using western blots and relative protein levels are shown after normalization to corresponding actin
levels on each lane. We explored differential protein expression profiles in the rat brain at different stages of postnatal development, whole brain at postnatal day 21 (P21) and regional expression in hippocampus, cerebellum, cortex and midbrain in postnatal day 60 (P60) rat. Protein expression in the embryo brains following administration of KMO inhibitor, Ro61-8048 was also examined at 5 h and 24 h after treatment. Apart from that, the ability of the administration of the novel compound 3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]benzenesulfomide or known as Ro61-8048 during gestation in altering the balance of kynurenine pathway through reducing kynurenine pathway flux to QUIN, either to cause a rise in brain KYNA levels or decreased formation of QUIN has been previously determined by our colleague, Dr Forrest C.M. (HPLC results shown in (Forrest et al., 2013a).

4.2 Research aims

What is the influence of early modulations to the kynurenine metabolism pathway on the functional expression of proteins important to brain development and to what extent does kynurenine-3-monooxygenase inhibitor (Ro61-8048) play a role in reducing kynurenine pathway flux? To address this issue we set out our aim to study the role of compound Ro61-8048 in influencing the regulation of normal neuronal development through modulation of the kynurenine pathway on the level of protein expressions in the embryo and subsequent changes in the brain development of offspring at P21 and P60. The aim was also to characterize the effects of the KP inhibition on CNS function in the offspring at various stages of development.

4.3 Experimental approaches

Our experimental strategy is to use an animal model similar to the protocol established for maternal immune activation by employing the western blotting technique to examine protein expression of selected markers that play a key role in neuronal development, synaptic plasticity, axon guidance and neurite outgrowth. For the first phase of the study, investigations into the effect of administration of compound Ro61-8048 during gestation on selected molecular
targets of neurodevelopmental relevance was carried out in the embryo brains at two time points, 5 hour and 24 hour post-injection of Ro61-8048 on embryonic day 18. A later phase of the project involved an examination of the effect of prenatal inhibition of kynurenine pathway on E14/16/18 in terms of changes to the protein expressions of a selected panel of neurodevelopmental targets in the offspring at postnatal day 21 and 60. It is important to determine whether any alterations in protein expression at an early stage of development either in embryo or at young age P21 would persist into adulthood.

4.3.1 Animal treatment regimens

4.3.1.1 Acute administration of Ro61-8048

This experiment was set up for the purpose of assessing the immediate effects of gestational modulation of the kynurenine pathway on the levels of protein expression in the embryo brains measured 5 h and 24 h post administration. For this purpose, gestational day 18 timed-pregnant rats were assigned to receive i.p. injections of the following treatment regimens: (1) 5 h group: saline (0.9% NaCl) and (2) 5 h group: 100mg/kg Ro61-8048. After the injections the animals were left undisturbed for 5 hours and then anesthetized with an overdose of sodium pentobarbital and decapitated after blood was obtained by cardiac puncture. The blood was collected in a heparinised vacutainer tube and kept ice cold. The animals were dissected and the uterine horn containing embryonic day 18 pups were surgically removed. Foetal whole brains were dissected and immediately frozen on dry ice. In another set of experiment, two groups of pregnant rats were injected at gestational day 18 with single injection of vehicle solution and compound Ro61-8048 (100mg/kg), respectively. Twenty- four hours later, the animals were sacrificed and dissected to obtain foetal whole brains along with the mother’s brain. In addition, this set up was partly to demonstrate the effect of selected dosages in producing changes in the levels of several metabolites of the kynurenine pathway through HPLC analysis. The levels of kynurenine and kynurenic acid were measured by high performance liquid chromatography (HPLC) with electrochemical detection as previously described (HPLC data not shown). HPLC analysis was performed by Dr Forrest C.M. (Forrest et al., 2013a).
4.3.1.2 Postnatal day 21 and 60

For gestational treatment, groups of pregnant dams were administered intraperitoneally (i.p.) with compound Ro61-8048 (100mg/kg) at the dose indicated on days E14, E16 and E18 in order to extend the temporal impact of kynurenine pathway modulation on the embryos, with no apparent ill effects on the dam or her behaviour towards her litter after birth. Group of pregnant dams were injected on E14, 16 and 18 between 9am and 11am to minimise variation in responses. Control females were injected with the same volume of 0.9% NaCl. Gestation was allowed to proceed normally and the offspring were undisturbed until weaning on P21 when they were taken from the home cage for euthanasia followed by removal of the brain. Each brain was divided into the two cerebral hemispheres and frozen immediately on dry ice before being transferred to storage at -80°C until required for analysis.

In a separate experiment to evaluate the extended effect of prenatal exposure of compound Ro61-8048 in more mature animal, the neonates were allowed to survive until around 60 days of age (young adult) at which time all litters were euthanized and their brains were removed. The brain was then sectioned into four main regions i.e. hippocampus, cerebellum, cortex and midbrain and preserved for further western blot (WB) analysis.

4.3.2 Western blot analysis

Tissue samples were prepared as described in the chapter 2. Briefly, samples were homogenized in RIPA buffer containing protease inhibitors and total protein concentration was determined in the resulting supernatant using the Bio-Rad Coomassie Blue Protein assay. Samples were normalised to 10-20µg per lane and subjected to 4-12% Bis-Tris gel electrophoresis and run at 150V for 80 min with SeeBlue pre-stained as standard. Each gel contained samples from three separate litters (two pups from each litter), n=6. Proteins were separated according to their molecular weight and transferred onto PVDF membrane and blocked in 5% non-fat milk/TBST and then incubated (overnight at 4°C) with the primary antibodies (see Antibodies section in chapter 2, Table 2-5). Membranes were then washed 3 times for 15 min in TBST and incubated for 1 h at room temperature with the appropriate secondary antibody (prepared in 5%
milk/TBST). After incubation, blots were washed 3 times for 15 min and developed using enhanced chemiluminescence plus detection kit and immediately exposed to film.

The measurement of protein level for the selected molecular targets for western blot analysis includes NMDA receptor subunits NR2A and NR2B, an ephrin receptor EphA4, NMDA receptor anchoring protein post synaptic density (PSD95), inflammatory markers NFkB and COX2, the key signalling molecule for organogenesis sonic hedgehog (SHH), proliferating cell nuclear antigen (PCNA) as a proliferation marker and stem cell marker SOX2. Actin was used as a control for transfer and loading and was unchanged throughout the experiment.

**4.3.3 Data analysis and statistics**

Immunoreactive bands for all the markers were analysed using ImageJ software. To control for accuracy of tissue loading and efficiency of transfer, all sample bands were examined after staining with Ponceau S stain. Data were normalised to actin detected on the same blots. The final data are expressed in standard protein unit and presented as a ratio of [intensity of target protein]/[intensity actin]. The data were analysed statistically using unpaired $t$ tests to examine differences between groups of pups born to mothers treated with Ro61-8048 and groups born to mothers injected with saline. A $p$ value $<0.05$ was considered significant and actual $P$ values are indicated when $>0.0001$. All data are presented as means ± SEM.

**4.4 Results**

Studies on the effects of inhibitors of enzymes particularly enzyme kynurenine-3-monooxygenase (KMO) in the kynurenine pathway are interesting, since such molecules could on the one hand increase kynurenine availability and kynurenic acid (KYNA) production and on the other, decrease the synthesis of 3-hydroxykynurenine and of QUIN, 2 toxic compounds which originates from a different pathway of kynurenine metabolism.
To examine whether a single or triple injections of Ro61-8048 during the late gestational period influenced the level of expressions of several proteins important to brain development, either acutely or chronically in young and adult animals, we performed semi-quantitative western blot on brain tissue collected either at 5 h and 24 h after injection (acute) or on P21 whole brain (chronic). Another set of animals using much older animals at P60 is important to determine whether any changes seen in developing brains would be extended to a later age. The effect of each marker might be masked in the whole brain, thus we hope to able to pick up any subtle changes in regional samples of P60 which includes hippocampus, cerebellum, cortex and midbrain.

4.4.1 Effects of prenatal modulation of kynurenine pathway on embryonic brains following prenatal exposure to Ro61-8048

In order to provide a better understanding of the actions of this inhibitor in the central nervous system and to assess the possible mechanism of action, we examined the protein expression of selected markers, namely NMDA receptor subunits NR2A and NR2B, NMDA receptor post synaptic density protein PSD95, type A ephrin receptor EphA4, inflammatory markers NFKB and COX2, sonic hedgehog (SHH), stem cell marker SOX2 and proliferation marker PCNA in the embryo brains measured after 5 h and 24 h. The levels of the protein expression in all the markers were determined by immunoblot analysis with the specific antibody as listed in the chapter 2 (Table 2-5). The protein level of these markers was examined by using western blot and relative protein levels are shown after normalization to corresponding actin on each lane. Levels of other proteins, in this case, actin were not significantly modified for the duration of the treatment.

4.4.1.1 Effects of prenatal Ro61-8048 on embryonic brains after 5 h exposure.

The protein expression profile of NR2A-and NR2B-containing NMDA receptor subtypes and one of the NMDA receptor-interacting proteins, PSD95 following maternal administration of Ro61-8048 was examined in the embryo brains. For both NMDA receptor subunits and PSD95, immunoreactive bands corresponding to
molecular masses of 180kDa and 95kDa, respectively, were observed in brain homogenates. In the embryo brains, an obvious reduction in the expression of NR2A subunit (Figure 4-1 (A), \( p=0.005 \)), and a significant increase in expression of the other subunit NR2B (Figure 4-1 (B), \( p<0.0001 \)) were observed at the early stage following 5 hour post administration of Ro61-8048. There were no significant changes in the level of expression for PSD95 (Figure 4-2 (A), \( p=0.10 \)), a major postsynaptic protein interacting with the C-terminal domains of the NR2 subunits.

Antibodies directed against the ephrin receptor type A (EphA4), sonic hedgehog (SHH), proliferating cell nuclear antigen (PCNA) and sex determining region of Y-chromosome related-HMG box 2 (SOX2) all detected single prominent bands at the appropriate molecular weights (120kDa for EphA4, 45 kDa for SHH, 36 kDa for PCNA and 34 kDa for SOX2). We have performed western blot analysis to determine whether protein expression of the molecules that have important roles in several aspect of neuronal development particularly neurogenesis processes, guidance of migrating cells and neuronal growth cones to specific destinations and synapse formation. Involvement of EphA4, one of the Eph receptor tyrosine kinases is seen as a key player in controlling cell movements in many tissues and at multiple stages of patterning that underlie the establishment, maintenance and remodelling of cellular organisation. However, level of EphA4 protein revealed no changes (Figure 4-2 (B), \( p=0.34 \)) after 5 h prenatal administration of Ro61-8048. The groups treated with Ro61-8048 also produced no significant changes in the levels of the rest of these proteins. As shown in Figure 4-3 (A), western blot analysis of the embryo brain lysate by using anti-SHH antibody showed that regulation of precursor form of SHH protein was not affected by single administration of Ro61-8048 during late gestation (\( p=0.57 \)). PCNA is an auxiliary protein of DNA polymerase delta necessary for DNA replication and its expression has been shown to elevated during G1/S cell cycle phase (Kurki et al., 1986). As shown in Figure 4-3 (B), strong signals were seen in whole embryo lysates. Our immunoblot analysis showed that PCNA expression was not changed following 5 h of Ro61-8048 treatment (Figure 4-3 (B), \( p=0.73 \)). Five hour after treatment with KMO inhibitor also made no differences in the levels of SOX2 protein although this protein was found to be strongly expressed in the brain homogenates prepared from foetal brain (Figure 4-3 (C), \( p=0.48 \)).
We detected immunoreactive NFKB p65 subunit and COX2 expression in embryo as shown in Figure 4-4 (A & B). However, no changes in immunoreactive levels of both markers were observed after 5 h injection with the enzyme inhibitor, Ro61-8048 (NFKB: p= 0.53; COX2: p= 0.51).
Figure 4-1  Expression of NMDA receptor subunits NR2A and NR2B proteins in the embryo brains after prenatal Ro61-8048.
Pregnant dams were administered with Ro61-8048 i.p. on embryonic day 18 (E18) and 5 h later, embryo brains were obtained. Homogenized tissues of the brains from 6 embryos in each group were used for analysis. Blots were probed for NR2A and NR2B as marked, showing the presence of NR2A/NR2B containing complexes in the whole brain tissue. Representative immunoblots showing protein expression levels of NR2A (M.W. 180 kDa) and NR2B (M.W. 180 kDa) in the embryo brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NR2A and NR2B protein expression normalized to actin expression. Data were expressed as mean ± SEM. (A) Compared with saline-treated animals(S) (n=6), there was a significant decrease in NR2A protein expression in Ro61-8048 (R) (n=6)(**p<0.01). (B) Prenatal treatment with 100mg/kg Ro61-8048 showed significant increase in NR2B expression compared to control animals (n=6)(***p<0.001).
Figure 4-2  Expression of PSD95 and EphA4 proteins in the embryo brains after prenatal Ro61-8048.

Pregnant dams were administered with Ro61-8048 i.p. on embryonic day 18 (E18) and 5 h later, embryo brains were obtained. Homogenized tissues of the brains from 6 embryos in each group were used for analysis. Blots were probed for PSD95 and EphA4 as marked, showing the presence of PSD95 and EphA4 containing complexes in the whole brain tissue. Representative immunoblots showing protein expression levels of PSD95 (M.W. 95 kDa) and EphA4 (M.W. 120 kDa) in the embryo brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative PSD95 and EphA4 protein expression normalized to actin expression. Data were expressed as mean ± SEM. The effect of the treatments were determined by performing student t-test and the differences between mean values were defined as significant when p<0.05.
Figure 4-3  Expression of neurodevelopmental proteins in the embryo brains after prenatal Ro61-8048.

Pregnant dams were administered with Ro61-8048 i.p. on embryonic day 18 (E18) and 5 h later, embryo brains were obtained. Homogenized tissues of the brains from 6 embryos in each group were used for analysis. Representative immunoblots showing protein expression levels of SHH (M.W. 45 kDa), PCNA (M.W. 36 kDa) and SOX2 (M.W. 34 kDa) in the embryo brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative SHH, PCNA and SOX2 protein expression normalized to actin expression. Data were expressed as mean ± SEM. The effect of the treatments were determined by performing student t-test and the differences between mean values were defined as significant when p<0.05.
Figure 4-4  Expression of inflammatory proteins in the embryo brains after prenatal Ro61-8048.

Pregnant dams were administered with Ro61-8048 i.p. on embryonic day 18 (E18) and 5 h later, embryo brains were obtained. Homogenized tissues of the brains from 6 embryos in each group were used for analysis. Representative immunoblots showing protein expression levels of NFκB (M.W. 65 kDa) and COX2 (M.W. 72 kDa) in the embryo brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NFκB and COX2 protein expression normalized to actin expression. Data were expressed as mean ± SEM. The effect of the treatments were determined by performing student t-test and the differences between mean values were defined as significant when p<0.05.
4.4.1.2 Effects of prenatal Ro61-8048 on embryonic brains after 24 h exposure.

It has been shown previously that single administration of Ro61-8048 at E18 was enough to significantly affect both subunits by a 43% reduction (NR2A) and an almost 400% increase in the level of NR2B protein expression following 5 h post injection. These changes were no longer observed by 24 h of treatment. It can be seen that after the first time point, the expression remained slightly lower compared to controls for the NR2A subunit (Figure 4-5 (A), \( p=0.49 \)) while the NR2B content returned to almost control levels after 24 h of treatment (Figure 4-5 (B), \( p=0.09 \)). Twenty four hour after treatment also had no effect on the level of PSD95, a major postsynaptic protein which interacts with the C-terminal domain of the NR2 subunits (Figure 4-6 (A), \( p=0.93 \)). Similar effect was also observed in the level of EphA4 protein in the whole cell extracts from the brain at 24 h (Figure 4-6 (B), \( p=0.60 \)).

The activity of SHH as a morphogen during CNS development involved regulating the patterning of cellular responses that is controlled by the concentration and duration of SHH exposure. The expression pattern of SHH is the result of the combined activity of enhancers that control SHH transcription and regulate SHH expression in different tissue (Traiffort et al., 1998). In order to further analyze the potential roles exerted by SHH signalling in the nervous system of foetal following gestational inhibition of kynurenine pathway, we investigated developmental characteristic of SHH expression in whole cell lysates prepared from embryo brain. As shown previously in Figure 4-3 (A) we show that the time course of Ro61-8048 mediated regulation of SHH protein expression revealed no alteration 5 h following single administration of the compound. Interestingly, when we examined the level of SHH protein 24 h after Ro61-8048 treatment, a significant increase of the protein level was observed (Figure 4-7 (A), \( p=0.04 \)). To further assess whether inhibition of the kynurenine pathway during gestation affects expression of intrinsic proliferation marker, the levels of PCNA protein in the Ro61-8048-treated group was compared with controls. Our immunoblot analysis showed that PCNA expression was not changed following 24 h of Ro61-8048 treatment (Figure 4-7 (B), \( p=0.18 \)). As SOX2 might represent a useful in vivo marker for neural progenitor cells in the CNS, we examined the expression
patterns of SOX2 protein in the embryo 24 h after maternal administration of KMO inhibitor. Western blot analysis with goat anti-SOX2 antibody of total protein in the brain homogenate resulted in a single band of 34 kDa. The antibody did not cross react with other SOX-B1 subfamily members i.e. SOX1 and SOX3. Thus the detection capability of this antibody allowed us to carefully examine SOX2 expression in the subsequent sets of experiments. As can be seen in Figure 4-7 (C), SOX2 was found to be strongly expressed, although no change was seen in the expression of this protein after 24 h post treatment with Ro61-8048 ($p = 0.60$). Five hour after treatment with Ro61-8048 had no significant effect on the level of inflammatory markers used in this study. However, prenatal Ro61-8048 treatment induced strong immunoreactivity for NFkB in the embryo brain 24 h post injection (Figure 4-8 (A), $p=0.01$) with no differences in COX2 protein level (Figure 4-8 (B), $p = 0.55$).
Figure 4-5  Expression of NMDA receptor subunits NR2A and NR2B proteins in the embryo brains 24 hour after prenatal Ro61-8048
Pregnant dams were administered with Ro61-8048 i.p. on embryonic day 18 (E18) and 24 h later, embryo brains were obtained. Homogenized tissues of the brains from 6 embryos in each group were used for analysis. Representative immunoblots showing protein expression levels of NR2A (M.W.180 kDa) and NR2B (M.W. 180 kDa) in the embryo brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NR2A and NR2B protein expression normalized to actin expression. Data were expressed as mean ± SEM. The effect of the treatments were determined by performing student t-test and the differences between mean values were defined as significant when \( p<0.05 \).
Figure 4-6  Expression of PSD95 and EphA4 proteins in the embryo brains 24 hour after prenatal Ro61-8048.

Pregnant dams were administered with Ro61-8048 i.p. on embryonic day 18 (E18) and 24 h later, embryo brains were obtained. Homogenized tissues of the brains from 6 embryos in each group were used for analysis. Blots were probed for PSD95 and EphA4 as marked, showing the presence of PSD95 and EphA4 containing complexes in the whole brain tissue. Representative immunoblots showing protein expression levels of PSD95 (M.W. 95 kDa) and EphA4 (M.W. 120 kDa) in the embryo brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative PSD95 and EphA4 protein expression normalized to actin expression. Data were expressed as mean ± SEM. The effect of the treatments were determined by performing student t-test and the differences between mean values were defined as significant when p<0.05.
Figure 4-7  Expression of neurodevelopmental proteins in the embryo brains 24 hour after prenatal Ro61-8048.
Pregnant dams were administered with Ro61-8048 i.p. on embryonic day 18 (E18) and 24 h later, embryo brains were obtained. Homogenized tissues of the brains from 6 embryos in each group were used for analysis. Blots were probed for SHH, PCNA and SOX2 as marked, showing the presence of SHH, PCNA and SOX2 containing complexes in the whole brain tissue. Representative immunoblots showing protein expression levels of SHH (M.W. 45 kDa), PCNA (M.W. 36 kDa) and SOX2 (M.W. 34 kDa) in the embryo brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative protein expression of these proteins normalized to actin expression. Data were expressed as mean ± SEM. (A) Prenatal treatment with 100mg/kg Ro61-8048 (R) showed significant increase in SHH expression compared to control animals (S) (n=6)(*p<0.05).
Figure 4-8  Expression of inflammatory proteins in the embryo brains 24 hour after prenatal Ro61-8048.
Pregnant dams were administered with Ro61-8048 i.p. on embryonic day 18 (E18) and 24 h later, embryo brains were obtained. Homogenized tissues of the brains from 6 embryos in each group were used for analysis. Blots were probed for NFkB and COX2 as marked, showing the presence of NFkB and COX2 containing complexes in the whole brain tissue. Representative immunoblots showing protein expression levels of NFkB (M.W. 65 kDa) and COX2 (M.W. 72 kDa) in the embryo brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative protein expression of these proteins normalized to actin expression. Data were expressed as mean ± SEM. (A) Prenatal treatment with 100mg/kg Ro61-8048 (R) showed significant increase in NFkB expression compared to control animals (S) (n=6) (*p<0.05).
4.4.2 Effects of prenatal Ro61-8048 in P21 neonates

In addition to the expression of proteins studied for the acute response to Ro61-8048 in embryos, the levels of expression were also examined at P21. In the brains from P21 pups exposed to Ro61-8048, the greatest changes noted in these animals were of the NMDA receptor subunits, NR2A and NR2B. The expression of both subunits NR2A and NR2B showed a highly significant upregulation producing approximately 16% (Figure 4-9 (A), p = 0.0045) and 27% (Figure 4-9 (B), p = 0.03) increases, respectively. Analysis of the levels of both subunits showed a progressive increase in the full-length proteins, indicative of both NR2 subunits being regulated in a time-dependent way. We also characterized the expression of PSD95, a major postsynaptic protein interacting with the C-terminal domains of the NR2 subunits. Five and 24 hours after maternal administration of Ro61-8048 had no effect on the level of this protein. However, at P21 subsequently altered the development of PSD95 protein expression, Ro61-8048-treated animals had significantly greater expression than saline controls (Figure 4-10 (A), p = 0.03). This finding altogether suggests an influence on the kynurenine pathway and involvement of NMDA receptor function to maintaining normal regulation during brain development. During postnatal period as examined at P21, expression of the EphA4 receptor for secreted ephrin molecules remained unchanged in the treated pups (Figure 4-10 (B), p = 0.95).

The sonic hedgehog protein (SHH), which is intimately involved in the patterning and orientation of tissues and cell growth, was reduced to a significant extent after exposure to Ro61-8048 (Figure 4-11 (A), p = 0.02). To further assess whether inhibition of the kynurenine pathway during gestation affects expression of intrinsic proliferation markers at a later developmental age, PCNA, a protein often studied as a marker of the earliest phases of DNA synthesis, was more highly expressed in the treated animals compared with controls (Figure 4-11 (B), p = 0.02) while levels of the SOX2 protein were unaffected (Figure 4-11 (C), p = 0.45). Finally, levels of the inflammation transcription factor, NFκB and the inducible form of cyclooxygenase (COX2), showed a significant induction of NFκB reactivity in the total homogenates of P21 brains (Figure 4-12 (A), p = 0.02) with no change in COX2 level as observed in treated animals (Figure 4-12 (B), p = 0.64).
Figure 4-9  Expression of NMDA-receptor proteins in the brains of P21 offspring after prenatal Ro61-8048. Pregnant dams were administrated with Ro61-8048 i.p. on gestation days E14, E16 and E18, after which parturition proceeded naturally and the young were allowed to develop up to the time of weaning at postnatal day 21 (P21). The brains were then removed and frozen immediately until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Blots were probed for NR2A and NR2B as marked, showing the presence of NR2A/NR2B containing complexes in the whole brain tissue. Representative immunoblots showing protein expression levels of NR2A (M.W. 180 kDa) and NR2B (M.W. 180 kDa) in the P21 brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NR2A and NR2B protein expression normalized to actin expression. Data were expressed as mean ± SEM. (A) Compared with saline-treated animals (n=6), there was a significant decrease in NR2A protein expression in Ro61-8048 (n=6)(**p<0.01). (B) Prenatal treatment with 100mg/kg Ro61-8048 (R) showed significant increase in NR2B expression compared to control animals (S) (n=6)(*p<0.05).
Figure 4-10  Expression of PSD95 and EphA4 in the brains of P21 offspring after prenatal Ro61-8048.
Pregnant dams were administered with Ro61-8048 i.p. on gestation days E14, E16 and E18, after which parturition proceeded naturally and the young were allowed to develop up to the time of weaning at postnatal day 21 (P21). The brains were then removed and frozen immediately until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Blots were probed for PSD95 and EphA4 as marked, showing the presence of PSD95 and EphA4 containing complexes in the whole brain tissue. Representative immunoblots showing protein expression levels of PSD95 (M.W. 95 kDa) and EphA4 (M.W. 120 kDa) in the P21 brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative PSD95 and EphA4 protein expression normalized to actin expression. Data were expressed as mean ± SEM. (A) Compared with saline-treated animals (S) (n=6), there was a significant increase in PSD95 protein expression in Ro61-8048 (R) (n=6) (p<0.05).
Figure 4-11 Expression of neurodevelopmental proteins in the brains of P21 offspring after prenatal Ro61-8048.

Pregnant dams were administered with Ro61-8048 i.p. on gestation days E14, E16 and E18, after which parturition proceeded naturally and the young were allowed to develop up to the time of weaning at postnatal day 21 (P21). Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Representative immunoblots showing protein expression levels of SHH (M.W. 45 kDa), PCNA (M.W. 36 kDa) and SOX2 (M.W. 34 kDa) in the P21 brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative neurodevelopmental protein expressions normalized to actin expression. Data were expressed as mean ± SEM. (A) Compared with saline-treated animals (n=6), there was a significant decrease in SHH protein expression in Ro61-8048 (n=6) (*p<0.05). (B) Prenatal treatment with 100mg/kg Ro61-8048(R) showed significant increase in PCNA expression compared to control animals(S) (n=6) (*p<0.05).
Figure 4-12  Expression of inflammatory proteins in the brains of P21 offspring after prenatal Ro61-8048.

Pregnant dams were administered with Ro61-8048 i.p. on gestation days E14, E16 and E18, after which parturition proceeded naturally and the young were allowed to develop up to the time of weaning at postnatal day 21 (P21). The brains were then removed and frozen immediately until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Blots were probed for NFκB and COX2 as marked, showing the presence of NFκB and COX2 containing complexes in the whole brain tissue. Representative immunoblots showing protein expression levels of NFκB (M.W. 65 kDa) and COX2 (M.W. 72 kDa) in the P21 brains, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NFκB and COX2 protein expression normalized to actin expression. Data were expressed as mean ± SEM. (A) Compared with saline-treated animals(S) (n=6), there was a significant increase in NFκB protein expression in Ro61-8048(R) (n=6) (*p<0.05).
4.4.3 Effects of prenatal Ro61-8048 in P60 offsprings

Another set of animals using much older animals at postnatal day 60 is important to determine whether any changes seen in developing brains would be extended to a later age. The effect of each marker might be masked in the whole brain in which the juvenile study was erformed on whole cerebral hemispheres, thus we hope to be able to pick up any subtle changes in regional samples of P60 that includes hippocampus, cerebelum, cortex and midbrain. The range of proteins examined in this aspect was similar to that targetted in the previous set up of animals at P21 days of age.

4.4.3.1 Protein expression in the hippocampus

In the P60-treated groups the effect of maternal administration of Ro61-8048 on the level of NR2A subunit protein expression was markedly reduced (Figure 4-13(A), p=0.003) whereas NR2B level was not affected (Figure 4-13 (B), p=0.69). By adulthood as measured regionally in hippocampus, there was no longer effect on PSD95 protein expression as seen in Figure 4-14 (A) (p=0.14). In view of the role of NMDA receptors for both synaptic development and function, the expression of both subunits, NR2A and NR2B and one of its post synatic protein complex, PSD95 also had been observed to be significantly affected at P21. This suggest that the levels of several proteins that are influential to NMDA receptor function are dynamically influenced by the kynurenine pathway. Similarly, no significant difference was evident by the age of P60 by the the proinflammatory transcription factor, NFκB (Figure 4-16 (A), p= 0.25) and PCNA (Figure 4-15(B), p= 0.19) in which changes seen at P21 had been resolved or compensated by adulthood. While in other cases, proteins which had shown no change at P21 remained unchanged at P60. These included the axon guidance and synapse formation receptor, EphA4 (Figure 4-14(B), p=0.70) and one of the importany enzyme that accompanies tissue inflammation, COX2 (Figure 4-16(B), p= 0.43). There was eminently differences in the expession of SHH, a protein concerned with cell differentiation and maturation, which had shown modified expression in animals at the time of weaning (P21). Expression of this molecule was decreased significantly in the Ro61-8048-treated animals compared with controls (Figure 4-15 (A), p= 0.05). The changes seen during adulthood in the expression level of SHH hippocampus region was comparable to that seen at P21.
We found very negligible expression of SOX2 in the hippocampus (data not calculated). The absence of SOX2 expression in this study is intriguing. It might be due to the processing of the sample during laboratory preparation. Since there have been a number of studies that indicate the presence of SOX2 in the hippocampus by using immunofluorescent techniques and indicate the expression was largely due to proliferating neural progenitors in the neurogenic region of hippocampus (Brazel et al., 2005, Komitova and Eriksson, 2004). Therefore, repeating the experimental approach in a similar setting is important to characterize the expression level of this marker, or re-examining using another source of commercially available antibody for SOX2, though we cannot exclude the possibility of differential expression pattern as each commercial antibody has its own specific localization expression.
Figure 4-13    Expression of NMDA receptor subunits in the hippocampus of control and Ro61-8048-treated animals.

Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed and whole hippocampi were snap frozen until required for analysis. Homogenized tissues of the brains were used for analysis. Blots were probed for NR2A and NR2B as marked, showing the presence of NR2A/NR2B containing complexes in the whole hippocampi tissue. Representative immunoblots showing protein expression levels of NR2A (M.W. 180 kDa) and NR2B (M.W. 180 kDa) in the hippocampus, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NR2A and NR2B protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6) for Ro61-8048, n=4 for control). (A) Compared with saline-treated animals(S), there was a significant decrease in NR2A protein expression in Ro61-8048(R) (**p<0.01).
Figure 4-14 Expression of PSD95 and EphA4 in the hippocampus of control and Ro61-8048-treated animals. Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed and whole hippocampi was snap frozen until required for analysis. Homogenized tissues of the brains were used for analysis. Blots were probed for PSD95 and EphA4 as marked, showing the presence of PSD95 and EphA4 containing complexes in the whole hippocampi tissue. Representative immunoblots showing protein expression levels of PSD95 (M.W. 95 kDa) and EphA4 (M.W. 120 kDa) in the hippocampus, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative PSD95 and EphA4 protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6 for Ro61-8048, n=4 for control).
Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed and whole hippocampi was snap frozen until required for analysis. Homogenized tissues of the brains were used for analysis. Blots were probed for SHH and PCNA as marked, showing the presence of SHH and PCNA containing complexes in the whole hippocampi tissue. Representative immunoblots showing protein expression levels of SHH (M.W. 45 kDa) and PCNA (M.W. 36 kDa) in the hippocampus, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative SHH and PCNA protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6) for Ro61-8048, n=4 for control. (A) Prenatal treatment with 100mg/kg Ro61-8048(R) showed significant reduction in SHH expression compared to control animals(S) (*p<0.05).
Figure 4-16  Expression of inflammatory proteins in the hippocampus of control and Ro61-8048-treated animals.

Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed and whole hippocampi was snap frozen until required for analysis. Homogenized tissues of the brains were used for analysis. Blots were probed for NFκB and COX2 as marked, showing the presence of NFκB and COX2 containing complexes in the whole hippocampi tissue. Representative immunoblots showing protein expression levels of NFκB (M.W. 65 kDa) and COX2 (M.W. 72 kDa) in the hippocampus, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NFκB and COX2 protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6 for Ro61-8048, n=4 for control).
4.4.3.2 Protein expression in the cerebellum

The cerebellum exhibited fewer changes of protein expression than either the hippocampus or cortex, with only one protein examined showing significant modification. Of all the proteins examined, the majority showed no difference between control and drug-exposed animals. Levels of the NR2A and NR2B subunits and PSD95 of the NMDA receptor were not significantly different in animals exposed in utero to Ro61-8048 compared with controls [NR2A: Figure 4-17(A), p = 0.42, NR2B: Figure 4-17(B), p = 0.40, PSD95: Figure 4-18 (A), p = 0.61]. Similarly, there was also no evidence of change for EphA4 receptor for secreted ephrin molecules in the treated pups (Figure 4-18(B), p = 0.45). The most striking was the finding of very significant changes in one of the proteins involved in neurogenesis. Significant difference was noted in the expression of SHH, which is intimately associated with cell growth and maturation. In the nervous system of young and adult animals, SHH is known to stimulate the proliferation of neuronal precursor cells in the cerebellum and other brain regions, such as the hippocampus and forebrain (Fuccillo et al., 2006). Prenatal Ro61-8048 exposure resulted in significant decreases in the protein level in the cerebellum (Figure 4-19(A), p = 0.02), a result again in a similar pattern to that of hippocampus. The other proteins that are linked with neurogenesis, PCNA binds to a nuclear protein in mature cells and transcription factor SOX2, which regulates and maintains the undifferentiated state of progenitor cells, were both unchanged following prenatal treatment with Ro61-8048 (PCNA: Figure 4-19(B), p = 0.61, SOX2: Figure 4-19(C), p = 0.26). In another case, two proteins intimately reflecting inflammatory processes were also examined, with no changes were noted in the expression of NFkB and COX2 in the treated animals (Figure 4-20).
Figure 4-17 Expression of NMDA receptor subunits in the cerebellum of control and Ro61-8048-treated animals. Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed and whole cerebellum was snap frozen until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Blots were probed for NR2A and NR2B as marked, showing the presence of NR2A/NR2B containing complexes in the whole cerebellum tissue. Representative immunoblots showing protein expression levels of NR2A (M.W. 180 kDa) and NR2B (M.W. 180 kDa) in the cerebellum, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NR2A and NR2B protein expression normalized to actin expression. Data were expressed as mean ± SEM.
Figure 4-18  Expression of PSD95 and EphA4 in the cerebellum of control and Ro61-8048-treated animals.

Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed and whole cerebellum was snap frozen until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Blots were probed for PSD95 and EphA4 as marked, showing the presence of PSD95 and EphA4 containing complexes in the whole cerebellum tissue. Representative immunoblots showing protein expression levels of PSD95 (M.W. 95 kDa) and EphA4 (M.W. 120 kDa) in the cerebellum, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative PSD95 and EphA4 protein expression normalized to actin expression. Data were expressed as mean ± SEM.
Figure 4-19  Expression of neurodevelopmental proteins in the cerebellum
Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18
of gestation while groups of control animals were injected with saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed and whole cerebellum was snap frozen until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Blots were probed for SHH and PCNA as marked, showing the presence of SHH, PCNA and SOX2 containing complexes in the whole cerebellum tissue. Representative immunoblots showing protein expression levels of SHH (M.W. 45 kDa), PCNA (M.W. 36 kDa) and SOX2 (M.W. 34 kDa) in the cerebellum, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative SHH and PCNA protein expression normalized to actin expression. Data were expressed as mean ± SEM. (A) Prenatal treatment with 100mg/kg Ro61-8048(R) showed significant reduction in SHH expression compared to control animals(S) (n=6) (*p<0.05).
Figure 4-20  Expression of inflammatory proteins in the cerebellum of control and Ro61-8048-treated animals. Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed and whole cerebellum was snap frozen until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Blots were probed for NFκB and COX2 as marked, showing the presence of NFκB and COX2 containing complexes in the whole cerebellum tissue. Representative immunoblots showing protein expression levels of NFκB (M.W. 65 kDa) and COX2 (M.W. 72 kDa) in the cerebellum, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NFκB and COX2 protein expression normalized to actin expression. Data were expressed as mean ± SEM.
4.4.3.3 Protein expression in the cortex

In order to compare the changes occurring in the cortex with those observed previously in the hippocampus and cerebellum, a similar range of proteins was examined in this study, including proteins involved in neuronal migration, axon guidance, neurogenesis and dendrite formation. Most of the proteins included in this analysis showed no difference between control and drug-exposed animals. However, a significant difference was noted in the expression of the NR2A subunit for which expression was increased 20% (Figure 4-21(A), $p = 0.008$) although there were no changes detected in the GluN2B subunits (Figure 4-21(B), $p = 0.74$), or in the NMDA receptor-associated post-synaptic density protein PSD-95 (Figure 4-22(A), $p = 0.73$). In addition, no significant differences were detected in the expression of PCNA (Figure 4-23(B), $p = 0.31$) and SOX2 (Figure 4-23(C), $p = 0.15$) in brains from Ro61-8048-exposed compared to controls at P60.

On the other hand, expression of the morphogenetic protein SHH, was found to be substantially decreased by about 24% (Figure 4-23(A), $p = 0.0003$). This pattern of expression was similar to that observed in the cerebellum and also in the hippocampus. Axon guidance and synapse formation receptor, EphA4 exhibited a significant alteration in the pups exposed to Ro61-8048, with about 13% increase in expression compared to saline treated animals (Figure 4-22(B), $p = 0.05$). Two proteins intimately reflecting inflammatory processes were examined, with a highly significant, 30% decrease observed in COX2 expression (Figure 4-24(B), $p = 0.01$) but no change in NFkB (Figure 4-24(A), $p = 0.38$).
Figure 4-21  Protein expression of NMDA receptor subunits in P60 cortex after prenatal Ro61-8048.

Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed, sectioned and snap frozen until required for analysis. Homogenized tissues of the brains were used for analysis. Blots were probed for NR2A and NR2B as marked, showing the presence of NR2A/NR2B containing complexes in the cortex tissue. Representative immunoblots showing protein expression levels of NR2A (M.W. 180 kDa) and NR2B (M.W. 180 kDa) in the cortex, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NR2A and NR2B protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6 for Ro61-8048, n=6 for control). (A) Compared with saline-treated animals (S), there was a significant increase in NR2A protein expression in Ro61-8048 (R) (**p<0.01).
Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed, sectioned and snap frozen until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Blots were probed for PSD95 and EphA4 as marked, showing the presence of PSD95 and EphA4 containing complexes in the cortex tissue. Representative immunoblots showing protein expression levels of PSD95 (M.W. 95 kDa) and EphA4 (M.W. 120 kDa) in the cortex, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative PSD95 and EphA4 protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6 for Ro61-8048, n=6 for control). (B) Compared with saline-treated animals(S), there was a significant decrease in EphA4 protein expression in Ro61-8048(R) (*p<0.05).
Figure 4-23  Protein expression of neurodevelopmental proteins in P60 cortex after prenatal Ro61-8048.

Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed, sectioned and snap frozen until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Representative immunoblots showing protein expression levels of SHH (M.W. 45 kDa), PCNA (M.W. 36 kDa) and SOX2 (M.W. 34 kDa) in the cortex, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative SHH, PCNA and SOX2 protein expression normalized to actin expression. Data were expressed as mean ± SEM. (A) Prenatal treatment with 100mg/kg Ro61-8048(R) showed significant reduction in SHH expression compared to control animals(S) (n=6) (**p<0.01).
Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). After euthanasia the brains were then removed, sectioned and snap frozen until required for analysis. Homogenized tissues of the brains from 6 pups (two pups from 3 litters) were used for analysis. Blots were probed for NFkB and COX2 as marked, showing the presence of NFkB and COX2 containing complexes in the cortex tissue. Representative immunoblots showing protein expression levels of NFkB (M.W. 65 kDa) and COX2 (M.W. 72 kDa) in the cortex, with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NFkB and COX2 protein expression normalized to actin expression. Data were expressed as mean ± SEM. (B) Prenatal treatment with 100mg/kg Ro61-8048(R) showed significant reduction in COX2 expression compared to control animals(S) (n=6)(**p<0.01).
4.4.3.4 Protein expression in the midbrain

Maternal exposure to 100mg/kg Ro61-8048 had no effect on the level of proteins in the midbrain regions which included the striatum and caudal regions projecting to it. The majority of the markers examined in this study showed no difference between control and drug-exposed animals. No significant differences in the expression of proteins between control and Ro61-8048-treated animals were noted for NMDA receptor subunit in this region (NR2A: Figure 4-25(A), \( p = 0.24 \); NR2B: Figure 4-25(B), \( p = 0.98 \)). Similar expressions were seen in PSD95 (Figure 4-26(A),\( p = 0.30 \)) and EphA4 (Figure 4-26(B), \( p = 0.14 \)). Three of the proteins linked to neurogenesis, namely SHH (Figure 4-27(A), \( p = 0.39 \)), PCNA (Figure 4-27(B), \( p = 0.83 \)) and SOX2 (Figure 4-27(C), \( p = 0.88 \)) remained unchanged after prenatal Ro61-8048. While no change was seen in the levels of NFκB (Figure 4-28 (A), \( p = 0.15 \)) or COX2 (Figure 4-28(B), \( p = 0.45 \)).
Figure 4-25 Expression of NMDA receptor subunit proteins in the midbrain of control and Ro61-8048-treated animals. Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). Offspring were subsequently euthanized for the removal of brains. Homogenized tissues of the brains were used for analysis. Blots were probed for NR2A and NR2B as marked, showing the presence of NR2A/NR2B containing complexes in the midbrain tissue. Representative immunoblots showing protein expression levels of NR2A (M.W. 180 kDa) and NR2B (M.W. 180 kDa) in the midbrain with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NR2A and NR2B protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6 for Ro61-8048, n=6 for control).
Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). Offspring were subsequently euthanized for the removal of brains. Homogenized tissues of the brains were used for analysis. Blots were probed for PSD95 and EphA4 as marked, showing the presence of PSD95 and EphA4 containing complexes in the midbrain tissue. Representative immunoblots showing protein expression levels of PSD95 (M.W. 95 kDa) and EphA4 (M.W. 120 kDa) in the midbrain with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative PSD95 and EphA4 protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6 for Ro61-8048, n=6 for control).
Figure 4-27  Expression of neurodevelopmental proteins in the midbrain of control and Ro61-8048-treated animals.
Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). Offspring were subsequently euthanized for the removal of brains. Homogenized tissues of the brains were used for analysis. Blots were probed for SHH, PCNA and SOX2 as marked, showing the presence of SHH, PCNA and SOX2 containing complexes in the midbrain tissue. Representative immunoblots showing protein expression levels of SHH (M.W. 45 kDa), PCNA (M.W. 36) and SOX2 (M.W. 34 kDa) in the midbrain with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative SHH, PCNA and SOX2 protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6 for Ro61-8048, n=6 for control).
Compound Ro61-8048 was administered i.p. to the pregnant dam at days E14, E16 and E18 of gestation while groups of control animals were injected with the saline vehicle. Parturition was allowed to proceed naturally and the young were allowed to develop up to postnatal day 60 (P60). Offspring were subsequently euthanized for the removal of brains. Homogenized tissues of the brains were used for analysis. Blots were probed for NFkB and COX2 as marked, showing the presence of NFkB and COX2 containing complexes in the midbrain tissue. Representative immunoblot showing protein expression levels of NFkB (M.W. 65 kDa) and COX2 (M.W. 72 kDa) in the midbrain with actin (M.W. 42 kDa) shown as a loading control. Histogram showing relative NFkB and COX2 protein expression normalized to actin expression. Data were expressed as mean ± SEM (n=6 for Ro61-8048, n=6 for control).
4.5 Discussion

In the current study, we have demonstrated that the protein expression of some of the important molecules involved during neurodevelopmental events in the brain undergoes a differential pattern of regulation. We investigated possible alterations in the inhibitory activity of compound Ro61-8048 on acute and long term changes in the expression of proteins in the brain as well as regional areas of the brain (P60) of animals at various stages of development. Evaluation of region-specific protein expression presented in P60 animal allows us to make some speculations on the involvement of relevant brain regions that provide a guide for further investigation. Several findings are of interest in this study and will be discussed further.

We demonstrate that pharmacological manipulation of the kynurenine pathway during the prenatal period can be accomplished by treating pregnant dams during late gestation. Our previous data indicate that the levels of KYNA in maternal plasma and embryo brain can be elevated by the systemic administration of KMO inhibitor, Ro61-8048 which deflects kynurenine degradation towards enhanced KYNA formation. Administration of this compound to pregnant rats at three time points during late gestation caused comparable substantial increases in maternal plasma and embryo brain of KYNA level (Forrest et al. 2013b). In accordance with this, increases in the concentrations of brain KYNA has been reported following administration of the KMO inhibitor, Ro61-8048 to immune-stimulated mice and this was accompanied by reduced concentrations of 3-hydroxykynurenine and QUIN, suggesting that in pathological situations, KMO is a rate limiting enzyme for both 3-hydroxykynurenine and also QUIN (Chiarugi et al., 2001). Administration of Ro61-8048 also provoke an increase in brain KYNA concentrations and reduced the severity of dystonia (Richter and Hamann, 2003). Beneficial effects of KMO inhibition have been shown in a murine model of cerebral malaria, where an increase in KYNA and anthranilic acid in R061-8048-treated infected mice would protect against the activation of NMDAR. However, when Ro61-8048 was given to the controls, no alteration in QUIN concentrations was observed(Clark et al., 2005). The effect of maternal KMO inhibition on embryo brain KYNA and neurochemical alterations seen in later stages of developmental suggests evidence for acute endogenous
changes in KP metabolism which might be potentially responsible for the changes seen in this study.

4.5.1 Protein expression of NMDA receptor subunits and associated protein

To date, no study has examined the influence of maternal administration of KMO inhibitor on NMDA receptor subunits expression in the embryo and also in the postnatal brains. To examine the possibility that NMDA receptors contribute to the kynurenine-mediated regulation of NMDA subunits level in the brain, the influence of Ro61-8048 on the inhibition of the kynurenine pathway was addressed. The present study demonstrates that prenatal modulation of the kynurenine pathway during pregnancy is associated with alterations in NMDA receptor subunits protein levels as early as 5 hour following single injection. In addition, we found that both NMDA receptor subunits and PSD95 followed a different profile of expressions during postnatal development in the brain.

The NMDA receptors modulate glutamate postsynaptic neurotransmission by generating long lasting calcium channel opening for calcium influx through the channel and causing longer depolarizations. This process is responsible for mediating synaptic plasticity changes such as LTP and LTD that underpins many higher functions including learning and memory. Activation of the receptor in the mechanism of LTP is considered and understood to be the synaptic model of learning and memory (Bliss and Collingridge, 1993, Gladding and Raymond, 2011). The subunits NR2A and NR2B were chosen for examination in this study because they are the most predominant NMDA receptor subunit subtypes in their respective subunit families and would therefore be the most likely receptor subunits responsible for changes. Our results demonstrate that both these major subunits of the NMDA receptor, NR2A and NR2B, are differentially expressed during pre-and postnatal development in the rat brain. Both NMDA receptor subunit proteins were already expressed in the embryo brains as observed at E18 (following 5 hour post injection). Developmental expression using immunoblot analysis showed that the NR2B proteins were detectable as early as E14, continued increasing at P10, P20 and into late adulthood P60. It also pointed out that environmental insults during prenatal development may result in
morphological abnormalities in which aberrant co assembly of the NMDA receptor complex may reflect the differentiation of synaptic rearrangement and receptor affinities (Babb et al., 2005). Five hours of Ro61-8048 administration to the pregnant dams had different effects on two major NR2 subunits of NMDA receptor, with reduced protein expression for NR2A and increased levels of NR2B protein at embryonic day 18. A consistent set of chronic changes in the protein levels of both subunits was observed in young animals at P21. It is important to note that a change in the NR2A expression in the hippocampus and cortex after Ro61 treatment was maintained into adulthood and likely to have influenced structural and functional aspects of synaptic plasticity at the receptor. The observed increase in NR2A subunits in the 21 day postnatal offspring produced by prenatal Ro61 exposure may therefore be a compensatory mechanism for a potential decrease in the receptor subunit function at earlier time points in development. This may also hold a similar explanation for the differential pattern of NR2A expression observed in mature animals. There may be brain regional and developmental age selectivity for the effects of prenatal kynurenine inhibition on NMDA receptors. For PSD95, we showed that acute exposure to the enzyme inhibitor produced no alteration in the level of protein expression in the whole brain lysate. However, the level of protein detected was progressively increased at P21. In accordance with this, we did observe significant differences in the level of NR2A and NR2B proteins in the same age group of postnatal rats. Concerning the expression of the major subunit NR1 (results presented in (Forrest et al., 2013a), no significant alteration was observed in all ages of development. The present study demonstrates that there are differential long term-effects of prenatal modulation of the kynurenine pathway on the NMDA receptor complex in the offspring. This implies that a change in NMDA receptor number or function could underlie altered neurotransmission and receptor pharmacology both during normal physiological activity and in response to neuroactive metabolites. The fact that we observed reduced NR2A subunit, but not NR1 and NR2B protein levels in homogenates prepared from hippocampus isolated from P60 rat in the presence of unaltered mRNA expression of the subunits (data presented in (Forrest et al., 2013a) indicates that the reduction in NR2A protein may be the result of differential post-transcriptional processing of the protein in the Ro61-treated animal. This observation is consistent with other studies showing that, in some cases, NMDA
receptor subunit mRNA levels do not correlate with protein levels (Gazzaley et al., 1996). The exact mechanisms for how NR2A expression is decreased in the hippocampus was not formally addressed in our study, thus reduced translation or increased degradation of the NR2A protein in the hippocampus of exposed animals should be evaluated in future studies. Up regulation of NR2A subunits in the cortex has been noted suggesting that prenatal kynurenine inhibition enhanced neural activity in the cortex. Therefore, changes in NR2A protein levels seen in only two regions could be a compensatory response to prolonged NMDA receptor inhibition in utero that persist into adulthood. As our western blot analysis indicates, protein expression of NMDA subunits NR2A and NR2B and also PSD95 is enriched in the hippocampus compared with other regions. This is supported by the evidence demonstrating that in the hippocampal region, the expression of NR2A, NR2B and PSD95 increased with age. During the development of the hippocampus, the pyramidal cells in CA1 and CA3 grow rapidly after birth and are gradually stabilized after postnatal week 3. Since the granule cells had self-renewal capacities for their lifetime, the number of granule cells in the dentate gyrus gradually increases with age and maintain the ability to proliferate in adulthood. The obviously different expression patterns of the subunits implicated their distinct roles in the postnatal development of hippocampus (Al-Hallaq et al., 2007). NMDA receptor function is regulated mainly through association with many signalling proteins that are concentrated with the postsynaptic density (PSD) compartment. Of the many PSD proteins that can potentially modulate NMDA receptor properties, PSD95 has gained the most attention because this molecule associates with various signalling protein and exerted its effect by recruiting kinases or phosphatases to the NMDA receptor complex (Li et al., 2003, Tezuka et al., 1999). However, the present study could not justify the exact functional composition and localization of NMDA subunits since we used a total pool of proteins that included intracellular pools of assembled and unassembled NMDA receptors expressed at the cell membrane. The homogenates are heterogeneous, containing a percentage from the synaptic region itself, as well as from extrasynaptic membranes, the soma and the axons. Apart from that, interaction between the major subunits with its associated protein, like PSD95 using immunoprecipitation studies was not examined in this study. Although the distribution of NMDA receptor compositions across brain region were not addressed in this study, these data indirectly support a
regulated mechanism of subunit assembly because NMDA receptor subunit composition differs across regions in which NR2A and NR2B are the predominant NR2 subunits, such as in hippocampus and cortex (Wenzel et al., 1997). In addition, physical interaction between NR2 subunits and PSD proteins have been shown to play an important role in regulating the localization of the NMDA receptor and this interaction is mediated by the cytoplasmic tail of NR2 subunits (Al-Hallaq et al., 2007, Delint-ramirez et al., 2010).

The present studies add to a growing body of evidence that environmental insults during pregnancy are associated with alterations in NMDA receptor subunit levels. Our results report changes in the protein levels of NMDA receptor subunits in different developmental ages as a result of either acute or persistent effects of kynurenine modulation during pregnancy. The persistence of these alterations into adulthood indicates that modifications of glutamatergic neurotransmission is highly involved in the adaptive responses that underlie homoeostatic and compensatory changes in the function of the receptor complex that contribute to the neurochemical imbalance associated with learning and memory deficits that are observed in adult animals that were exposed prenatally to KMO inhibitor.

4.5.2 Differential expression of developmental molecules following prenatal Ro61-8048 administration

We have examined the expression level of developmental molecules responsible for some important processes including neuronal proliferation and migration, axon guidance and synapse formation in the embryo and postnatal brains. The increasing number of developmental events associated with these molecules has generated considerable interest in studies regarding their molecular mechanisms as well as putative involvement in a range of neurodegenerative diseases. Our study showed that EphA4 was expressed in the developing brain from embryonic day 18 with reduced levels detected 24 hour after Ro61 administration and also at P21. In P60 brain, there was a dynamic pattern of expression, with a persistent but differential pattern of expression in the several regions of the mature brain. Although no significant changes was seen in the hippocampal level of EphA4 as compared to control levels, this molecule has been found to be
highly expressed in the hippocampus and involved partly in synaptic plasticity events such as long term potentiation. While many of the molecular mechanisms regulating the induction of LTP remain unclear, Ephs receptor protein kinase activity is known to be involved in this process (Pasquele, 2008). We demonstrate here that the regional pattern of EphA4 expression changes markedly during postnatal development as shown at P60, with the observation of lowered expression in the cortex suggesting that prenatal modulation of the kynurenine pathway may preferentially affect particular regions of the brain. These data suggest that EphA4 expression is regulated in a NMDA-dependent synaptic response through regulation of kynurenine metabolites in the brain and that EphA4 kinase activity may play a functional role in maintaining the organization of dendritic spines postnatally. The fact that EphA4 regulates axon guidance during embryonic development and postnatal synaptic function emphasizes the varied and complex roles for this receptor family in regulating cell-cell interactions within the CNS. The molecular mechanisms of many Ephs and ephrin related synaptic functions are still largely unknown, thus a more elaborate examination of the roles and mechanisms of this receptor complex will be needed to further establish them as important synaptogenic molecules in the nervous system (Lai and Ip, 2009).

The physiological function of PCNA is well described, being necessary for DNA replication and repair in proliferating cells. Changes in cell proliferation dynamics in response to various insults have been assessed previously using multiple cell cycle markers, in which the pattern of changes was reflected in double/triple immunolabelling using a combination of PCNA with other proliferation markers including 5-bromo-2′-deoxyuridine (BrdU) and/or Ki-67. The combination of different cell cycle regulators permits study of the proliferative, migratory and survival characteristics of neuronal progenitors cells in the developing and adult rodent brain (Halbach, 2011, Valero et al., 2005). We showed that in the early developmental period, kynurenine pathway modulation during gestation caused no changes in the proliferative event, as demonstrated by PCNA expression in the embryo. However, it progressively appeared that the resistance of the immature brain has its limitations, in which we noted an elevated extent of proliferation as shown by PCNA expression at later stage of development only at P21 and regionally unaffected when examined at P60.
Upon binding to its receptor Patched (Ptc), SHH activates the highly conserved hedgehog (HH) signalling cascade, resulting in activation of the Gli family of transcription factors. The SHH signalling cascade is involved in the development and patterning of several vertebrate embryonic structures, anteroposterior patterning of the limbs and dorsoventral patterning of the neural tube (Ruiz i Altaba et al., 2002, Palma et al., 2005). Analysis of SHH protein expression after administration of enzyme inhibitor Ro61-8048 has paved a way for the characterization of the potential roles for this molecule in the normal and treated brain. Robust SHH expression was found in the embryo, suggesting localized signalling in the developing brain. Interestingly, the expression is high before birth and markedly reduced at postnatal day 21. Application of antibodies specific for SHH led to a pattern of regional protein expression in the adult rat brain. In protein extracts isolated from animals exposed to Ro61-8048, western blot analysis indicated that a 45kDa SHH protein SHH was broadly expressed in all brain areas examined with reduced expression found in three main areas of cerebellum, cortex and hippocampus. Such a pattern of expression was found conserved for the SHH mRNA, albeit a low expression was detected within these brain regions, suggesting that this molecule may be implicated in local signalling circuitry (Forrest et al., 2013b). Although in this study, we did not focus on the regulation of neural stem cells, a number of reports have demonstrated that in the nervous system of young and adult animals, SHH is known to regulate the proliferation of the cerebellar granule cell precursors, neural progenitors lining the subventricular zone of the forebrain and in the dentate gyrus of the hippocampus. These studies partly demonstrate that concerted cell proliferation, migration and differentiation in response to SHH protein are important for normal embryonic developmental and postnatal homeostasis (Palma et al., 2005, Lai et al., 2003, Machold et al., 2003, Bertrand and Dahmane, 2006). Expression studies of SHH and other genes involved in hedgehog signal-transduction have indicated a function of hedgehog signalling in the adult brain in addition to its roles during early development (Traiffort et al., 1999, Charytoniuk et al., 2002). A number of approaches including in vitro cell culture and animal model have been used to unravel the SHH signalling pathways linking relationship between various perturbations or exposure to the regulation of the expression of SHH signalling cascade in the embryonic and adult rodent brain. It has been shown that this powerful developmental morphogen is
upregulated in adult organisms after injury and involved in a repair mechanism in which it enhances adult hippocampal neurogenesis following electroconvulsive seizure (Banarjee and Rajendran, 2005). It appears that the expression of SHH also increased during ischemia (Sims et al., 2009) and under oxidative stress induced by hydrogen peroxide (H$_2$O$_2$) (Dai et al., 2011). Other molecular events that have influence on the modulation of the SHH pathway includes exposure to caffeine in primary neuronal and astroglial cultures (Sahir et al., 2004) and in the presence of teratogen valproic acid (VPA), which altered the expression of some regulatory genes reported to be involved in the serotonergic neuronal development, and that SHH and its components expression were all decreased in the VPA-treated cells compared to the control (Miyazaki et al., 2005). Exposure of zebrafish embryos to alcohol during gastrulation impaired SHH signal transduction and suggesting a role for defective SHH signalling in the pathogenesis of alcohol-induced birth defects (Li et al., 2007). Our results demonstrate that in the adult brain, this pathway remains active and has a continuous role in the adult neural stem cells, and mature neurons are likely to be the source of SHH (Ihrie et al., 2011). We show that the SHH pathway, one of a key developmental signals in the brain, may be differentially regulated following kynurenine modulation. At present we are not entirely certain of the exact mechanisms that underlie the effects seen in this study. There remains room for further exploration to gain a greater understanding of the processes of SHH signalling mechanism.

We analysed the expression of SOX2 in brain homogenates prepared from foetal and also from rat offspring at P21 and regional areas at P60 to determine whether this transcription factor was affected after modulation of kynurenine pathway during gestation. The biological roles of SOX2 have been thoroughly studied in the context of embryonic developmental regulation as it is known as one of the key factors in the regulation of embryonic stem cell self-renewal and is associated with neural stem cell (NSC) populations in the mature rodent brain (Brazel et al., 2005, Komitova and Eriksson, 2004). It is worth mentioning that all proteins of the SOX-B1 group (SOX1,SOX2,SOX3) are expressed in the developing brain to a different extent during development and many of the SOX proteins coexist throughout the CNS (Bylund et al., 2003). Our study shows that expression of SOX2 is abundant in the control embryonic brains, during a time
window in which the pool of neural progenitors expands extensively which in agreement with a previous study that indicate an essential role for SOX2 in early development (Avilion et al., 2003). Acute treatment with Ro61-8048 caused no alteration in the SOX2 levels suggesting the important role of SOX2 in neural stem cell maintenance in the developing brain. The prominent expression of SOXB1 protein in general, in the neurogenic regions of adult brain has prompted many researchers to analyse the expression pattern of these markers in the neurogenic lineage using a culture model of different types of dividing precursor cells with the aim of comparing the expression level of the markers that are expressed at different stages of adult neurogenesis. The reason for the lack of expression in the hippocampus of P60 animal, even in a control setting might arise from the hippocampal sections used in this study. The sections were obtained after it had been exposed to electrophysiological procedures. Thus, it is likely to reflect the overall cellular contents of the homogenates. As SOX2 proteins bind to specific DNA sequences via their HMG box domains and synergizing with other transcription factors to fulfil variable roles, their mode of action may depend both on the cellular context and on the target gene functionally regulating active transcriptional complexes (Lefebvre et al., 2007). It has been shown that SOX2 is expressed mainly in dividing neural stem cells and transient amplifying progenitor cells in the hippocampus which indicates that SOX2 is expressed stage-specifically in cells of the adult neurogenic lineage (Suh et al., 2007, Ellis et al., 2004). In another series of experiments, studies have shown that SOX2 continues to be expressed in specific populations of adult cells of other brain regions including cerebellum, where SOX2 positive cells have been observed in Bergmann glia which is not considered as a stem cell population (Alcock et al. 2009). Our results show a differential expression pattern in the adult brain regions after kynurenine pathway regulation as we demonstrate that endogenous concentrations of SOX2 varies between distinct classes of neural progenitor cells in those regions. The present findings only indicate that SOX2 is differentially expressed between regions. It is worth mentioning that isolated populations of these types of progenitor cells based on their SOX2 expression and subsequent gene expression profile of each sorted population can be used to accurately ascertain distinct neural progenitors (Hutton and Pevny, 2011), though at this stage we cannot identify the stem cell population pool in vivo as the antibody used in this study resulted in both
cytoplasmic and nuclear localization. As SOX2 represents a molecular signature for neural stem cells, this finding is particularly interesting and highlights the need to fully characterize the cell populations that express this marker in the adult brain.

These observations suggest that prenatal modulation of the kynurenine pathway might lead to changes in the expression levels of several molecules that are involved in the regulation of cell cycle, proliferation as well as the neurogenesis process in developing and adult brains. An increased expression of the transduction molecule SHH was observed 24 h after treatment with enzyme inhibitor during late gestation. However, when repeated challenges of the inhibitor were given, a different pattern of expression was seen with reduced levels of expression demonstrated at developmental age P21 and it remained repressed in some regional areas of the brain including the hippocampus, cerebellum and cortex. Substantial studies have gathered evidence supporting the idea that dysregulation of the cell cycle in neural cells plays an important role in diverse pathological processes in the CNS. Further study on the characterization of cell cycle properties in this process and comparative studies on the expression of individual members of each related proteins under physiological conditions, including various developmental stages and in exposure to pathological stimuli may provide better and conclusive explanations.

4.5.3 Expression profiles of inflammatory associated proteins

As both NFκB and COX2 are critical to host defense to various pathological stimuli, their common activating conditions represent either a direct threat to cells or that they are endogenous signals produced in response to pathogens. In this respect, administration of foreign substances might induce an inflammatory response; we explored the expression profiles of relevant inflammatory markers following administration of an enzyme inhibitor of the kynurenine pathway, Ro61-8048. The impetus of the present study was to test the hypothesis that prenatal inhibition of KP by Ro61-8048 would have impacts on the transcriptional activity of NFκB and COX2 expression in the offspring. The ability of Ro61-8048 to shift the production of kynurenine metabolites towards greater levels of KYNA has been reported and we show a substantial increase of KYNA in the maternal
plasma and in the brains of the embryos after 5 h of Ro61 administration. Level of KYNA remained significantly elevated in the embryonic brains after 24 h post administration (Forrest et al. 2013b). This effect was accompanied by increased NFKB activity in the embryo brains 24 h post treatment, indicating a role for this TF in inducing activity of other relevant target genes in the presence of the kynurenine pathway metabolite, KYNA. The effect of Ro61-8048 in shifting the balance of KP as seen in the embryonic brains also suggests that there is some constitutive NFKB activity that normally influences activity of KP during embryonic development. Increased activity of NFKB in gestational tissue has been thought to be an integral part of the final phase of pregnancy and involved in the biochemical changes associated with labor (Choi et al., 2007).

It has been reported that NFKB mediates the increased expression of both IDO and TDO in the human placenta following infection (Dharane et al., 2010). IDO is considered to be an important immunoregulator, where its induction during pregnancy has led to the suggestion that it has as essential role in suppressing the maternal immune response to the presence of the fetus (Munn et al., 1998). NFKB is a primary transcription factor specialized in cytoplasmic-nuclear signaling and coordinate the activation of various genes following extracellular stimulation (O’Neill and Kaltschmidt, 1997). Extracellular signals, in particular neurotransmitters might regulate gene expression through activation of NFKB. In this regard, glutamate and NMDA caused an induction of NFKB binding activity in the nucleus of mouse cerebellar cells through activation of NMDAR. It seems that NFKB appears to be a good candidate as a mediator for glutamate induced immediate early gene transcription in the CNS (Guerrini et al., 1995). Sustained expression with significant increased of the level of NFKB protein level was observable in the total brain homogenates from P21 offspring whose mother had been injected with Ro61-8048. However, the levels of this TF were detectable with no apparent changes in term of protein levels when observed in the hippocampus, cerebellum, cortex and midbrain of P60 brains. Nuclear translocation of activated NFKB must be demonstrated in order to determine its DNA binding activity. However, current findings applied to the quantification of expression levels generated from whole extracts of whole brain or selected regional areas i.e. hippocampus, cortex, cerebellum and midbrain. Demonstration of an activation of transcription factors (described before in the
within these specialized brain sites is therefore a useful tool to evaluate inflammatory activation of the brain to a given insult. Owing to the fact that these transcription factors are constitutively present in the cytoplasm and migrate and accumulate in the nucleus of a stimulated cell, the activation of NFκB within specific brain sites can be further analyzed by means of immunohistochemistry. Given that the TF also appear to be expressed throughout CNS in both neurons and in non-neuronal cells, we sought to demonstrate the presence of NFκB complexes within brain areas following maternal administration of Ro61-8048. It has been found that the majority of NFκB present in whole cell extracts (not separated into subcellular fractions of nuclear and cytosolic fractions) is bound to its inhibitory unit IκB, thus, specific DNA binding activity was not determined in this study. It warrants further investigation for the functional consequences of differential subcellular localization of NFκB following KP modulation. In conclusion, the presence of a well-known primary transcription factor in the brains throughout developmental ages and its induction at different times during development in response to prenatal KP modulation by enzyme inhibitor suggests a possible involvement of NFκB in the regulation of gene expression during CNS development and also represents possible physiological consequences from stimulation.

Different reports have demonstrated the involvement of NFκB in the transcriptional regulation of COX2 expression after stimulation with LPS or cytokines i.e. TNF-α. Coordinated increases in COX2 expression were associated with enhanced PGE2 production. Functional relevance of NFκB activation in this aspect was seen using NFκB inhibitors in the transfected macrophages prior to stimulation with agent (Diaz-Munoz et al., 2010). In addition, rapid activation of NFκB upon stimulation with IL-1 has been demonstrated in human myocyte tissue from term pregnant women and this factor is functionally linked to the expression of COX2 mRNA and protein that NFκB can induce COX2 enzyme (Choi et al., 2007). Our results show that the expression of COX2 protein in the embryonic brain was not affected following acute stimulation of Ro61-8048. Increased expression of NFκB in the total protein from embryo brain after 24 h treatment (correspond to E19) and P21 offspring was not accompanied by any apparent changes in COX2 levels. Differential expression of COX2 was presented in the P60 animals while a relatively strong repressive effect of COX2 were
observed in the cortex of Ro61-treated rats than control animals. Neurons of various regions including the hippocampus, cerebral cortex and hypothalamus express COX2 which is thought to play a role in fundamental brain functions, such as synaptic activity and memory formation. COX2 expression has been shown to be rapidly upregulated in neurons in response to excitatory synaptic transmission mediated by NMDAR activation or neurotoxic stimuli (Yamagata et al., 1993). Taken together with our findings, these characteristic localizations suggest regional and tissue-specific roles for COX2 in cellular signaling.

4.6 Conclusion

A number of mechanisms modulating kynurenine metabolism in the brain during early life can be envisioned. In this respect, we noted that the in vivo use of Ro61-8048, not only caused KYNA levels to be pharmacologically enhanced, but also confirmed the ability of this compound to inhibit KMO, thus demonstrating the intimate relationship between maternal and foetal KP metabolism. Administration of Ro61-8048 to pregnant rats during the late gestation period caused comparable increases in maternal environments (plasma and brain) and fetal brain KYNA, demonstrating that the endogenous changes in KP metabolism were compromised by this pharmacological intervention. In the context of the study, we provided evidence of how variations in brain development mechanisms are affected by regulation of the kynurenine pathway during pregnancy. The brain is very sensitive to noxious environmental influences, in particular during early stages of life. Severity and duration of the insults/agents are of critical importance for the brain outcome of any detectable lesions. One implication of this finding is that exposure to high levels of KYNA may begin early in life, as we have previously shown an increased endogenous KYNA concentration in the maternal plasma and foetal brain, and that increases in KYNA concentration and the resulting inhibition of NMDAR during early developmental stages could have lasting impacts on brain development.
5 Molecular effects in the adult rat brain following prenatal inhibition of the kynurenine pathway

5.1 Introduction

Kynurenine pathway metabolites, particularly the pivotal QUIN and KYNA (Zhao et al., 2008) concentrations in the foetal brain are controlled locally and are also responsive to maternal infections and pharmacological manipulations. Moreover, the pathway is intimately associated with excitatory amino acid neurotransmission systems that play central roles in neuronal growth, differentiation and synapse formation. It has been shown that various anomalies of glutamate-mediated synaptic transmission can lead to excessive activation of neurons (excitotoxicity), which may in turn initiate their death or compromise their survival in some neurological disorders. Considering that the plasticity of glutamate-mediated receptors could be affected by changes in the balance between endogenous levels of KP metabolites, several pharmacological agents have been successfully used to probe the physiological and pathological roles of kynurenines (Schwarcz et al., 2012, Schwarcz and Pellicciari, 2002, Carlsson et al., 2001). It is well know that NMDA subtypes of glutamate receptors are involved in plasticity processes during brain development and excessive activation of this receptor during early stages of brain development may manifest its effects only later in life. It is therefore important to consider the possibility that pharmacologically induced prenatal increases in brain KYNA levels might induce neurochemical alterations which could interfere with developmentally essential glutamatergic neuronal function in the offspring.

Generation of new neurons (known as neurogenesis), that persists lifelong postnatally in the subventricular zone of the lateral ventricle and subgranular zone of the dentate gyrus in the hippocampus forms crucial neuronal networks responsible for cognitive, emotional and memory function. The brain regions that are capable of producing new neurons contain pools of neural progenitor cells (NPC) which are functionally integrated into the existing neuronal circuitry and regulate hippocampus-dependent learning and memory processes. These tightly regulated processes takes place within an appropriate time frame and sequence and consists a balance between NPC proliferation, migration, differentiation and survival. Any defect along these processes could have some
implications and lead to neurological and psychiatric disorders (Zhao et al., 2008). Study on the regulation of these processes are continually researched as they represent prominent forms of brain plasticity, potentially involved in learning and memory and partly associated with cognitive deficits of many neurodevelopmental disorders. Various neuropsychiatric disorders such as depression, schizophrenia, Huntington’s disease and also in stroke and drug addiction patients are characterized by alterations in neurogenesis in the hippocampus as indicated by the increased cell proliferation and the presence of newly generated neuron in the neurogenic regions of the brain (Curtis et al., 2003b, Curtis et al., 2003a, Jin et al., 2004). There is also growing interest on the evidence that neurodevelopmental disorders are associated with cerebellar deficits and learning impairments. Thus, the cerebellum is likely one of the structures of choice to investigate the relationship between regional development defects and learning. Its main role not only has been primarily associated with motor control, but also in motor learning and cognition. The presence of cognitive deficits and structural changes together with functional cerebellar alterations have also occasionally been implicated in neuropsychiatric pathology (Hoppenbrouwers et al., 2008).

In the present study, to elucidate the effects of developmental exposure to an enzyme inhibitor of the KP on neurogenesis, the distribution of granule cell lineages in the hippocampus formation were analysed in the offspring of rats exposed to Ro61-8048 during pregnancy. The hippocampus represents a unique structure for ongoing neurogenesis throughout postnatal life and is crucial for higher brain functions such as learning and memory. Moreover, NMDA receptors are abundant in this region and it is suggested that hippocampal NMDA receptors are closely related to the cause of various psychiatric disorders such as anxiety and depression as well as the functions of learning and memory. Apart from the hippocampus, the cerebellum with its well-defined cell types positioned in a layered fashion also makes the region particularly amenable for the study of morphogenesis. An increase in cerebellar volume during development is due to the expansion and proliferation of the granule cell precursor (GCP) during early postnatal periods. Various stimuli are involved in the regulation and induction of granule cell proliferation. Among these SHH, a secreted factor expressed in Purkinje cells from the embryonic period is the most efficacious endogenous
mitogen known to be involved in the regulation of GCP in the cerebellum in which inhibition of SHH signalling has been shown to reduce GCP proliferation (Dahmane and Ruiz i Altaba, 1999, Lewis et al., 2004).

5.2 Research aims

In the present study, to detect a key molecular event reflecting permanent disruptions of neuronal development due to modulation of the kynurenine pathway during gestation that can interfere with NMDA receptor signalling and function, we examined cellular distribution and localization of several markers that have been implicated in neurogenesis process of adult rats. We hypothesized that early modulation on NMDA receptor function via kynurenine pathway metabolites may affect ongoing neurodevelopmental processes including postnatal hippocampal neurogenesis in the offspring. The aim was to address whether prenatal inhibition of the kynurenine pathway regulates the expression of key components of the cell genesis in the hippocampus and also in the cerebellar cortex and to demonstrate whether the patterns of morphological findings were consistent with those of protein expression levels. Moreover, we also sought to corroborate if the changes described in these regions are representative of the whole brain or whether prenatal exposure to Ro61-8048 induced region-specific changes in the adult offspring.

5.3 Experimental approaches

5.3.1 Prenatal treatment and operative procedures

Timed pregnant Wistar rats were injected intraperitoneally (i.p.) with 100mg/kg of Ro61-8048, a compound inhibiting a key enzyme in the kynurenine pathway which shifts the balance of tryptophan metabolism towards the production of kynurenic acid (Forrest et al., 2013a), or saline (0.9% NaCl) at E14, 16 and 18 (n=3 per group). Then, gestation was allowed to proceed normally and at postnatal day 60 (adult) their brains were perfusion-fixed for immunocytochemical processing (Figure 5-1). Animals were deeply anesthetized with Euthatal and intracardially perfused with mammalian Ringer solution followed by 4% paraformaldehyde in 0.2M PB.
Figure 5-1  Timeline for prenatal treatment of Ro61-8048 for immunocytochemical experiments.

5.3.2 Tissue processing and immunocytochemistry

The brains were then dissected, post-fixed for 4 h in the same fixative at 4°C and transferred to a 30% sucrose solution until equilibrium was reached. The brain fixative contained 30% sucrose as a cryoprotectant, preventing large ice crystal formation. The regions of the brains were embedded in 5% agarose gel and coronal sections were cut with a vibratome at 60 µm in 0.1M PBS. Free floating sections through the entire rostral to caudal hippocampal axis were serially collected in bottles filled with the same buffer with 5-6 sections/bottle.

For analyses of hippocampal neurogenesis, serial free-floating sections from one bottle of coronally cut sections were first washed in 0.3M phosphate buffered saline (PBS) before being incubated with optimally diluted primary antibodies in 0.1% Triton in PBS (PBST; pH 7.4) for 72 h at 4°C under continuous shaking conditions. Primary antibodies used in our study were monoclonal mouse anti-NeuN (1:500), polyclonal goat anti-Doublecortin (DCX) (1:250) and polyclonal rabbit anti-SHH (1:100). In this study, immature and developing neurons in the
dentate gyrus of the hippocampus were identified with a goat antiserum (Sc8066, Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:250) directed against a synthetic doublecortin peptide corresponding to amino acids 385-402 at the C-terminus of human doublecortin and specific for DCX of mouse, rat and human origin. Postmitotic mature neurons were identified by using a mouse monoclonal antibody that recognizes the neuron-specific nuclear protein (MAB377 Millipore, Temecula CA92590, USA; diluted 1:500). It reacts with most neuronal cell types throughout the nervous system of rats and is primarily localized in the nucleus of neurons with lighter staining in the cytoplasm. In this study, the NeuN antibody provided specific labelling of neurons throughout the hippocampal formation and the staining pattern was very similar to that seen in other studies in which this antibody was used. Rinses were performed between all steps using 0.3M PBS before or after the primary and secondary antibody incubation. Following primary antibody treatment, sections were subsequently incubated overnight at 4°C in species specific Alexa Flour-tagged secondary antibodies: Alexa 488, Alexa 647 and Rhodamine-conjugated secondary antibody. Sections were mounted and cover-slipped with an aqueous mounting medium for fluorescence H-1000: Vectashield. This application of method is dependent on antibody specificity and is not a direct measurement of protein amount. However, it has the advantage of cellular localization within cell bodies and neuronal processes.

5.3.3 Confocal microscopy and data analysis

All analyses were conducted by the researcher blind to experimental condition. Sections were initially examined with a 20× objective lens using a fluorescent microscope. The regional areas of CA1, CA3 and dentate gyrus of hippocampal sections (from bregma -2.50 mm to -3.50mm) were delimited by morphological criteria and only immunopositive cells showing a stained unit area sufficient to ensure that the majority of their volume was contained within the selected area were scored. For the cerebellum, at least 3 coronal sections were immunolabelled and in every section, two to four images were taken followed by image processing to optimally visualize the staining area. These sections were scanned with a Bio-Rad Radiance 2100 confocal system through a 40×oil-immersion lens to produce image stacks with a 1 μm z-separation. The counting
of the DCX, SHH and NeuN positive cells were conducted in a total of 15 grids of area (50µm x 50µm) for the dentate gyrus and CA1 regions and grids of 5x5 (50x50µm each grid size) for the CA3 region per section. Cell staining was assessed in the granule cell layer (GCL) including the germinative SGZ and molecular layer of the DG within the grid area. While the number of NeuN immunoreactive neurons in the internal granular layer of the cerebellum were quantified in one hemisphere at approximately the middle region of the cerebellum in a total grid size of 4x4 (50µm x 50µm). Three dimensional reconstructions of the z-series were performed to assess the overlap between cell bodies. The cell number analysis was done on a total of 3 sections per animal with at least 2-3 observations per section using ImageJ software (Version 1.43M; National Institute of Health). Immunopositive cell counts of 3 sections were averaged to obtain a single value for the number of positive-stained cells per section. Quantification of immunopositive cells were analysed using data generated from individual animal as the experimental unit. A mean ± standard error (SE) was calculated from the data in each group (n= 7-9 animals). Images were generated as projections of z-stacks to illustrate the extent of all the markers staining (DCX+ve cells, NeuN+ve cells and SHH+ve cells) within a 60µm section. Representative images were not altered except for overall brightness and contrast. For statistical analysis, a student’s t test was performed to examine the differences in the mean number of cells per region per animal between treatment groups. P<0.05 was deemed significant.

5.4 Results

To determine the cell fate of the hippocampal cells, we did triple immunoflourescent labelling using cell specific markers, including DCX, a marker of immature and developing neurons, SHH and one of the neuronal markers, NeuN.

5.4.1 DCX-expressing cells in hippocampus

Doublecortin is a microtubule binding protein that is transiently expressed in proliferating progenitor cells of adult neural progenitor cells (Francis et al., 1999). On tissue sections of the adult hippocampus dentate gyrus overviews (Figure 5-3) showed the band of DCX-positive cells in the granule cell layer
proper. Labelling in both groups were generally in cell bodies aligned in a single row along the base of the granule cell layer while its dendrites of various lengths were visible and reach into the molecular layer. In both blades of the DG, we observed some DCX-positive neurons with extensive and orientated vertical dendrites which extended into the molecular layer. There were also a number of cells having horizontally orientated dendrites. The nucleus appears usually devoid of immunoreactions and it looks like an envelope. The immature neurons in the control group are more often found clustered and extending as rows in to granule cell layer. In addition, the absence of basal dendrites was another feature that we noticed in our study.

The number of DCX-positive cells representing newborn granule cells in both control and Ro61-8048-treated brains appeared to progress through similar pattern of expression with no difference in term of general localization which were more abundant in the granule cell layer as seen in the dentate gyrus. DCX immunopositive cells in the DG were mostly dispersed with few clustered cells (Figure 5-3). DCX expression can be visualised within the soma and dendrites suggesting that the newly born cells differentiate into neurons. The average number of DCX-positive cells located in the SGZ of dentate gyrus was not statistically different between control and Ro61-8048 treated group. Ro61-8048 treatment had no effect on the number of cells staining for DCX ($p=0.93$) when compared to their control offspring. We then compared the overall dendritic maturation of newly generated neurons between the groups during the DCX expression phase which was based on the complexity and orientation of dendrites. The DCX positive neurons with vertical dendrites emanating from the soma and extending into the GCL were counted. Quantification of neurons with dendritic projections that are relatively more mature showed significantly fewer neurons bearing these longer dendrites in tissue from animals exposed prenatally with Ro61-8048 (Ro61: $8.29 \pm 1.19$ vs Saline: $14.30 \pm 1.19$, $p=0.0029$, Figure 5-3(F)). Furthermore, most of the newly generated neurons with vertically orientated dendrites in Ro61-8048-treated sections exhibited short terminal branches and the spread of the dendritic tree in these newly generated granule cells appeared narrower. This modification of DCX immunoreactivities were restricted to the dentate gyrus and not observed in any other hippocampal subfield. In the hippocampal formation area, CA1 and CA3, we observed
essentially no DCX-expressing dividing cells in both areas following exposure to Ro61-8048.

Figure 5-2 Coronal section through the rat brain indicating the position and extend of the hippocampal formation.
Figure 5-3  Arrangement of newly generated neuron (DCX+ve immunoreactivity) in the dentate gyrus of P60 rat.
Expression of DCX positive newly generated neurons were visualized with green fluorescence (A-D). Overview pictomicrograph showing the hilus, molecular layer (ML) and subgranular zone (SGZ) which shows the distribution of newly born neurons with dendrites in the two blades of the control dentate gyrus (10x magnification)(A). A higher power (40x magnification) representation of DCX expression in control dentate gyrus which shows DCX labelling of dendrites around the apex of dentate gyrus reaching into molecular layer (C). Representative image of DCX expression in the dentate gyrus of P60 offspring after prenatal administration of Ro61-8048 (10x) (B). A higher power (40x) DCX expression in Ro61-8048 treated rats which shows relatively fewer and simple unbranching processes (D). Note that the distribution of new neurons is heterogeneous and dendrites from many newly generated neurons cross the inner and middle molecular layer and reach the outer molecular layer. Arrows indicate examples of DCX+ve cells which appeared to envelope the somata and dendrites. The number of DCX expressing cells was unchanged following prenatal treatment with Ro61-8048 when compared to control saline(E), whereas the proportion of DCX+ve cells with dendritic projections in the dentate gyrus were greatly reduced in Ro61-8048 animals [n=8] when compared to control [n=8-9 from each group] (F). Asterisks indicate statistical significance between experimental groups (**P<0.01; Student’s test). Data are presented as mean ± SEM. SGZ, subgranular zone; GCL, granule cell layer; ML, molecular layer. Scale bar= 100µm (A and B), 10µm (C and D).
5.4.2 Immunoreactivity of NeuN in the hippocampal formation at P60

Using NeuN-immunoreactivity as a neuron-specific marker, we examined whether neuronal density in the hippocampus was affected by in utero exposure to Ro61-8048. The distribution of NeuN-immunoreactive cells in the hippocampal formation that included the DG, CA1 and CA3 regions is shown in Figure 5-4 and Figure 5-5. To see whether early modulation of the kynurenine pathway during pregnancy affects the late neuronal differentiation process and the survival of neuronal lineage committed cells during the late stage of adult hippocampal neurogenesis, a mature neuronal marker, NeuN was immunolabelled. Generally, the NeuN antibody labels the nucleus and cytoplasm of most neuronal cell types in all regions of the adult brain with no reactivity seen in astrocytes. The most cytoplasmic immunopositive staining was concentrated in the soma, rarely extending at a short distance into the dendritic processes. In all the regions, this extranuclear staining appeared finely granular and somewhat diffuse. We also noted with closer inspection of less well-stained dot-like subnuclear structure staining within cells. In this study the expression of NeuN was clearly observed in all subfields of the hippocampus. In the hippocampal dentate gyrus, the entire section of both control and treated animals were intensely stained with NeuN-immunoreactivity while some granule neurons of the hilar region were also immunoreactive as shown in Figure 5-4(A & B). Animals exposed to the kynurenine inhibitor exhibited a high survival rate of newly born neurons.

The maturity of newly born neurons indicated by NeuN were affected at P60, where NeuN reactivity was found to be more intense in the granular cells of dentate gyrus and the number of NeuN positive cells increased significantly when compared with controls (Ro61: 206.07 ± 7.65 vs Saline: 179.07 ± 4.00, p=0.0049, Figure 5-4 (E)). In the CA1 region, the expression of nuclear immunoreactivity to NeuN was predominantly seen in the pyramidal cells both in the nuclei and cytoplasm Figure 5-5 (A & B). At P60, a significant increase was observed in the number of NeuN positive cells in the CA1 region (Ro61: 140.71 ± 17.31 vs Saline: 94.91 ± 6.57, p=0.0168, Figure 5-5 (E)), whereas there were no statistically significant differences in the number of immunoreactive cells to NeuN between the control and treatment groups in the CA3 which indicate that
existing mature neurons are not affected (Figure 5-5 (F); \( p=0.8071 \)). These results provide evidence that Ro61-8048 administration during pregnancy has a stimulatory effect on the development of new neurons and also demonstrate that this effect is long lasting, being evident in pubertal offspring 9 weeks after their first exposure to the compound.
Prenatal Ro61-8048 exposure increases the number of NeuN immunoreactive neurons in the dentate gyrus of P60 rats. Representative photomicrographs showing morphology of NeuN expression in the saline control dentate gyrus (10x magnification) (A) and at higher power (40x magnification) (C). Low magnification confocal image of the immunohistochemical localization of NeuN in P60 dentate gyrus coronal sections after prenatal administration of Ro61-8048 (B). A higher power (40x) DCX expression in Ro61-8048 treated rats which shows the granule cell layer is slightly thicker than that of control animals (D). Expression of nuclear immunoreactivity to NeuN was more intense in the granular cell layer (GCL), and some granule neurons in the hilar region were also immunoreactive. The size of the hippocampal formation in the Ro61-treated rats appears to be larger and the granule cell layer of the dentate gyrus is somewhat thicker than that seen in the control animal. Quantification of NeuN immunoreactivity in the dentate gyrus in a subset of animals prenatally exposed to Ro61-8048 suggested a significant increased of mature neurons [n=7-9 from each group] (E). Asterisks indicate statistical significance between experimental groups (**P<0.01; Student’s test). Scale bar=100µm in A and B and 10µm in C and D.
Immunocytochemical localization of NeuN in hippocampal coronal sections of CA1 and CA3 regions

Representative photomicrographs of NeuN positive neurons from control and Ro61-8048 animals are shown. NeuN immunopositive pyramidal cells in CA1 region in the control (A) and Ro61-8048-treated rats with an apparent neuronal increase in the hippocampal CA1 region (B). Immunostaining of NeuN in the CA3 region of the hippocampus in the control (C) and Ro691-8048-treated sections (D). Bar graphs show the quantified immunopositive cells to NeuN in CA1 and CA3 regions. The quantified score based on the number of cells showed significantly increased number of NeuN+ve neurons in the CA1 hippocampus of Ro61-8048-treated rats while no differences in terms of immunoreactive cells were observed in the CA3 region (E & F). Values are expressed as mean ± SEM [n=8-9 from each group]. Asterick indicates statistical significance between experimental groups (* P<0.05; Student’s test). Scale bar= 20μm (A,B,C and D).
5.4.3 Immunocytochemical analysis of SHH distribution in the adult rat hippocampus

The present study indicates that early modulation of kynurenine pathway which leads to changes in the levels of neuroactive metabolites in the brain could impose a long lasting effect on the regulation of expression of one of the cell genesis morphogen in the adult hippocampal subfields. Immunostaining studies using anti-SHH antibody revealed cellular localization of SHH which was predominantly observed in cells within the subgranular layer of the dentate gyrus, the hilar region and CA1 and CA3 regions (Figure 5-6 and Figure 5-7). Our immunocytochemical analysis demonstrates that endogenous SHH in the hippocampus were distributed in puncta extracellularly and intracellularly at the soma of the hippocampal neurons. The Ro61-8048-treated offspring compared with control exhibited a marked increase in the number of immunopositive cells for SHH in the hippocampal dentate gyrus (Ro61: 84.33 ± 7.49 vs Saline: 52.56 ± 9.02, \( p=0.029 \), Figure 5-6 (E)). This stimulatory effect on the generation of new cells was also detected in the CA1 region of the hippocampus of Ro61-8048-treated animal (Ro61: 90.17 ± 5.58 vs Saline: 51.48 ± 5.92, \( p=0.0003 \), Figure 5-7 (E)). In contrast to these changes in SHH positive cells as seen in DG and CA1, prenatal Ro61-8048 treatment failed to produce a significant increase in the number of SHH-labeled cells in CA3 region (Ro61: 73.48 ± 7.55 vs Saline: 48.17 ± 10.08, \( p=0.0678 \), Figure 5-7 (F)). We showed that SHH is predominantly expressed in cells within the subgranular layer of the dentate and CA1 region. These results suggest that neurons upregulate SHH in response to early changes in the endogenous levels of kynurenine metabolites. Based on immunohistochemistry analysis, neurons within the dentate gyrus and CA1 region may be accounted for by the increased SHH.
Figure 5-6  Representative pictures of SHH immunoreactivity in the dentate gyrus of P60 rat.

SHH expression in the saline control dentate gyrus (DG) at 10x magnification (A). A higher power (40x magnification) representation image of SHH expression in control dentate gyrus (C). SHH expression in P60 dentate gyrus offspring after prenatal administration of Ro61-8048 (10x) (B). A higher power (40x) SHH expression in Ro61-8048 treated rat (D). Note that the majority of SHH+ve cells were identified within the granular cell layer and hilus regions of the DG in both control and Ro61-8048-treated rats. A small fraction of immunopositive cells could also be detected in the molecular layer of the hippocampus. Analysis of the number of SHH immunoreactive cells in the DG revealed a significant increase in the SHH+ve cell number after prenatal inhibition of the kynurenine pathway as depicted in (E). Data is represented as mean ± SEM [n=8-9 from each group]. Asterisks indicate statistical significance between experimental groups (*P<0.05; Student’s test). Scale bar=50µm (A and B), 10µm (C and D).
Figure 5-7  Immunocytochemistry for SHH in the CA1 and CA3 region of the hippocampus.

SHH immunolabelling of neurons in coronal sections through the hippocampal formation subfield CA1 and CA3 region in P60 animals after prenatal treatment with Ro61-8048. SHH immunopositive staining shows increased expression within the CA1 region following kynurenine pathway inhibition. Representative photomicrograph shows distribution of SHH at 40x magnification in the CA1 region of saline control animals (A). Representative image of SHH expression in Ro61-8048-treated animals in the CA1 (B). SHH expression in the CA3 region of P60 offspring in control animals (C). A higher power (40x) SHH expression in the CA3 region of Ro61-8048-treated animals (D). Analysis of the number of SHH immunoreactive cells in CA1 revealed a significant increased in the SHH+ve cell number after prenatal inhibition of the kynurenine pathway as depicted in (E) while no differences were observed in the CA3 region (F). Asterisks indicate statistical significance between experimental groups (**P<0.001; Student’s test). Data represented as mean ± SEM (n=8-9). Scale bar=20µm (A,B,C and D).
5.4.4 Cerebellar NeuN-positive cells after prenatal Ro61-8048

We examined on closely spaced triple-labelling coronal sections of the adult cerebellum in an attempt to identify and characterize the expression of these developmental markers following Ro61-8048 treatment during the late phase of gestation. Inspection of these sections revealed no evidence of staining for DCX which marks postmitotic, migrating neurons, while high levels of diffuse staining were observed for SHH in the granule cell layer which made it impossible to count individual neurons for both markers. Previous studies have demonstrated that SHH expression is confined to the purkinje layer, in line with its production in the purkinje cells (Traiffort et al., 1998). However, observations in our sections exhibited a very faint reactivity for SHH antibody in the purkinje layer, which on the basis of its distribution might reflect labelling of purkinje cells. In this respect, increased secretion or reduced removal and altered metabolism of SHH could possibly be attributable to the indistinct diffuse staining intracellularly and on the extracellular surface of the soma. Immunoreactivity for NeuN was observed in the small granule neurons located within the granular layer, showing cytoplasmic reactivity in addition to nuclear immunolocalization. As for the number of NeuN-positive cells in this area, there were no statistically significant differences in both control and treatment groups (Ro61: 137.85 ± 4.39 vs Saline: 148.76 ± 5.29, p=0.1348, Figure 5-8 (E)).
In the adult cerebellar cortex, most NeuN-positive cells could be seen in the internal granule layer (IGL) with only a few stained nuclei could be found in the molecular layer (ML).

Representative photomicrographs showing morphology of (A) NeuN expression in the saline control IGL of the cerebellum (40x magnification). (C) A higher power (40x, zoom3 magnification). (B) NeuN expression in the IGL of P60 offspring after prenatal administration of Ro61-8048 (40x). (D) A higher power (40x, Z3) DCX expression in Ro61-8048-treated rats. Values are expressed as mean ± SEM, and n=8 animals per group. Quantification of NeuN immunoreactivity in the IGL in a subset of animals prenatally exposed to Ro61-8048 showed no changes between treatments. (E). Scale bar=20µm in A and C and 10µm in B and D.
5.5 Discussion

Confocal microscopy was used to determine the phenotype of all the immunoreactive cells in selected region of interest. Immunolabeling with DCX, SHH and NeuN allowed visualization of newly generated cells that were developing and maturing according to a neuronal fate in both treatment groups. The observation of variable conditions including physiological, pathological and pharmacological stimuli could modulate adult neurogenesis. Therefore the present study was designed to identify possible changes in granule cell number, proliferation and maturation in the hippocampus as to corroborate the molecular data at the morphological and protein level. We also sought to examine whether the modified expression of these proteins are associated with structural changes at the cellular level that might contribute for the modification of neuronal structure and development.

We studied the effects of modulation of NMDA receptor function by administering a known enzyme inhibitor, Ro61-8048 on the localisation of neuronal structure with respect to neurogenesis processes that includes neuroblast differentiation using DCX staining, neuronal migration and orientation using SHH and the maturity of neurons using NeuN immunocytochemistry, in the hippocampus at P60. During early development of the brain, NMDA subtypes of glutamate receptors and α7NCR receptors of glutamatergic and cholinergic mechanisms are critically involved and play central roles in neuronal growth, differentiation and synapse formation (Bard and Groc, 2011, Cull-Candy et al., 2001). It has been established that endogenous KYNA functions as an endogenous agent capable of directly affecting both NMDARs and α7NCRs. Antagonistic properties of KYNA at the NMDA receptor account for its ability to reduce excitation and are responsible for its neuroprotective and anticonvulsant properties (Foster et al., 1984). However, these effects may also pose for a pathophysiologically relevant function in neuropsychotic diseases such as schizophrenia, which includes abnormal brain KYNA levels (Muller and Schwarcz, 2006). As endogenous KYNA might also be involved in the processes underlying learning and more generally synaptic plasticity, fluctuations in KYNA levels can be envisioned to influence these processes. As elaborated in previous studies, a range of pharmacological agents have been used to probe the physiological and
pathological roles of KP metabolites. One approach to influence the levels of KP metabolites is based on the pharmacological blockade of KMO, the enzyme responsible for the formation of 3-HK and QUIN (Stone et al., 2012, Stone, 2000). This concept is supported by our previous studies showing that the administration of Ro61-8048 during late gestation of rats were reported to alter the relative concentrations of endogenous KP metabolites and resulted in disordered neuronal development.

We have demonstrated an increased in the levels of kynurenine and KYNA in the maternal plasma and also in the embryo brains 5 hour post-injection. The maternal levels of these metabolites normalize after 24 hour but remain elevated in the embryonic brain. It seemed that the outcome of administering pharmacological compounds that block KMO somehow shunted the metabolism of KP toward the production of KYNA. It has been suggested that the changes in the protein expressions of some of the target molecules observed at developmental ages of P21 and P60 were likely to be caused by the increased levels of these KP metabolites, particularly KYNA (Forrest et al., 2013a, Forrest et al., 2013b).

5.5.1 Protein localisation in the hippocampus

To test the hypothesis that an early modulation in the endogenous levels of KYNA induced by blocking one of the main enzymes in the KP, would produce long lasting changes in the generation and development of neurons, we focused on the hippocampus because it possesses a substantial capacity for regeneration and presents itself as a unique form of neural circuit plasticity. A substantial body of evidence regards a crucial role for hippocampal neurogenesis in mediating specific cognitive functions. In line with its well-recognised role in cognitive processes, any modification as in decreased or abnormal process is strongly correlated with deficits in hippocampal structure and function in various neurological disorders including epilepsy, ischemia and schizophrenia (Curtis et al., 2003b, Eriksson et al., 1998).

The dentate gyrus of the hippocampus is one of the few mammalian brain structures that continue to generate new neurons through adulthood. It has been demonstrated that adult-generated cells especially in the dentate gyrus of rats
develop the morphological characteristics of granule cells, receive synaptic input, then extend axons into the mossy fiber pathway and express a number of markers of mature neurons. These sequential steps of adult neurogenesis ranging from neural precursor proliferation, migration, differentiation to synaptic integration of newborn neurons are tightly regulated by a variety of extrinsic and intrinsic factors, most of which still remain uncharacterized (Zhao et al., 2008, Kempermann et al., 2004). The extended period of granule cells genesis and the ability for continual restructuring of neurogenic areas according to the current environment and involvement of signalling cues, represent a dynamic process that could be regulated by natural factors including physical activity, hippocampal-dependent tasks, NMDA receptor activity, hormones and growth factors. The late-generated neurons in this brain region might render the dentate gyrus particularly sensitive to environmental perturbations that may adversely alter hippocampal structure and function. In addition, it has been brought forward into consideration that treatments which typically enhance or normalize the cell generation process could ameliorate the behavioural alterations in animal models or humans. This data substantiates a ground cause that adult hippocampal neurogenesis may be part of intrinsic requirement of therapeutic targets for behavioural of distinct neurological diseases (Eriksson et al., 1998, McDonald and Wojtowicz, 2005, Kempermann et al., 2004).

In most studies to examine morphological details of newborn neurons have been made possible by the application of either retrovirus-mediated gene transfer or the use of thymidine analogue, 5'-bromodeoxyurindine (BrdU) which incorporates into the DNA of dividing cells during the S-phase of the cell cycle (Zhao et al., 2006, Rao and Shetty, 2004). The identification of newly born neurons has also been accomplished by immunostaining with DCX. In animal studies of adult rodents, DCX has been identified as a promising candidate for the identification of newborn immature neurons in which its expression specifically persists in the hippocampus with clear labelling detected in both cell bodies and the dendritic processes that extend into the granule cell and molecular layers of the dentate gyrus. Previous studies have showed that the expression of DCX appears to be persist for a prolonged period and overlapped with both immature as well as mature neuronal markers (NeuN), indicating that DCX expression in postmitotic granule cells is quite extensive during the early
growth period of the neurons including dendritic and axon growth. Counting of
eurons that are positive for DCX in hippocampal sections particularly in the
dentate gyrus gives a good estimate of the newly generated neurons that exhibit
the phenotype of differentiated granule cells. Thus, transient expression of DCX
during specific times of cell genesis justifies the ability of DCX as a reliable
marker of newly generated neurons in the adult hippocampus (Rao and Shetty,

In our study, DCX positive staining exhibits the phenotype of neurons with
staining around the soma and also in dendrites. DCX immunostaining revealed
newly formed neurons in the DG and the overall production of new neurons were
maintained at the same levels as controls. Characterization of DCX-positive cells
having the phenotype of differentiated granule cells showed that dendritic
process in the control group showed a comparatively complex branching of
apical dendrite in the molecular layer which presumably indicate more mature
new neurons while neurons in Ro61-8048-treated specimens exhibited either no
or with short processes which did not reach deep into the molecular layer.
Analysis of the dendrites revealed that the new granule cells in the Ro61-8048-
treated sections exhibit fewer number of cells with a complex dendritic spread.
The shorter apical dendrites with narrower dendritic spread in newly born
granule cells of the adult, most likely reflect changes in differentiation of the
cells having a relatively immature morphology. Although the precise functions
that depend on the production of new neurons in the DG throughout life are not
defined, a range of studies have implied that the cells might be involved in
aspects of normal hippocampal function, such as learning and memory. Along
this line, studies reported an age-related decrease in adult neurogenesis and has
further been proposed as a factor in the age-related decline of cognitive ability
(Ben Abdallah et al., 2010, McDonald and Wojtowicz, 2005, Farioli-Vecchioli et
al., 2008).

Stressful experiences during development may also potentially be involved in
impaired performance of hippocampal-dependent learning in adulthood by
suppressing the production of granule neurons. Elevation of glucocorticoid
corticosterone and stimulation of glutamate release following stress diminishes
the number of proliferating cells in the DG. This evidences suggested that stress
exhibits a suppressive effect of glucocorticoid and NMDA receptor activation which decreases the production of granule neurons in the developing brain and might possibly have a negative impact on learning and memory in adulthood (Gould and Tanapat, 1999). In contrast, several other types of brain injuries such as traumatic brain injury, ischemia, epileptic seizure and stroke enhance the production of new neurons in the adult DG (Wennstrom et al., 2003, Yu et al., 2008). Similarly, studies have suggested that the therapeutic mechanisms of antipsychotics are related to their stimulatory effect on neurogenesis. The effects on proliferation in the neurogenic regions of the adult rat brain have been observed by incorporating the proliferation marker BrdU, while neurogenesis has been described following an increase in markers of neuronal differentiation and survival such DCX and NeuN (Toro and Deakin, 2007).

The secreted factor SHH is a signalling protein that plays multiple roles in the regulation of early patterning of neural tube and subsequently has been found to stimulate the production of progenitor cells by actively being involved in the regulation of neuronal precursor proliferation, migration, differentiation and cell survival. This indicates that SHH is a powerful regulator of stem cell maintenance in the developing embryonic brain (Traiffort et al., 2010). Involvement of this morphogen in the control of stem cell properties in the adult brain have been studied in vivo and in vitro. SHH has been shown to regulate the proliferation of adult hippocampal neural stem cells and represents its function in the adult nervous system. This proliferative effect of SHH through activity-dependent synthesis or secretion within the hippocampal neurogenic area may indicate a mechanism for regulating neurogenesis and could be implied as one of the therapeutic potential for the regeneration of neural tissue in neurodegenerative disorders (Lai et al., 2003, Palma et al., 2005). Several pathological conditions for instance ischemia, hypoxia and seizures have been shown to induce a rapid regulation of key genes of the SHH signalling cascade and that SHH signalling is required for adult neurogenesis. This implies that activation of SHH and its receptor components through activity-dependent neural plasticity may be involved in the regulation of adult brain neurogenesis (Banarjee and Rajendran, 2005, Sims et al., 2009).
Another major finding of this study was the localization of SHH in the hippocampal formation. In the study described here, we found that prenatal modulation of kynurenine pathway by inhibiting KMO enzyme upregulated the expression of SHH. Most notable is our finding that the newly generated neurons produced by in utero exposure to Ro61-8048 are able to express SHH, as demonstrated by a significant increase in the number of cells expressing SHH in the hippocampus. This stimulatory effect has been observed in the dentate gyrus and CA1 region. Although it is unclear by what mechanism NMDA antagonistic properties of KYNA up regulates SHH, the increase may likely involve other intermediate regulatory steps that regulate the expression of SHH. It is possible to suggest that SHH produced in the hippocampal subregions particularly the CA3 and dentate gyrus, might play a role in regulating axonal guidance from the newborn granular neurons of the dentate gyrus as the CA3 region receives the mossy fiber output from the granular neurons of the dentate gyrus (Charytoniuk et al., 2002).

Our results show that despite high levels of SHH expression seen in the CA3 region, it did not reach statistical significance. In addition to that, we also observed a diffuse staining of granule cells throughout the hilar region of the hippocampus. As the function of secreted protein SHH has generally been attributed in relation to cell proliferation and migration, the expression of SHH in fully differentiated neurons in this area could be associated with errors or modification in cell migration process. These data demonstrate that an in utero challenge with Ro61-8048 has a significant, stimulatory effect on the generation and development of new neurons in the selected areas of hippocampal formation. The increased activation of the SHH pathway in this region could potentially increase the growth and survival of newly generated granule cells and their developing axons. These findings raise the possibility that the early modulation of NMDA receptor function through KYNA on the expression of SHH and its unidentified receptor components could contribute to the influence on adult neurogenesis. It also suggest that the SHH pathway may continue to have an important role in adulthood in regulating structural plasticity. Further studies are thus required to address whether the effects of KP inhibition also involve other SHH receptor components which could further regulate the response of SHH signalling mechanisms. Unlike our protein expression study (chapter 4)
which showed significant decreases of SHH protein in the hippocampus after exposure with Ro61-8048 while no changes were seen in mRNA levels may suggest a possible explanation for this discrepancy. We assumed that the reduced protein level seen in whole hippocampal lysates of adult animals is possibly due to decreased translation or altered stability or degradation of the protein at the time of examination.

Of several molecular markers that could be linked to neural development, NeuN has gained widespread acceptance as a reliable immunocytochemical marker for neurons in neurodevelopmental research. It is also has been incorporated as a useful tool in diagnostic neuropathology where its specific expression in postmitotic neurons has made it possible to distinguish and localize neuronally differentiated cells. Immunostaining analysis showed that NeuN was primarily localized to the nuclei of postmitotic neurons throughout the CNS and the immunoreactivity can be detected in multiple vertebrate species suggests a considerable conservation of this antigen (Mullen et al., 1992). The present findings are consistent with previous studies that indicate preferential nuclear localization of the protein, but also show NeuN immunoreactivity in the cytoplasm (Lind et al., 2005). The hippocampal granule cells and pyramidal cells of the CA1 and CA3 regions are finely stained by this antibody and the dendrites emanating from these cells are clearly devoid of staining.

### 5.5.2 Protein localisation in the cerebellum

Numerous studies have confirmed that the process of generating new neurons in the adult brain, after the foetal and early postnatal development has ceased is a robust and continual step that occurs within specific neurogenic areas of the adult brain. Although it remains controversial, recent findings have proposed that adult neurogenesis could also occur in brain areas such as the cortex and the substantia nigra (Gould et al., 1999, Zhao et al., 2003, Magavi et al., 2000). It is conceivable that addition of new neuron to these areas where neurogenesis is not normally detected in adulthood play a special role in functions associated with cognitive processes. For instance, in one study on adult maraque by using a combined technique of BrdU labelling and fluorescent retrograde tracing, the research suggested that the SVZ is primarily the source for an additional population of new neurons that migrate through fiber tracts to neocortical
regions (Gould et al., 1999). Moreover, cells with proliferative properties also appear to reside in some other adult CNS regions where neurogenesis occurs rarely under non-pathological conditions. The neurogenic potential of these cells could only be demonstrated after injury, such as stroke and ischemia (Lichtenwalner and Parent, 2006). This assumption is based on a finding that injuries to the brain accompanied by responses that also include mitogenic recruitment of progenitor cells besides other local activation such as astrocytes and angiogenic response. Although a low level of neurogenesis in other brain regions is detected, appropriate combination of multiple stimulatory exogenous and endogenous factors may be necessary for inducing the regeneration potential of neurons at lesions in areas with slow turn-over rates (Yamamoto et al., 2001).

Certainly, elucidation of the molecular nature and mechanisms of the actions of these signals will be essential for further understanding the regeneration capacity of the adult CNS. In this study, we examined the expression of selected developmental markers in relation to cell genesis in the adult cerebellum. We demonstrated regional specificity for DCX, SHH and NeuN immunoreactivity in the adult brain. Here, no detectable expression of DCX was seen in the cerebellar cortex. The lack of expression for this marker could be explained as part of adaptation or resistance to change during cerebellar development. Another possibility is that, since the DCX expression reflects ongoing neurogenesis and migration of immature cells in the adult, the failure for staining and no effect in the overall number of mature neurons at this point of developmental age might suggest that adult born cells have long past both neurogenesis and the neuronal migration stage. Despite no quantification of SHH expression in the adult cerebellum, the presence of SHH staining within small granule neurons and extracellular area demonstrate that SHH is required for granule cell development. In support of this, a number of lines of evidence have indicated the role of mitogenic factor SHH in regulating cerebellar growth and development. This is based on the findings that SHH control granule neuron precursors proliferate during embryogenesis and postnatally, and highlight a mechanism responsible for modulating rapid proliferation of GCP in the germinative layer, external granular layer (EGL). Inhibition of SHH antibodies results in reduced GCP proliferation and caused reduction in the formation and
growth of the folia (Wechsler-Reya and Scott, 1999). SHH, secreted by purkinje cells and binds to Ptc, a receptor on GCPs, thereby unleashing the Smo transmembrane protein to cause the activation of downstream intracellular events. It has been demonstrated that GCP proliferation is dependent on interactions with purkinje cells, as demonstrated by the observed decrease in granule cell number when purkinje cells are lost because of genetic mutations (Charytoniuk et al., 2002, Corrales et al., 2006). In this regard, identification of other components of this signalling pathway during postnatal cerebellar development may provide some better understanding on the functional mechanism and interaction between the Purkinje cell and granule neurons development.

As NeuN has been used widely to identify postmitotic neurons and is found to be expressed by neurons throughout the nervous system, we sought to examine the developmental progression of the rat cerebellum by including NeuN. Based on initial observations which in agreement with previous studies that NeuN is not expressed in cerebellar purkinje neurons, it might be useful to include NeuN as a marker to contrast developmental expression of cerebellar neurons for example excitatory granule cells to that of purkinje neurons (Mullen et al., 1992). We observed that expression of this antigen was restricted to granule neurons of the adult cerebellum. The heterogeneity of NeuN immunoreactivity among cerebellar granule cells, as also evident in our specimens might suggest a subpopulation of distinct postmitotic cells which could be related to granule cells activity, synaptic input or NMDA receptor subunits (Vallano et al., 1996). We have shown earlier that the expression of NMDA receptor subunits in this region were not affected by the prenatal inhibition of the KP, thus might explain the lack of changes on the number of these neurons seen within the cerebellar cortex.
5.6 Conclusion

Our study supports the evidence that cell genesis occurs and retains the potential for self renewal throughout life, especially in the adult as part of the brain’s adaptive cellular plasticity to changes. We report here that gestational exposure to a compound that is able to modulate the balance of kynurenine metabolites, has a long lasting stimulatory effect on the generation and development of new neurons in the hippocampal formation, particularly in the dentate gyrus. This indicates that these effects of in utero exposure to a pharmacological substance induced alterations in protein levels which were accompanied by changes in cell numbers and survival that were anatomically localized and involved specific subpopulations of neurons. Our results indicate that maternal exposure to Ro61-8048 maintained the numbers of proliferative progenitors and immature granule cell populations in the hippocampal dentate gyrus. As the DCX immunopositive neurons with vertically orientated dendrites are relatively more mature among the overall DCX-expressing newly born neurons, a smaller fraction of such neurons in the Ro61-8048-treated specimens suggests that the early production and dendritic growth of newly born neurons happened at a much slower rate and also suggest that developmental exposure to Ro61-8048 suppresses the maturation of late stage granule cell lineages. It can be assumed at this point of study that the increased activation of growth and survival pathways as indicated by SHH, could lead to multiple changes in granule cell morphology that might include dendritic as well as axonal growth.

What is striking is that the regulation of the cell genesis was affected months after the animals were initially exposed to Ro61-8048 in utero, suggesting that the neural stem cell or progenitor cell populations that give rise to new hippocampal granule cells are impaired early on at the time during which the injections take place. It remains unclear whether this alteration represents a slowing of the cell cycle or maturation process. However, it does appear that these effects are at least in part mediated by the KP metabolites imbalance produced by administration of Ro61-8048. Future studies will be needed to determine the contributions of other SHH signaling molecules and the mechanism involved in the increase in metabolism and growth of cells. Such studies could prove to be particularly useful in the context of neurogenesis in determining the relationship between cellular growth pathway and modulation.
of NMDA receptor. The possibility that the present changes in the postnatal brain could also be interpreted as the brain’s attempt to compensate for the receptor’s decreased activity must be considered. However, the present findings of an increased numbers of postmitotic cells and SHH-labeled granule cells suggest that, under certain conditions, increased generation of granule cells can occur in the more commonly recognized dentate gyrus of the hippocampus and also in other hippocampal subfields of adult animals. Although our results demonstrate that cells in the adult brain undergo cell division and that some of the newly generated cells can survive and differentiate into cells with morphological and phenotypic characteristics of neurons as indicated by NeuN expression, we have not proven that these newly generated cells are functional. However, this does provide a basis to investigate the influence of environmental stimulation on the fate as well as rate of neurogenesis.

Our interest was to further examine any alteration in the expression of neurogenesis markers in the cerebellum as it has a unique developmental profile. The cerebellum plays a pivotal role in motor function including speech, muscle tone, posture and coordination of movement. However, there is currently growing awareness that neurodevelopmental disorders are also associated with cerebellar deficits and learning impairments. Cognitive impairments are also occasionally related to cerebellar pathologies and it has been suggested that functional connections between the cerebellum and other regions of the CNS are linked with its function in emotional processing and cognition (Hoppenbrouwers et al., 2008). The results of our immunocytochemical analysis showed that Ro61-8048 administration had no effect on the pattern and distribution of granule neurons in specimens examined at P60 when compared to control. However, these observations were in contrast by our western blot results which indicate a significant downregulation of SHH and also DCX at the same developmental age in the whole cerebellar tissues. qPCR analysis also found that SHH mRNA levels remained unchanged in these Ro61-8048-prenatally exposed groups by comparison to control counterparts.
6 Prenatal modulation of the kynurenine pathway alters the morphology of neurons in the hippocampus of the adult rat brain.

6.1 Introduction

Relatively modest elevations of extracellular KYNA, caused by the direct application of kynurenine or KYNA itself or by systemic kynurenine-3-monooxygenase (KMO) inhibition have remarkable biological consequences. Moreover, KYNA levels are significantly elevated in the brain and the cerebrospinal fluid of individuals with schizophrenia and Alzheimer’s disease patients. The most influential evidence of the involvement of NMDA receptors stemmed from studies in which blockade of the receptor prevented neuronal degeneration, raising the possibility that enhanced inhibition of NMDA receptors and also αNCR by KYNA has a causative role in the defining cognitive deficits seen in these diseases (Erhardt et al., 2009, Erhardt et al., 2007).

It is important to note that dendrites are not completely static structures and its diversity is a striking characteristic of the brain. The size and shape of dendrite trees determine where it can receive inputs and how it can integrate into neuronal networks. Evidences have highlighted continual dynamic changes of the dendrites as part of the establishment of neuronal circuitry during development. Neuronal activity including membrane depolarization, electrophysiological stimulation and NMDA receptor-mediated activity are among potent stimulator of dendritic remodelling and stabilization. As dendrite branches are becoming more stable in mature neurons and through with combination of neurotrophic signalling factor and associated protein, this stability is essential for the proper connectivity of neuronal networks. In addition, dendrites can also undergo rapid structural changes in response to a variety of stimuli and this plasticity is essential to the cellular response of learning and memory. By contrast, any alteration in dendrite growth and structure can result in impaired brain function, and contributes to the pathology of various illnesses such as schizophrenia and Alzheimer’s disease (Koleske, 2013, Cline and Haas, 2003).
The Golgi impregnation method has been utilised in many morphological studies that have attempted to describe cellular changes. Structural changes provoked by external stimuli later in life are very much related to plasticity. Changes in dendritic morphology represent one persisting form of plasticity that is an important neural correlate of NMDA-mediated neural activity. Plasticity of dendritic morphology has been demonstrated in hippocampal formation as implicated in NMDA receptor activity. One key idea is that the hippocampus is one of the most plastic regions in the brain and plays an essential role in inducing plasticity enabling certain forms of learning and memory consolidation (Kempermann et al., 2000, Bannerman et al., 2014). In this regard, the long term effects of KMO inhibition were assessed as structural modifications of dentate granule cells which include somata size and dendritic segments and length, since these anatomic parameters can be correlated with NMDA receptor mediated synaptic responses in the hippocampus.

6.2 Research aims

The aim of the present study was to assess the consequences of prenatal inhibition of the kynurenine pathway on the morphology of dentate granule cells in the hippocampus by performing a quantitative study of dendritic arborizations using the Golgi impregnation method. For the quantification of neuron morphology, the total length and segments of dendrites in individual granule cells were measured, and the complexity of the dendritic branches were determined by Sholl analysis.

6.3 Experimental approaches

6.3.1 Prenatal treatment

Pregnant rats were injected with the compound Ro61-8048 (100mg/kg) on embryonic days 14, 16 and 18, and gestation was allowed to proceed normally. Both male and female pups were included in this study and they remained with their mothers until weaning at postnatal day 21 (P21) and were assessed at postnatal day 60. Control animals were born from saline-treated mothers and were evaluated at the same age. At postnatal day 60, the pups from the
respective groups of litters (4 pups per litter, 3 litters per group) were deeply anesthetized with sodium pentobarbital before cervical dislocation.

6.3.2 Golgi-impregnation method

Golgi staining was performed using the FD Rapid Golgi Stain Kit according to the manufacturer’s instructions. Blocks from experimental and control brains were immersed in a freshly prepared solution of equal parts of Solutions A and B, and stored at room temperatures for 2 weeks. This solution was replaced with fresh solution after the first 24 h of immersion. Then, the brain tissues were immersed in Solution C and stored at 4°C for at least 48 h and shielded from light. Solution C was also refreshed after the initial 24 h of immersion. Brain blocks were blotted dry with tissue paper and sectioned at 200 µm and mounted with Solution C on gelation-coated microscope slides. The slides were allowed to dry naturally at room temperature while being shielded from light. They were then stained with using a solution containing one part of Solution D, one part of Solution E and two part of distilled water for 10 min after which the slides were rinsed with dH₂O twice for four min each. A dehydration process with graded concentrations of ethanol were done, and cleared with Histoclear solution. Finally, the sections were cover slipped with Histomount.

6.3.3 Morphometric analysis

From the time of killing the rats, all histological, drawing and Sholl analysis scoring were carried out on rats coded as not to reveal the treatment condition in order to preclude bias. Neurons were sampled from the dentate gyrus of the hippocampus. The exact localization was registered for the granular cell layer with a 40x objective lens. By considering all the morphological criteria for the selection of neurons as described earlier, drawings of the Golgi-impregnated granule cells from both treatment groups were made manually by hand with the aid of a camera at a final magnification of 40x to illustrate the pattern of dendritic segments. Each neuron was therefore examined for soma size, for number of primary dendrites, for number of dendritic arborizations (branch points) and for number of dendritic intersections crossing each concentric ring. The number of impregnated granule cells that fulfilled the morphological criteria varied in a given coronal section from any brain, ranging from one to
four cells per section. As a whole, 137 neurons (control: 69 neurons/11 animals, Ro61-8048: 68 neurons/9 animals) were drawn individually and used for Sholl analysis. The Sholl analysis evaluates the amount and distribution of the dendritic material at increasing distances from the cell body. This is accomplished by placing a template comprising a series of concentric circles of diameters at equidistant farther (20µm) intervals from the soma and quantifying the numbers of intersections of the branches with each of the circles (Sholl, 1953). Morphometric parameters included were the total number of branches and total dendrite length and were used to assess the overall magnitude of morphological changes following prenatal modulation of the kynurenine pathway. An estimate of mean total dendritic length (in µm) was made by multiplying the mean total number of intersections by 20. While for branch order analysis (to indicate primary and secondary dendrites), branches emerging from cell bodies were considered primary segments. After the first bifurcation, branches were considered second order of segments. Sholl analyses were conducted on the mean values per animal for the above morphometric parameters. Data on mean group differences in soma size, total dendritic length and number of branching points were analysed by Student’s t test. The analysis of dendritic complexity was subjected to a two-way analysis of variance (ANOVA) followed by Tukey’s post hoc analysis to examine the contributions of drug treatment, sample number and distance from the cell soma. The results were expressed as mean ± S.E.M. and significance was set at p<0.05.

6.4 Results

6.4.1 Morphology of granule cells in the dentate gyrus

Our aim was to characterize the possible morphological alterations of granule cells in hippocampal slices taken from control animals and from experimental rats subjected to prenatal administration of the KMO inhibitor, Ro61-8048. To this end, rapid Golgi method was adapted to study the morphology of granule cells in these rats and their age-matched controls. We chose to study the morphology of granule cells, as this neuron represents the principal neurons of the dentate gyrus. In the adult rodent brain, these neurons are arranged in a densely packed layer and show a highly uniform morphology of their V-shaped apical dendritic trees (Rahimi and Claiborne, 2007). In the Golgi preparations,
granule cells in the DG were easily identified according to their characteristic soma location and dendritic morphology. Granule cell bodies were round or oval shaped with a greater diameter oriented perpendicular to the granule cell layer. Typically, a granule cell gave rise to an apical dendrite that extended into and arborized within the molecular layer. Whereas the axons of granule cells originated from the hilar pole of the soma and projected into the hilus. The dendritic trees of impregnated neurons were visualized in photomicrographs of the upper and lower blade of the dentate gyrus as seen in Figure 6-1 (A&C). In general, the appearance of granule cell bodies and the arborisation pattern of their apical dendrites within the molecular layer of the DG in treated animals were similar to those of the control group which seemed to be evenly distributed and formed a relatively homogenous cell population. Granule cells with well-branched dendrites could be seen extending into the molecular layers, especially in the upper blade of the DG (Figure 6-1 (B&D)). Our observation in the specimens showed that granule cells from control and treated adult rats have no thick processes arising from the hilar side of their somata that could be interpreted as basal dendrites. An example of camera drawings used for Sholl analysis of dendritic tree complexity is shown in Figure 6-2. Even though overall observations of dendritic tree shapes for granule cells are very much alike and similar for both control and treated rats, there is a relatively significant decrease in the analysis of the general growth parameters of the dentate granule cells (Figure 6-3).

Several aspects of the dendritic tree including soma size were analysed. The area of cell bodies (soma) and branching points of the neurons were not affected by prenatal administration of Ro61-8048. We noted that the cell body of an adult granule neuron in the rat is approximately in a range between 14-17 µm wide and 15-20 µm long. Although in general, the appearance of granule cell bodies and the arborisation pattern of their dendritic trees in the control group were somewhat similar to those of Ro61-8048, the Sholl analysis on the total dendritic number which represents an average dendritic segments in each neuron was significantly lower in the Ro61-8048-exposed specimens than in controls (Ro61: 6.36 ± 0.22 vs Saline: 7.39 ± 0.05, p= 0.01). The number of dendrite segments of each order characterizes the degree of branching arborization. When the data were subdivided into distinct parts of the dendritic segment, it was apparent
that there were reductions in dendrite number in tissue from Ro61-8048-exposed rats only in the secondary dendrites. In this regard, separate measurement on the relative contributions of the primary and secondary dendrites in this region demonstrates significant differences between the groups, with fewer numbers of secondary dendrites in the apical dendritic regions of hippocampal sections from rats exposed with Ro61-8048 (Ro61: 3.77 ± 0.06 vs Saline: 4.38 ± 0.17, p=0.029).

Whereas, there were no differences in the number of primary dendrites in the same region (p=0.1019) (Figure 6-3 (C)). Our data indicate between 1-4 primary apical dendrites exit from the apical pole of the cell body and branch out relatively close to the soma. We noted that most branching occurs within 60-100 µm of the soma. Based on Golgi-impregnated cells, when the data were compared with controls, the estimated overall length of dendrites for the Ro61-8048- exposed rats was significantly decreased by 14% (Ro61: 904.65 ± 28.0 µm vs Saline: 1051.93 ± 33.72, p=0.03). However, the length of the primary and secondary dendrites did not differ between treated and control animals (p=0.076 for primary and p=0.075 for secondary) (Figure 6-3 (D)). Further analysis on overall dendritic branching complexity between the control and Ro61-8048-exposed animals were made using a two-way ANOVA, taking into account the elements of treatment, number of samples and number of intersection levels. The analysis revealed no interaction between treatment and dendritic distance from the cell body p=0.49 (Figure 6-4) but a highly significant difference between the treatments (control vs Ro61-8048) with p<0.0001. However, post-hoc analysis between individual data points showed no significant differences, although the possibility that there are small differences of this analysis cannot be excluded. Generally, as the complexity of the dendrites can reflect a propensity for neuron connectivity, this might suggest that kynurenine modulation as well as NMDA receptor activity appears to be associated with a loss of dendrite arbour complexity.
Figure 6-1  Morphology of dentate granule cells in the hippocampus at P60
(A,C) Low magnification of the hippocampus of control and Ro61-8048-treated rats showing Golgi-impregnated neurons (4X, scale bar=200µm).  (B,D) Photographs of representative dendritic trees of Golgi-impregnated granule cells in the dentate gyrus of control and Ro61-8048-treated rats in 200µm thick sections. Generally, light microscopic examination of Golgi-impregnated tissue from control and experimentally treated brains revealed reliable and consistent staining of granule cells. We noted granule cells with well-branched and bifurcated dendrites extending into the inner and outer molecular layers of the dentate gyrus and almost reaching the hippocampal commissure (10X, scale bar=100µm).
Figure 6-2  Reconstructions of Golgi-impregnated granule cells from control and Ro61-8048-treated rats

Representative drawings of dendritic trees of a granule neuron traced at P60. Granule cells were drawn from the upper and lower limbs of the fascia dentate of the hippocampus with approximately equal selection from each of these areas. Overall observation on the dendrites shows that the dendrites form a cone-like shape that spread across the molecular layer. The appearance of granule cell bodies and the arborisation pattern of their dendritic trees in the control group (A&B) were somewhat similar to those of Ro61-8048 (C&D). Scale bar =20µm.
Figure 6-3  Morphological measurements of dentate granule neurons following prenatal modulation of the kynurenine pathway

Quantitative assessment of neuronal morphology by Sholl analysis showed (A) soma diameter (B) mean branching point per neuron, (C) dendritic number, (D) total length of apical dendrites and (E) number of intersections of dendrites with concentric spheres surrounding the soma. A mean value of each morphometric parameter based on five to eight neurons per animal (saline: n=69, Ro61-8048: n=68). t-test comparison with age-matched controls, * p<0.05.
Quantitative assessment by Sholl analysis revealed prenatal kynurenine modulation reduced dendritic complexity in the number of intersections of dendrites with concentric spheres surrounding the soma. Data are expressed as mean ± S.E.M. (n= 69 cells/11 animals for control, 68 cells/9 animals for Ro61-8048). Sholl analysis revealed reduced dendritic branching complexity in Ro61-8048 compared with age-matched littermate controls at Sholl radii 20-280 µm from soma.
6.5 Discussion

The granule cells are the principal neurons of the dentate gyrus. In the adult rodent brain, these neurons are arranged in a densely packed layer and show a highly uniform morphology of their V-shaped apical dendritic trees. The dendrites which represent the main receptive structures of neurons are branched and all dendritic segments are covered with spines. The primary contribution of dendrites to a neuron’s mode of connectivity is through their characteristic branching and extension into specific domains. This branch ordering schemes wherein dendrites emerging from the cell soma are primary and their respective branches are considered secondary and so on, have been used to characterize the density of dendritic arborization (Rahimi and Claiborne, 2007).

The use of Golgi method which requires complete impregnation of neurons and separation between stained neurons to permit the visualisation of dendrites had enabled us to study the general morphological changes of granule neurons in the adult rat. The results of the present study have shown that prenatal modulation of the kynurenine pathway which alters the balance of its neuroactive metabolites regulates the dendritic remodelling and stabilization in the developing neuron. The results indicate that the dendritic morphology of the selected hippocampal area present important changes in rats prenatally exposed to Ro61-8048. Overall observations on the morphological characteristics of the granule cells in term of soma size and branch points did not differ between saline and Ro61-8048-treated animals. In our study, a simpler scheme of counting the number of branch points in the entire dendritic arbour provides a sense of how branched a neuron is, but this does not indicate the degree to which they fill the space and its functional connectivity. The main changes found were reductions in the number of dendritic segments and in the total dendritic length of neurons from dentate granule cells. The complexity of the dendritic system in the treatment group also appeared to be less robust than that seen in the control rats. In this study, we also aimed to determine whether basal dendrites appear on granule cells of adult rats as a consequence of KP modulation. Such an analysis would provide information about the formation of dendrites. The lack of basal dendrites in the dentate granule cells of control animals in this study confirmed previous finding that showed the adult rat
hippocampal granule cells do not display basal dendrites. In contrast to humans and monkeys, hippocampal formation basal dendrites which are studded with spines appear to be a functional and integral part of normal granule cells (Seress and Mrzljak, 1987).

In the treated specimens, no appearance of basal dendrites were observed. However, formation of basal dendrites have been observed in granule cells in response to epileptogenic stimuli and this may represent one of the adaptive cellular changes of neuroplasticity of granule cells (Spigelman et al., 1998). Although we did not measure the spine density for dentate granule neurons, due to limited numbers of good impregnated neurons which could produce clear identifiable spines or spine heads, the data obtained with this technique could indicate that Ro61-8048 produced a reduced number, length and complexity of the dendritic system. Based on the increased KYNA concentration in the embryonic brain in response to Ro61-8048 administration during late gestation (Forrest et al., 2013b), we suggested that NMDA receptor blockade could be a possible mechanism underlying these effects.

Previous reports have demonstrated that early dendritic arbour growth relies on NMDA receptor activation and that this receptor also involved in the refinement of synaptic connections during postnatal development, suggesting an intimate association between glutamatergic transmission and dendritic arbour development. In this regard, NMDA receptor activity might affect dendritic arbour development during an early developmental window, in which coincide with the occurring of the granule cell genesis in late embryogenesis when they started to contribute to the synaptic current (Cline, 2001). The role of this receptor might change as the granule neurons in the dentate gyrus undergo periods of development and maturation. In the rat, the neurons go through a period of maturation during which the dendritic tree is refined and the dendritic remodelling occurs with elongation of some branches and the loss of others between days 14 to 60 (Rahimi and Claiborne, 2007). Data from our lab demonstrated that granule cells have less dendritic segments which are largely attributable to a significant decrease in the number of secondary dendrites and in turn have slightly shorter dendritic trees. This indicates that dendritic branch loss in response to Ro61-8048 may be affected by incoming neuronal activity.
The changes in dendritic structure seen here are likely to have effects on neuronal function. To correlate with this finding, our recent findings demonstrated a reduction in the amplitude of LTP and paired-pulse inhibition which could be in part, a consequence of the altered expression of one of the NMDA receptor subunits in the offspring following Ro61-8048 exposure where we noted earlier a significant reduction in expression of the NR2A subunit in the hippocampus (Forrest et al., 2013a).

Established roles for NMDARs in dendrite development have been mentioned earlier and this suggests specific roles for particular subunits especially NR2A and NR2B in determining the functional properties of the NMDAR. In this context, subunit composition of synaptic NMDAR controls normal development of synapses by regulating the rate of synapse formation and overall dendritic morphological outcome (Ewald et al., 2008, Vastagh et al., 2012). Contribution of specific NMDAR subunits during synaptogenesis are still not clearly defined, but in vitro experiments have showed that reduced glutamatergic synaptic transmission following upregulation of NR2A might be associated with a reduction in the number of synapse. Whereas expression of NR2B provides greater structural plasticity by establishing appropriate synaptic contacts (Gambrill and Barria, 2010). Following exposure to KP modulation during late gestation, NR2A protein level decreased significantly in the foetal brain and remained low in the hippocampus at P60. A different observation was noted in NR2B expression, with significantly increased expression after 5h and P21 (Forrest et al., 2013a, Forrest et al., 2013b). Interestingly, out results also suggest that the observed variation of the levels of both subunits expression during development following prenatal exposure to Ro61-8048 might underlie the neuronal modification changes seen in the hippocampus. Total dendritic branch length and segment numbers provide a good indication of the growth behaviour of a neuron. Notably, both indicators in the treated group were comparably fewer than those in control. These data demonstrate that shift or imbalance in the synaptic NR2A and NR2B levels and the persistence of these alterations into adulthood suggests that modification of glutamatergic neurotransmission leads to structural changes in the general growth parameters of the dentate granule neurons in the hippocampus. Since the results emphasize the interdependence of receptor subunits, selectivity
effects of Ro61-8048 on synaptic NMDAR transmission by utilising respective subunits antagonist is important for future consideration of in vitro assessment.

It seemed that blockade of the NMDA receptor (in this case, by KYNA) could have some implications on the dendritic branch dynamics. It is well established that the glutamate receptors of the NMDA subtype play a central role in the process of LTP and also synaptic plasticity. The effects of NMDA receptor-mediated synaptic plasticity on dendritic arbour growth have been well studied. It has been shown that NMDA-mediated responses promote dendritic arbour growth and also causes a transient increase in both the size and density of dendritic spines, whereas blocking the receptor reduces the growth of dendrites (Lin et al., 2004a, Wedzony et al., 2005). In congruent with our data which support an association between synaptic transmission and dendritic development, it has been shown that glutamate receptor antagonists (either competitive or non-competitive antagonists) decrease dendrite growth and led to a corresponding reduction of the number of synaptic contacts, as well as to changes in synaptic morphology (Wedzony et al., 2005).

We showed that prenatal kynurenine pathway inhibition led to long term changes in neuronal dendritic morphology of the P60 offspring as a reduction in the number and length of apical branches of dentate granule cells. Changes in the endogenous levels of KYNA during early development induced dendritic retraction, indirectly through NMDA receptor blockade.
7 General discussion

7.1 Neurochemical alterations in rats offspring following maternal inflammation during pregnancy

Epidemiological studies have indicated that exposure to wide range of viruses during early development has been implicated in the pathogenesis of neuropsychiatric disorders (Adams et al., 1993, Brown et al., 2004, Mednick et al., 1988). The precise mechanisms associating the virus and prenatal neurodevelopmental consequences are not yet fully defined. Common mediators of maternal immune activation are suspected to be involved in producing a range of responses which are likely to contribute to the neuropsychiatric disturbances. In this respect, intrauterine elevations of proinflammatory cytokines are thought to play a role. The outcomes on the developing brain following the maternal immune activation includes alteration in gene and protein expression relating to various neurodevelopmental processes, excitotoxicity or changes in the molecular pathways involved in the generation of cells and also in the neurotransmitter functions (Damman and Leviton, 1997, Fatemi et al., 2008, Patterson, 2002). Here, we presented the relative contributions of prenatal viral infections to the emergence of neurochemical imbalances including glutamate-related pharmacological molecules after systemic administration of poly(I:C), a synthetic double-stranded RNA used to mimic viral exposure and is known to elicit immune responses by inducing the release of proinflammatory cytokines.

Pregnant rats were exposed to the inflammatory agent, poly(I:C) (10mg/kg) or saline during late pregnancy (E14,16 and 18). Our preliminary experiments showed that the indicated dose given to pregnant dams during the last seven days of gestation imposed no apparent adverse effects on the dam or her behaviour towards her litter after birth. We have demonstrated in previous report the ability of poly(I:C) to activate maternal immune system. The associated changes in which we observed an increase in MCP-1 with the absence of other proinflammatory cytokines at 5 h post injection may therefore exhibit the specificity of maternal host’s response to an immunological challenge which could modulate and precipitate related aberrations in the expression of selected neurodevelopmental proteins (Forrest et al., 2012).
Our results show a number of significant changes in the expression of several proteins important to early brain development following inflammatory challenge with poly(I:C). Some of the developmental markers showed changes during the time of insult takes place and disappeared or diminished as animal grew up. This may be an outcome of the compensatory capacity of the CNS to alleviate such changes. In light of fundamental role of NMDA receptor during brain development which include neuronal migration and plasticity, we found that prenatal poly(I:C) exposure affected the subunits composition of the receptor 5 h post treatment. Transient changes in the levels of NMDA receptor subunit NR2A and NR2B in the fetal brain could signify a potential mechanism of altering synaptic development and may impinge on the establishment of neuronal circuits and connection.

On the other hand it is also possible that prenatal poly(I:C) treatment manifested its effects on the postnatal brain that are independent from its effect at the time of exposure. The acute inflammatory response induce by prenatal poly(I:C) administration somehow induce further long term changes in factors regulating cell proliferation and migration, in order to produce altered neurogenesis in offspring later in development at P21. We show that changes in markers involved in neurogenesis, SOX2 and SHH were observed at later stage of postnatal development (P21). This could imply that prenatal immune activation elicited by poly(I:C) in rodents may have long term effects on neurogenesis in young postnatal offspring. The pattern of changes seen in SOX2 and SHH suggests such deficits or changes in neurogenesis could conceivably contribute to the alterations in hippocampal-mediated function that could have some implications in neuronal development. It seems important to note that activation of the immune system during gestation can precipitate brain mal-development, in which changes in the NMDA receptor subunits composition appear to be a key factor and more likely to be associated with long term developmental brain and relevant behavioural outcome in the offspring (Forrest et al., 2012, Khalil et al., 2013).
7.2 Regulation of kynurenine pathway metabolism in the brain after prenatal inhibition with Ro61-8048

Involvement of the KP occurs in a variety of CNS disorders, infection and inflammatory conditions. Moreover, the pathway has been found to be an important determinant of immune response which is thought to be related primarily to the activation of IDO by IFN-γ and other immune-active molecules and subsequent depletion of tryptophan leading to impairment of protein synthesis and modification in the cell proliferation and differentiation by a number of downstream KP metabolites such as QUIN. This supports evidence on the importance of the KP as a key link between inflammatory reactions and immune response (Stone and Darlington, 2013). The presence of neuroactive KP metabolites and their role in regulating excitatory amino acid receptors such as NMDA, AMPA and αNCR has been implicated in a number of diseases, thus raising out a possible intervention of manipulating the pathway by modulating the balance of the metabolites (Stone et al., 2012, Stone and Addae, 2002). Indirect approach to influence the metabolic function of NMDA receptor of glutamate subtype by stimulating endogenous KYNA formation may provide advantages compared to blocking the receptors indiscriminately with exogenous receptor antagonists. In this respect, to probe the physiological roles of KP metabolites experimentally, it appear sensible to inhibit enzyme kynurenine 3-monooxygenase (KMO) by administering known compound Ro61-8048 which would affect the synthesis of endogenous KYNA in accordance with changing physiological condition and indirectly reduce the degradation of the pathway along the QUIN branch (Chiarugi and Moroni, 1999, Richter and Hamann, 2003).

Our previous study has been carried out to examine the effect of a prenatal exposure to the KP enzyme inhibitor on the maternal, fetal and postnatal levels of major KP metabolites i.e. kynurenine and KYNA, considering that an imbalance in the production of these substances could have profound implications in cerebral neurotransmission and brain development. We have shown that administration with Ro61-8048 at an early stage of embryonic development elevated the levels of kynurenine and KYNA in both maternal plasma and brain within a few hours of administration (Forrest et al., 2013a, Khalil et al., 2013). The presence of kynurenine, a common parent compound of all neuroactive kynurenine metabolites, has shown that the pathway is readily
available in the CNS in normal physiological level. In the CNS, this substrate is then either transaminated to form KYNA or hydroxylated to produce 3-HK and other downstream metabolites including QUIN. Although we did not measure the level of QUIN, previous investigations have failed to show changes in QUIN concentrations following administration of Ro61-8048 in mice (Clark et al., 2005). The level of KYNA kept rising in the embryonic brain while maternal brain’s KYNA levels had returned to basal levels when observed 24 hour later. When we observed the levels of KYNA and kynurenine in the offspring at P21, the levels of both substrates had returned to normal control level as evidenced by its effect on the brain content. Importantly, the dose of Ro61-8048 caused the expected increment in the formation of KYNA in the brain during early period of development (Forrest et al., 2013a). Taken together with biochemical data showing alteration in the protein expression levels of various developmentally target molecules, these results suggest that the increased level of KYNA in the brain caused by the administration of Ro61-8048 in utero can influence the extent of excitability and viability of NMDA receptor function that could be partly responsible for the observed molecular and functional changes we have reported earlier.

We have shown in our previous study that raising brain tissue KYNA concentrations has lead to pronounced biochemical and neurochemical consequences in the rat offspring. It is noteworthy that the prenatal exposure to KP regulator, Ro61-8048 in rodents has been shown to induce a range of neurochemical alterations in the offspring detectable at various developmental ages. Considering that the plasticity of glutamate receptors could be affected by changes in the endogenous ligands of NMDA receptors and the role of the glutamatergic system in synaptic transmission, neuronal plasticity and also in the learning and memory processes, we focussed our attention on this paradigm and demonstrated that the exposure in utero to Ro61-8048 during late stage of gestation in the rodent was able to influence the level of expression of NMDA receptors and in particular those of NR2 subtype family in the brain. There were highly significant changes in the levels of both subunits NR2A and NR2B as observed 5 h post injection with Ro61-8048.
It is important to note that acute changes in NMDA receptor subunits expression in the developing brain immediately after injection were largely transient, the levels of both subunits were not different with control levels when observed 24 h later. The differential pattern of expression in the embryo brain (around late gestational period) for both subunits might reflect a compensatory adaptation of specific balance between these subunits. That is, NR2A expression is generally low at birth but increases over several weeks, whereas a relatively high NR2B expression at birth and declines slightly through adulthood (Janssen et al., 2005, Truman et al., 2002). In this aspect, appropriate NR2A:NR2B ratio and fundamental shifts in receptor properties based on subunit representation clearly have the capacity to influence neuronal function. We observed long term changes in the NR2 subunits when the offspring has reached young adulthood (P21). Alteration in the expression level of NR2A was maintained into adulthood (P60) as observed in the hippocampus and cortex regions. In fact, changes in the hippocampus in particular appeared to be the opposite of those observed earlier which might indicate regional specificity of the ratio between both subunits. We have previously shown that in the process of LTP, relative ratio of NR2A and NR2B may play a part in the molecular machinery of synaptic plasticity and contribute to the functional synaptic changes described in the hippocampus (Forrest et al., 2013b).

Given these differing developmental profiles, early life challenge with KP inhibition might be expected to have differential effects on subunit expression levels. This continuing change with respect to subunit representation in the receptor would impose overall dynamic regulation in NMDA receptor (Janssen et al., 2005). Furthermore, it has been shown that NMDA-mediated responses promote dendritic arbour growth and also cause a transient increase in both the size and density of dendritic spines, whereas blocking the receptor reduces the growth of dendrites (Wedzony et al., 2005). We showed that prenatal kynurenine pathway inhibition led to long term changes in neuronal dendritic morphology of the P60 offspring as a reduction in the number and length of apical branches of dentate granule cells. Therefore, changes in the endogenous level of KYNA during early development could have some implications on overall neuronal structure and the dendritic branch dynamics by antagonistic properties at the NMDA receptor.
The mechanisms responsible for the Ro61-8048’s effects on neurogenesis remain unclear. It seems probable that the regulation of the neurogenesis was strikingly evident months after the initial treatment with KP modulator, suggesting the process could be affected early on. Increased KYNA following the inhibition of KMO has been expected to cause an effect on the NMDA receptor function. We have described changes in term of protein expression of several neurodevelopmental markers that are involved in range of developmental process including neurogenesis, neuronal plasticity and transmission, inflammatory related process among many others (Forrest et al., 2013b, Forrest et al., 2013a). Hippocampal neurogenesis in this study was notably affected which indicates that this cell genesis process might be influenced by elevated levels of one of the kynurenine metabolites, KYNA (Forrest et al., 2013a, Forrest et al., 2013b). As SHH is conserved and functions prominently in both developing nervous system and the germinal zones of the adult brain. Enhancement effect of SHH particularly on the proliferation of neural progenitor cells and neuronal differentiation within the entire course of differentiation has been shown in vitro. In support with this evidence, the presence of SHH receptor complexes including Ptc and Smo within adult neurogenic areas emphasises the role for SHH in adult neurogenesis (Traiffort et al., 1998).

Our results suggest that prenatal modulation of kynurenine pathway might lead to changes in the expression levels of several molecules that involved in the regulation of cell cycle, proliferation as well as neurogenesis process in developing and adult brains. An increased expression of the transduction molecule SHH was observed 24 h after treatment with enzyme inhibitor during late gestation. However, when repeated challenge of the inhibitor was given, different pattern of expression was seen with reduced levels of expression was demonstrated at developmental age P21 and remained repressed in some regional areas of the brain including hippocampus, cerebellum and cortex. Further analysis using immunocytochemistry indicates that maternal exposure to Ro61-8048 maintained the numbers of proliferative progenitors and immature granule cell presented by DCX immunoreactivity in the hippocampal dentate gyrus. Whereas the increased number of SHH immunopositive cells in this region could potentially affect the growth and survival of newly generated granule cells and their developing axons. These findings raise the possibility that the early
modulation of NMDA receptor function through KYNA on the expression of SHH could contribute to the influence on adult neurogenesis. It also suggest that the SHH pathway may continue to have an important role in adulthood in regulating structural plasticity. We show that these effects of in utero exposure to pharmacological substance induced alteration in protein levels which was accompanied by a subtle structural modification that can results in changes in the cell numbers and survival that was anatomically localized and involved specific subpopulations of neurons. We report here that gestational exposure to a compound that is able to modulate the balance of KP metabolites, has a long lasting stimulatory effect which occurs in concert with other events on the generation and development of new neurons in the hippocampal formation, particularly in the dentate gyrus. Overall, this scope of research explains on the effect of KP modulation which extends from prenatal exposure to postnatal outcomes. It provides a partial explanation for the exceptionally brain development on long lasting and delayed effects following gestational exposure.

7.3 Future directions of study

The alterations in neurodevelopmental proteins especially seen in NMDA receptor subunits and synaptic physiology may be responsible to account for behavioural abnormalities. It is quite uncertain based on data generated from this study, to elucidate which developmental processes are responsible for different profile of behavioural abnormalities which constitute a biological mechanism of cognitive performance. Our behavioural findings on simple step down-avoidance test may not be appropriate to reflect a more complex task of cognitive changes, thus raising some limitations on conclusive interpretation (Forrest et al., 2013b). Thus, further and elaborate studies on the behavioural paradigms in relation to antagonistic profiles of Ro61-8048 are warranted. It has been shown that increases in endogenous KYNA concentration resulted in prepulse inhibition deficits and interferes with spatial working memory in the radial arm maze task. These findings are in line with the assumption that enhanced inhibition of NMDA receptor and αNCR by KYNA may contribute to the observed deficits in working memory in persons with schizophrenia (Pocivavsek et al., 2011, Chess et al., 2007). As KYNA is known to act on both glutamatergic and cholinergic systems that are critically involved in cognitive function and normal brain development, thus alterations in KP neuroactive metabolites can
influence the levels of neurotransmitters i.e. glutamate that have been implicated in schizophrenia (Muller and Schwarcz, 2006, Stone and Darlington, 2013). Therefore, further animal studies investigating mRNA/protein levels of receptors, neurotransmitter levels and treatment effects of Ro61-8048 in a more complex behavioural testing should improve the validity of this animal model. In addition, elucidating mechanisms that underlie the differential effects of Ro61-8048 at P21 and P60 will require further studies on the anatomical and molecular changes with particular interest on some molecular targets that has been linked to both glutamatergic and dopaminergic systems.

It is unclear by what mechanism alteration in the KP metabolites which favour KYNA formation upregulates SHH. The increase in expression is likely to involve an intermediate regulatory step of transcription factor that transcribes SHH. As the SHH pathway is important in the maintenance and proliferation of neural progenitor cells in the adult rodent brain, the activation of SHH and its receptor components is responsible for the effects that occur during this process. The pathway begins when the secreted SHH peptide binds to its membrane-bound receptor Ptc, thus relieving its inhibition of Smo which then lead to a complex signalling cascade involving the transcription factors of the Gli family. Future studies, possibly involving the suppression of SHH pathway with its antagonist will be useful to determine whether SHH signalling is necessary for KP regulation to promote the proliferation of adult’s pool of progenitor cells in the hippocampus. In this respect, incorporating an application of cyclopamine, natural product of plant alkaloid that is selectively known to inhibit SHH signalling by directly binding to one of the signalling component of the SHH receptor complex, is warranted (Charytoniuk et al., 2002, Britto et al., 2002). Because high expression of Ptc and Gli1 is an indicator of activity in the SHH pathway, it is also important to analyse the expression patterns this signalling targets either at mRNA levels or in tissue specimen of immunolabelling technique. By incorporating quantitative analysis with other signalling components of SHH pathway and inhibition with antagonist may further support the evidence of the requirement of the SHH signalling for adult neural progenitor cell expansion and development and that KP metabolism effects on hippocampus dependent-activity could be mediated by SHH signalling pathway.
Substantial studies have gathered evidence supporting the idea that dysregulation of the cell cycle of neural cells plays an important role in diverse pathological processes in the CNS. Characterization the cell cycle properties in this process and comparative studies of the expression of individual members of related proteins under physiological condition, including various developmental stages and in exposure to pathological stimuli may provide better and conclusive explanation.
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List of Publications


