Examining the vulnerability of developing white matter to injury

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

By

Entisar Ahmed Elsaeedi

Department of Cell Physiology and Pharmacology

University of Leicester

August, 2016
Abstract

Examining the vulnerability of developing white matter to injury

Entisar A. Elsaedi.

Many neurodegenerative disorders such as Alzheimer’s disease and cerebral ischaemic stroke can be caused by excessive glutamate release. Although experimental models of cerebral ischaemia, using NMDA-R antagonists, have shown protection against acute brain damage clinical trials with such compounds have failed due to the unacceptable side-effects. Firstly, this study aimed to examine the effect of acute exposure to NMDA-R antagonists (MK-801 and memantine) on the ultrastructure features of developing and adult rat optic nerve (RON) using electron microscopy. These data showed that NMDA-R antagonists acutely damage developing white matter (WM), but not the adult WM. In addition, examination of the effect of oxygen glucose deprivation (OGD) on the ultrastructural features of P0 RONs showed a significant reduction in the viability of axons, axon density and a significant increase in glial cell injury.

Secondly, this thesis has also examined whether the morphology of white matter is sexually dimorphic in both neonatal and adulthood using RON and corpus callosum. The data from this chapter observed that gender did not affect the white matter (in terms of axon diameter, density, area) at either P0 or adulthood and in addition no gender differences were seen in response to OGD-injury. However, there were some differences in the response to NMDA-R block. Specifically, gender affected axonal density following exposure to MK-801 or memantine – such gender differences were seen at both P0 and adulthood.

Thirdly, this thesis investigated the expression of voltage-gated calcium channels (VGCCs) in peripheral axons. The data showed that L-type and P/Q-type channel subunits were present at low levels at P0 and increased by P10 after which they declined by P20. Both double and triple labelling (IHC) experiments demonstrated that Schwann cells express VGCCs during the myelin formation process which starts from around P10.
Acknowledgements

I would like to thank my supervisor Dr Claire Gibson, for the patience, guidance and encouragement. I have been extremely lucky to have her as a supervisor who cared so much about my work and responded to my questions and queries so promptly. I would also like to thank Miss Natalie Allcock for her assistance and guidance with electron microscopy technique and helpful comments and for the talking and easing the difficulties throughout my doctoral work.

Finally, I would like to say a special thank you to my family who have always supported and encouraged me, to my husband, without your help writing this thesis would not be possible. My special thanks also go to my parents, mom and dad, your support, praying, your kind words and comfort are forever appreciated.
Contents

Chapter 1 Introduction ................................................................. 1

1.1 The Central Nervous System .................................................... 1

1.1.1 Cell types within the CNS ...................................................... 2
1.1.2 Axons .............................................................................. 2
1.1.3 The morphology of white matter during development and adulthood .... 4
1.1.4 Rat Optic Nerve (RON) ....................................................... 6
1.1.5 Ischaemia ......................................................................... 8
1.1.5.1 Classification of Cerebral Ischaemia .................................. 8
1.1.5.2 Pathophysiology of cerebral ischaemia ............................. 8
1.1.6 Glutamate ......................................................................... 11
1.1.6.1 Glutamate receptors ....................................................... 11
1.1.6.2 Glutamate and ischaemia ............................................... 13
1.1.6.3 Glutamate release in white matter injury ......................... 13
1.1.7 Voltage-Gated Calcium Channels (VGCC) ............................. 16
1.1.8 VGCC Subunits and the function of Ca²⁺ channels .................... 16
1.1.9 Molecular terminology of VGCCs ......................................... 17
1.1.10 Expression of VGCCs in the central axons ............................ 18
1.1.11 NMDA receptors - distribution and structure ....................... 19
1.1.12 The role of NMDA-Rs in the CNS ....................................... 20
1.1.13 NMDA-R antagonists – the neuroprotection efficacy ................ 21
1.1.14 NMDA-R antagonists and white matter injury ..................... 22
1.1.15 White matter and gender differences .................................. 24
1.1.16 Sex differences and white matter injury .............................. 24
1.1.17 Objectives ....................................................................... 27

Chapter 2 Materials and Methods ............................................... 28

2.1 Animals .............................................................................. 28
2.2 Animal modules and dissection ............................................... 28
2.2.1 Rat Optic Nerve (RON) ....................................................... 28
2.2.2 Rat Sciatic Nerve (RSN) ....................................................... 29
2.3 Solution preparation ............................................................. 29
2.3.1 Artificial cerebrospinal fluid (aCSF) ................................. 29
2.3.2 Phosphate Buffer Solution (0.1M PBS) ........................................... 29
2.3.3 Phosphate Buffer Goat serum Triton (PBGST) ............................... 29
2.3.4 Paraformaldehyde fixative solution (PFA) ..................................... 30

2.4 Electron microscopy ........................................................................... 30
   2.4.1 Embedding .................................................................................. 30
   2.4.2 Making glass knives and trimming the resin blocks ..................... 30
   2.4.3 Microtome sectioning ................................................................. 31
   2.4.4 Staining ..................................................................................... 31
   2.4.5 Transmission electron microscopy imaging ................................ 32
   2.4.6 Scoring system .......................................................................... 33

2.5 Immunohistochemistry (IHC) ............................................................... 34

2.6 Image collection .................................................................................. 35

2.7 Statistics and data analysis ................................................................. 36

2.8 Magnetic resonance imaging (MRI) ................................................... 36
   2.8.1 Protocol ..................................................................................... 36
   2.8.2 Corpus Callosum output measures ............................................. 37
   2.8.3 MRI Hardware .......................................................................... 37

Chapter 3: Determining the ultrastructural changes in white matter following
   OGD or NMDA-R block ........................................................................ 40

3.1 Introduction ......................................................................................... 40

3.2 Results ............................................................................................... 44
   3.2.1 The ultrastructural features of neonatal RON and the effect of OGD ... 44
   3.2.2 Comparison between ultrastructure of neonatal RON - control axons and
       OGD.................................................................................................. 45
   3.2.3 Quantifying the effect of OGD on neonatal RON ............................. 50
   3.2.4 Examining the ultrastructural features of adult RON and the effect of
       OGD .................................................................................................. 54
   3.2.5 Quantifying the effect of OGD on adult RONs ............................... 59
   3.2.6 Determining whether NMDARs contribute to the development of
       RONs .............................................................................................. 62
   3.2.7 Examining the effect of NMDA-R blockers on the ultra-structural features
       of neonatal RONs ......................................................................... 63
   3.2.8 Quantifying the effect of NMDA-R block on neonatal RON .......... 70
3.2.9 Examining the effect of NMDA-R blockers on the ultra-structural features of adult RONs

3.2.10 Quantifying the effect of NMDA-R block on adult RONs

3.2.11 Comparison study between OGD injury and the effect of NMDA-R blockers on axons and glial cells of neonatal rat optic nerve

3.2.12 The localisation of NMDA-Rs subunits (NR1 and NR2A) in the RONs comparing neonatal and adulthood

3.3 Discussion

Chapter 4 Investigating the sexual dimorphism of white matter and it’s response to injury

4.1 Introduction

4.2 Results

4.2.1 Determining the n value for comparison

4.2.2 To determine the gender differences in control-aCSF P0 RONs

4.2.3 Determining gender differences in normal adult RONs

4.2.4 Determining the gender differences of P0 RONs following OGD-induced injury

4.2.5 Determining the gender differences of adult RONs following OGD-induced injury

4.2.6 Determining the gender differences of P0 RONs after exposure to memantine

4.2.7 Determining the gender differences of adult RONs after exposure to memantine

4.2.8 Determining the gender differences of P0 RONs after exposure to MK-801

4.2.9 Determining the gender differences of adult RONs after exposure to MK-801

4.2.10 Determining gender differences in central white matter of adult rat corpus callosum using magnetic resonance imaging

4.3 Discussion

Chapter 5: Investigation of the expression of voltage-gated calcium channels in peripheral nervous system during myelination

5.1 Introduction

5.2 Results
5.2.1 Expression of voltage-gated calcium channels (VGCC) in the peripheral nervous system (rat sciatic nerve) during development ........................................ 146

Triple labelling IHC experiment .................................................................. 157

5.2.2 Are voltage gated Ca²⁺ channels expressed in unmyelinated central axons as well as non-myelinated central axons? ...................................................... 159

5.3 Discussion .................................................................................................. 164

Chapter 6: Conclusions and future work ...................................................... 166

Bibliography .................................................................................................. 171
List of figures

Figure 1-1 Differentiation of Stem cells to produce different types of nerve cells ........3
Figure 1-2 longitudinal section of normal optic nerve. ............................................. 7
Figure 1-3 The pathophysiological cascades originated by a cerebral ischaemia ........ 10
Figure 1-4 Subtypes of glutamate receptors ......................................................... 12

Figure 2-1 Transmission Electron Microscopy ...................................................... 32
Figure 2-2 Laser Scanning Confocal Microscopy ................................................... 36
Figure 2-3 Pictures show scanner and mouse brain coil ......................................... 39

Figure 3-1 Electron micrographs showing normal cross sections of P0 rat optic nerve
axons .................................................................................................................... 46
Figure 3-2 Cross sections of P0 RONs show the abnormal appearance of axons after
ODG ...................................................................................................................... 47
Figure 3-3 Electron micrographs show typical glial cell from P0 rat optic nerves. ......48
Figure 3-4 Electron micrographs showing the effect of OGD exposure on the
ultrastructural features of glial cells .................................................................... 49
Figure 3-5 Examining the effect of OGD on axons taken from neonatal optic nerve... 53
Figure 3-6 Electron micrographs showing normal adult RON (control axons). ...... 55
Figure 3-7 Cross sections of adult RONs show the abnormal appearance of axons after
ODG ...................................................................................................................... 56
Figure 3-8 Electron micrographs show typical glial cell from adult rat optic nerves... 57
Figure 3-9 Electron micrographs showing the effect of OGD exposure on the
ultrastructural features of glial cells .................................................................... 58
Figure 3-10 Examining the effect of OGD on axons taken from adult optic nerve. ....61
Figure 3-11 Electron micrographs showing control-aCSF P0 RON (control axons)..64
Figure 3-12 Electron micrographs showing glial cells of P0 RON (control glial cells).
............................................................................................................................. 65
Figure 3-13 Electron micrographs showing the effect of memantine on P0 rat optic nerve
axons .................................................................................................................... 66
Figure 3-14 Electron micrograph shows the effect of memantine on glial cell of P0 rat
optic nerve .......................................................................................................... 66
Figure 3-15 Electron micrograph shows the damage that MK-801 caused to axons of P0
rat optic nerve. The arrows indicate the breakdown of some axons .................68
Figure 3-16 Electron micrograph shows the effect of MK-801 on glial cell of P0 rat optic nerve. 

Figure 3-17 Comparison study of neonatal rat optic nerve following exposure to control (i.e. control aCSF, MK-801 or memantine). 

Figure 3-18 Comparison study between the control and the effect of MK-801 and memantine on glial cell of P0 rat optic nerve. 

Figure 3-19 Electron micrographs showed that there was not any effect from memantine (1μM) (B), and MK-801(10μM) (C), on axons of adult rat optic nerve comparing to the control condition (A). 

Figure 3-20 Electron micrographs showed no effect of memantine (B) and MK-801(C) on glial cells of adult rat optic nerve compared to the control condition (A). 

Figure 3-21 Comparison study between control and exposure to MK-801 or memantine on axons of adult RONs. 

Figure 3-22 Comparison study between the control and the effect of MK-801 and memantine on glial cell of adult rat optic nerve. 

Figure 3-23 Comparison studies looking at the effects of MK-801, Memantine and OGD on the ultrastructure of neonatal rat optic nerve. 

Figure 3-24 Comparison study between the control and the effect of MK-801 and Memantine and OGD on glial cell injury in neonatal RON. 

Figure 3-25 localisations of NMDA-Rs subunit expression (NR1) in the RONs. 

Figure 3-26 localisations of NMDA-Rs subunit expression (NR2A) in the RONs. 

Figure 4-1 Gender differences of neonatal rat optic nerve. 

Figure 4-2 Gender differences of adult rat optic nerve. 

Figure 4-3 Comparison study between neonatal male and female RONs after 60 min OGD-induced axonal injury. 

Figure 4-4 Comparison study between adult male and female RONs after 60 min OGD-induced axonal injury. 

Figure 4-5 Comparison study between P0 male and female RONs after exposure to memantine. 

Figure 4-6 Comparison study between adult male and female RONs after exposure to memantine. 

Figure 4-7 Comparison study between P0 male and female RONs after exposure to MK-801.
Figure 4-8 Comparison study between adult male and female RONs after exposure to MK-801.

Figure 4-9 Corpus Callosum Masks. Agilent software (VnmrJ 4.0) was used to apply these masks to all the samples.

Figure 4-10 Fractional anisotropy (FA) maps derived from (A) adult male rat corpus callosum and (B) adult female rat corpus callous.

Figure 4-11 Apparent Diffusion Coefficient (ADC) maps derived from (A) adult male rat and (B) adult female rat.

Figure 5-1 Ca²⁺ Pan VGCC staining with axonal marker neurofilament light (70K Da, NF-L).

Figure 5-2 P/Q-type VGCC clusters (anti-α1A), and neurofilament marker (70K Da, NF-L).

Figure 5-3 L-type VGCCs labelling (anti-α1D subunits) with neurofilament light (70K Da, NF-L).

Figure 5-4 Ca²⁺ pan labelling with Na⁺ pan antibodies.

Figure 5-5 Ca²⁺ Pan VGCC staining with Schwan cell marker (GFAP).

Figure 5-6 Triple labelling staining of adult RSN with Ca²⁺ anti-Pan(green) and Schwan cell marker GFAP (red) and neurofilament light (blue) - (70K Da, NF-L).

Figure 5-7 Immunohistochemistry graph shows the head region of P0 rat optic nerve.

Figure 5-8 Cross reactivity control of P0 RON using Ca pan and NF-L.

Figure 5-9 Preadsorption control of P0 RON using Ca pan and NF-L.

Figure 5-10 Different area of head region of P0 RON showing different type of VGCCs.
List of tables

Table 1-1 Classification and pharmacology profiles of VGCCs. (Budde et al., 2002) 18
Table 4-2 The mean of axon area, diameter and density of adult male and female RONs…………………………………………………………………………………………………………………………103
Table 4-3 The mean of axon area, diameter and density of P0 male and female RONs after exposure to OGD…………………………………………………………………………………………………………………………107
Table 4-4 The mean of axon area, diameter and density of adult male and female RONs after exposure to OGD…………………………………………………………………………………………………………………………111
Table 4-5 The mean of axon area, diameter and density of P0 male and female RONs after exposure to memantine………………………………………………………………………………………………………………………………………………116
Table 4-6 The mean of axon area, diameter and density of adult male and female RONs after exposure to memantine………………………………………………………………………………………………………………………………………………120
Table 4-7 The mean of axon area, diameter and density of P0 male and female RONs after exposure to MK-801………………………………………………………………………………………………………………………………………………124
Table 4-8 The mean of axon area, diameter and density of adult male and female RONs after exposure to MK-801………………………………………………………………………………………………………………………………………………128
Table 4-9 Table shows the results for volume, FA and ADC………………………………………………………………………………………………………………………………………………137
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate.</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate.</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance.</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate.</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium ion.</td>
</tr>
<tr>
<td>CAP</td>
<td>Compound action potential.</td>
</tr>
<tr>
<td>CC</td>
<td>Corpus callosum.</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous system.</td>
</tr>
<tr>
<td>CP</td>
<td>Cerebral Palsy.</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporter.</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscope.</td>
</tr>
<tr>
<td>ESC</td>
<td>Extra cellular space</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FOV</td>
<td>Fields of view</td>
</tr>
<tr>
<td>G</td>
<td>Golgi apparatus</td>
</tr>
<tr>
<td>GABA</td>
<td>$\gamma$ aminobutyric acid.</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillar acidic protein.</td>
</tr>
<tr>
<td>GLT</td>
<td>Glutamate transporter.</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate Aspartate Transporter.</td>
</tr>
<tr>
<td>GLYT</td>
<td>Glycine transporter.</td>
</tr>
<tr>
<td>GLY</td>
<td>Glycine.</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>GluR</td>
<td>Glutamate Receptor.</td>
</tr>
<tr>
<td>GM</td>
<td>Grey matter</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G protein coupled receptors</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase.</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid.</td>
</tr>
<tr>
<td>HCO3⁻</td>
<td>Bicarbonate.</td>
</tr>
</tbody>
</table>
IHC  Immunohistochemistry.
iGluR  Ionotropic glutamate receptor
IHC  Immunohistochemistry
K⁺  Potassium ion.
LTP  Long term potentiation
LTD  Long term depression
MBP  Myelin Basic Protein
Mg²⁺  Magnesium ion.
mGluR  Metabotropic glutamate receptor
Mit  Mitochondria
MK  Dizocilpine.
MK-801  (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
MON  Mouse optic nerve.
mRNA  Messenger RNA.
MS  Multiple sclerosis
MT  Microtubules
N  Nucleus
Na⁺  Sodium ion.
NBQX  2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dion.
NF-L  Neurofilaments light chain.
NMDA  N-methyl-D-aspartate.
NMDA-R  N-methyl-D-aspartate receptor.
NO  Nitric oxide.
OGD  Oxygen and glucose deprivation
OPC  Oligodendrocytes precursor cells.
P  Postnatal day
PBS  Phosphate-Buffered Saline
PBSGST  0.1M PBS with 10% blocking goat serum and 0.5% Triton-X100
PD  Parkinson’s disease
pH  Power of hydrogen (decimal logarithm of the reciprocal of the H+ activity).
PKC  Protein kinase-C
PNS  Peripheral nervous system
PVL  Periventricular Leukomalacia.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RON</td>
<td>Rat optic nerve</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species.</td>
</tr>
<tr>
<td>RSN</td>
<td>Rat sciatic nerve</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>Sec</td>
<td>Second.</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean.</td>
</tr>
<tr>
<td>SER</td>
<td>Smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>VGCCs</td>
<td>Voltage-gated Ca²⁺ channels</td>
</tr>
<tr>
<td>WM</td>
<td>White matter</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction

The nervous system is the most complex system in the body. It is divided into two main divisions based on the location of the nervous tissues in the body - the central nervous system (CNS) which plays an essential role in the control of most physiological functions, and the peripheral nervous system (PNS) which comprises nerves that send information from organs to the CNS and receive information from the CNS to the organs. The PNS is divided into the somatic and autonomic systems. The autonomic system is further divided into sympathetic and parasympathetic branches. Schwann cells are responsible for myelinating axons in the PNS by forming the myelin sheaths. The myelin sheath is basically composed of many layers of Schwann cell membrane which contains lipids and proteins that play an important role in maintaining the myelin structure and adhesion to the axons. Large axons (more than 1-2µm) release signals to stimulate myelin production. A single Schwann cell starts to enwrap one axon and leaves some areas in which nodes of Ranvier are located. Schwann cells work as an insulating sheath; therefore, the electrical signals are transmitted from node to node along the axons, this process is called salutatorily conduction. Also, Schwann cells are important for providing nutrition and supports the axons.

1.1 The Central Nervous System

The Central Nervous System (CNS) consists of the spinal cord and the brain. It is mainly composed of a complex network of neural cells that interact with each other by means of electrochemical waves. The CNS has two types of tissue; grey matter and white matter. Grey matter consists of the nerve cell bodies (sometimes called nuclei or ganglia), axons terminals and dendrites. It appears pinkish in colour and covers the white matter in some places, as in the cortex, or appears as an island within the white matter as in the deep nuclei. White matter, which connects different regions of grey matter to each other, consists generally of axons, blood vessels and glial cells. It is called white matter due to the presence of myelinated axons which are ensheathed by the myelin sheath that contains lipids.
1.1.1 Cell types within the CNS

The CNS is made up of different types of cells called neurons and glial cells which include macroglia (astrocytes and oligodendrocytes) and microglia (immune cells in the CNS). The examination of the glial and neuronal networks was initially made possible using the Golgi staining method, established by Camillo Golgi in 1873 (Pannese, 1996), which revealed the complicated structure of nervous system. In the CNS, neurons and glial cells originate from neural precursor cells of a germinal layer called the ventricular zone that lines the lumen of the ventricles of the brain and the developing spinal cord (Jessen, 2004). During embryonic development, neuroectodermal matrix cells go through mitotic division and their daughter cells change into small glioblasts. Such cells grow until a critical size is reached and then either continue within the generation cycle to undergo additional mitotic divisions or begin to transform into young astrocytes (Vaughn, 1968). It is estimated that glial cells occupy half of the brain space (Kettenmann, 1995), but neurons have long been considered the main functional unit of the nervous system. Glia play an important role in the structure and function of the nervous system. For instance, glial cells are involved in; the formation of myelin to increase conduction velocity, regulation of the microenvironment surrounding the neuron, establishing the neuronal connections in development and after injury, and modulating synaptic activity (Demarest & Ruggiero, 2005). Glial cells are also involved in the production of cerebrospinal fluid (CSF) and of extracellular fluids that coat, support and protect neurons (Demarest & Ruggiero, 2005). Glial cells communicate with axons through neurotransmitter signals and evidence shows that glial cells modulate neurotransmission.

1.1.2 Axons

Axons are an extension of the neuron which arise from the axon hillock of the cell body at the initial segment and can extend for a distance before reaching the terminal branches, also called end-feet, which form a synapse or neuromuscular junction. The axons are specialised in transmission of information (messages) from the cell body to another cell body or axon of another neuron as all-or-nothing action potentials.
Figure 1.1 Differentiation of Stem cells to produce different types of nerve cells (NIH Publication No.11-3440d Last updated November 14, 2012 www.ninds.nih.gov)
1.1.3 The morphology of white matter during development and adulthood

White matter (WM) consists mostly of axons and glial cells and comprises around 50% of the total brain volume. CNS white matter is a firmly packed tissue with myelinated and unmyelinated axons and lacks neuronal cell bodies. For many years the WM has been studied as one of the brain compartments that connect different regions to each other (Concha, 2014). The white appearance of WM is due to the lipid content of the myelin sheath. In the PNS, the myelin sheath is composed of Schwann cells while the CNS myelin sheath comprises oligodendrocytes (Demerens, et al., 1996). However, not all axons in the WM are myelinated - actually very few WM tracts are completely myelinated. Most of the non-myelinated central axons have a diameter of less than 0.2 μm; while myelinated axons tend to have a larger diameter (Butt, et al., 1998). In addition, there is a noticeable difference in the number of myelinated axons between WM tracts and the number of myelinated axons is area dependent. For example, the corpus callosum has a much greater proportion of myelinated axons in the splenium (i.e. posterior part) rather than the anterior located genu (Sturrock, 1980).

Almost all oligodendrocytes exist within WM during the early postnatal period and continue throughout adulthood (Richardson, et al., 2011). During maturation of oligodendrocyte processes the myelin membrane wraps around an axonal segment many times and compacts to form the internode. Then, the axonal membrane separates into different regions including the node of Ranvier, juxtaparanode and paranode, which function together to allow fast action potentials (Rios, et al., 2003); (Susuki & Rasband, 2008). In fact, propagation of action potentials along unmyelinated axons is continuous whereas in myelinated axons, nodes of Ranvier transmit action potentials through the salutatory conduction from one node to the next node. The speed of conduction is faster in myelinated than unmyelinated axons and depends on axonal diameter, thickness of myelin sheath and the distance between the nodes of Ranvier.

During ageing, humans and other primates experience some myelin loss which is not seen in rodents. Some studies using diffusion tensor imaging (DTI) of rat corpus callosum have showed that fractional anisotropy (FA) is lower in middle aged rats compared to old rats (Peiffer, et al., 2010). Several studies using magnetic resonance imaging (MRI) and electron microscopy (EM) of mouse optic nerve showed that axons continue to undergo myelination until the age of approximately 4 months but did not
show any evidence of demyelination during ageing (Dangata & Kaufman, 1997; Bartsch, et al., 1997). Many studies have observed the cellular components of white and grey matter over the life span in order to detect changes that may be related to cognitive decline during ageing. Comprehensive studies in the brains of rhesus monkeys during ageing report a decrease in forebrain WM volume by around 12% and a decrease in grey matter volume by around 2% between adult and young monkeys (Wisco, et al., 2008). Moreover, there was no noticeable loss of primary motor neurons in visual or entorhinal cortex (Tigges, et al., 1990); (Vincent, et al., 1989); (Merrill, et al., 2000). In addition, during ageing, microglia and astrocytes showed no detectable alterations but the amount of oligodendrocytes increased by around 20 to 50% in the visual and prefrontal cortex (Peters, et al., 2008). This is an interesting finding showing how the size of WM is reduced and the WM integrity loss detected with ageing. A study by Baltan and others (2008) investigated the mechanisms of ischaemia in young and old adult mice. In addition, adult mice were more vulnerable to ischaemic injury than younger mice, and adult mice showed greater WM damage than younger mice after the ischaemic insult (Baltan, et al., 2008).

Many age-related alterations in WM may contribute to increased axonal vulnerability to ischaemia such as changing in concentration of Na⁺ ions and reduction of Na⁺/ATPase (Scavone, et al., 2005). Additional causes that may lead to increased vulnerability of ageing WM to ischaemia is the ATP depletion, formation of free radicals and oxidative stress (Schaller, 2007). Oxidative stress is the principal effect in ageing WM injury, since the mechanisms of antioxidant detoxification are gradually decreased with the lifespan (Scavone, et al., 2005). In developed WM, other factors such as glutamate transporters can contribute to glutamate excitotoxicity. An improved understanding of the central WM mechanism of injury is essential to the development of potential treatments for many brain disorders.

Any damage in the myelin sheath leads to the demyelination of myelinated axons which causes nerve impulses to stop or slow and may cause neurological problems such as multiple sclerosis (MS). Some studies showed that demyelination in mice leads to increased production of new mature oligodendrocytes via oligodendrocyte precursor cells (OPCs), which in turn may remyelinate the demyelinated axons (Tripathi, et al., 2010); (Richardson, et al., 2011); (Kang, et al., 16). This process has also been found to occur in humans (Kipp, et al., 2012). Remyelination of large axons often occurs
when a thin and short internode is added along the demyelinated axons (Blakemore, 1974); (Stidworthy, et al., 2003). The conduction of action potentials can be restored by remyelination, however, changes to the myelin structure leads to slower conduction when compared to the normal myelinated axons which have thicker and longer internodes (Lasiene, et al., 2008). In adulthood, the corpus callosum is one of the WM regions that contains a large number of unmyelinated axons and is an area which has helped our understanding of how new oligodendrocytes would be engaged in remyelination of the originally healthy unmyelinated axons (Sturrock, 1980).

1.1.4 Rat Optic Nerve

The rat optic nerve (RON) is a white matter tract of the CNS and has been used as a model for studying axons (see Fig.1-2). It has the advantages of lacking neuronal cell bodies, being dissected and maintained easily, and being small enough to fix by submersion. (Raff et al, 1987). The RON has been useful in understanding the function and role of glial cells within the CNS (Raff et al, 1987). The optic nerve forms from an extension of the neural tube called the optic stalk (Raff, 1989). During embryonic days (E) 14-16 the inner layers of the retina begin to differentiate and the ganglion cells are formed, leading to the formation of small axons which extend and invade the optic stalk forming groups of variable size (Kuwabara, 1975). The axons of the ganglion cells of the retina come together in the optic disk and enter the sclera of the eye to develop the optic nerve, which is sheathed with all three meninges of the brain. The first membrane is the pia mater and then the dura mater which is continuous with the sclera of the eye. The subarachnoid and subdural spaces are continuous up to the optic disk and the nerve then continues to the optic chiasma, which is situated in the middle cranial fossa (Nobck & Demarest, 1967).
Figure 1.2 longitudinal section of normal optic nerve - Gary, Henry Gary’s Anatomy: descriptive and applied (Philadelphia: Lea & Febiger, 1913).
1.1.5 Ischaemia

Cerebral ischaemia is a disorder defined by reduced blood flow to a specific region of the brain. Due to a lack of oxygen and other essential elements that are brought by the blood, the brain starts to go through gradual cell death. This condition can cause various neurological problems including stroke and the lesions underlying cerebral palsy. Exposure of cell cultures or brain slices to oxygen and glucose deprivation (OGD) is a widely used in vitro model of ischaemia which produces a quick progressive loss of function that can be irreversible unless these essential elements (i.e. nutrients) are resupplied within a limited time (Webster & Ames, 1965).

1.1.5.1 Classification of Cerebral Ischaemia

Cerebral ischaemia can be classified by cause into hypoperfusion, embolic, and thrombotic. It can also be divided into focal and global brain ischaemia. Focal ischaemia can be either thrombotic or embolic and occurs in a specific brain region when a blood clot has blocked a cerebral vessel and increases the possibility of cell death within that particular area (Sullivan, Jonathon, 2013). While global brain ischaemia (hypoperfusion) affects the brain globally and occurs when the blood flow to the brain is arrested or considerably reduced and this may happen in the case of cardiac arrest. However, if the blood flow is restored within a short time, clinical signs may be temporary. On the other hand, if a long period of time passes (more than four minutes) before blood flow restoration, brain injury could be permanent (Sullivan, Jonathon, 2013).

1.1.5.2 Pathophysiology of cerebral ischaemia

In the course of cerebral ischaemia due to the insufficient nutrients such as glucose and oxygen, the brain will not be able to operate aerobic metabolism. Thus, the brain switches to anaerobic metabolism and the level of adenosine triphosphate (ATP) falls significantly and reaches zero in a few minutes (Sullivan, 2013). At this stage, cells start to lose the ability to sustain ionic gradients. As a result, there is a substantial influx of calcium into the cytoplasm and also glutamate (see below) is released from synaptic vesicles and reversal of glutamate uptake occurs; calpains are subsequently activated and leads to cell membrane breakdown and then cell death (see Fig.1-3) (Sullivan, 2013). The CNS is very sensitive to any changes in energy supply and therefore is
vulnerable to injury due to the high energy demand of CNS tissue (Stys, 2005). Ischaemic injury occurs after an interruption of blood flow and causes damage in many parts of the CNS such as the retina, grey matter and white matter (Hansen, 1985). Any lack of ATP will lead to failure of the sodium/potassium ATPase pump (Na⁺/ K⁺ ATPase) and calcium-ATPase pump (Ca²⁺ ATPase) which are ATP dependent pumps and causes the moving of cations down their concentration gradient (Stys, 2005). This failure mediates Na⁺ influx into and K⁺ efflux from the axoplasm, which leads to anoxic depolarisation. Eventually, high levels of intracellular Na⁺ will reverse the Na⁺/Ca²⁺ exchanger and then induce Ca²⁺ influx (Stys, 1998). In addition, voltage-gated calcium channels (VGCC) play a role in releasing Ca²⁺ ions through some intracellular stores (Stys, 2005), (Muller & Ballanyi, 2003). Thus, membrane depolarisation activates and releases some of the excitatory neurotransmitters (e.g. glutamate). During an ischaemic event, glutamate can be also released from presynaptic vesicles and/or failure and reversal of glutamate reuptake, which may result in the accumulation of high levels of glutamate in the extracellular space. Such glutamate release will activate some, or all, of the glutamate receptors which are either ionotropic receptors (iGluR) or metabotropic receptors (mGluR). Activation of iGluRs can mediate Ca²⁺ influx which causes critical pathological events, also leads to overload of mitochondrial Ca²⁺ and activates some Ca²⁺ dependent enzymes including phospholipase (C and A2) and nitric oxide synthase (NOS), the latter will eventually produce nitric oxide (NO) which is one of the main factors that leads to mitochondrial dysfunction and causes damage to the DNA and leads to energy failure. Also activation of the phospholipase leads to breakdown of the cellular membrane and then cell death (Harukuni & Bhardwaj, 2006).
Figure 1.1 The pathophysiological cascades originated by cerebral ischaemia. Adapted from (Siesjo, 1992)
1.1.6 Glutamate

Glutamate is the main excitatory neurotransmitter in the brain and is involved in many aspects of brain functions such as memory and learning (Danbolt, 2001), (Collingridge & Lester, 1989) (Fonnum, 1984). It also has an important role in the development of the CNS including cell migration, cell differentiation and cell death (Danbolt, 2001) as well as playing a key role in peripheral tissues (Moriyama, et al., 2000). Nearly all neurons and glial cells have glutamate receptors (Shelton & McCarthy, 1999) (Bergles, et al., 2000). Enormous amounts of glutamate are present throughout the brain; around 5 – 15 mmol/kg depending on the region, however, very small (and tightly controlled) amounts are normally present in the extracellular space (Schousboe, 1981).

1.1.6.1 Glutamate receptors

Although glutamate receptors are involved in normal neurotransmission they are also involved in many neurological diseases and disorders which include epilepsy, ischaemia, Alzheimer’s and Parkinson’s disease (Dingledine, et al., 1999). Thus, these receptors have been considered as an important target to identify therapeutic strategies for several CNS disorders (Traynelis, et al., 2010). However, activation of these receptors leads to excitatory synaptic transmission and synaptic plasticity which play a role in long-term depression (LTD) and long-term potentiation (LTP), important processes for memory and learning.

1.1.6.2 Types of glutamate receptors

Glutamate receptors (GluRs) are generally divided into two subtypes (Fig. 1-4); metabotropic receptors i.e. G-protein coupled receptors (GPCR) and ionotropic receptors (ligand-gated ion channel) (Watkins & Jane, 2006). Metabotropic receptors are composed of eight mGluR subtypes which are divided into three groups: group I, comprising of mGlu1 and mGlu5, group II (mGlu2 and mGlu3) and group III (mGlu4, 6, 7 and 8), based on signal transduction pathways, agonist pharmacology and sequence homology (Watkins & Jane, 2006).

Ionotrophic receptors are comparatively larger than metabotropic receptors and consist of four large subunits (>900 residues) (Traynelis, et al., 2010). These subunits have an extracellular hydrophilic N-terminal domain and four (M1 to M4) hydrophobic membrane spanning segments (Hammond, 2008). Ionotrophic receptors can be
subdivided into three families; non-NMDA receptors, i.e. AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid and kainate, and NMDA receptors (N-methyl-D-aspartate acid), termed according to their selectivity to specific glutamate analogues (Hammond, 2001). AMPA receptors, the predominant form found on glial cells, are composed of four subunits, GluR1-4, that can be differentially expressed to allow various functional properties of the receptor (Kondo, et al., 1997). Kainate receptors are found on astrocytes (assumed to be involved in neuronal-astroglial signalling) and on the soma of oligodendrocytes (Verkhratsky & Kirchhoff, 2007), and are composed of five identified subunits GluR5-7, KA1 and KA2 (Lerma, 2003). The third family NMDA receptors, which require the binding of both glutamate and glycine for activation, are assembled from three gene families NR1, NR2 (A-D) and NR3 (A, B) (Watkins & Jane, 2006). Recently, a new receptor has been identified termed the ‘delta’ receptor which are considered to be iGluR and play role in some form of synaptic plasticity (Traynelis, et al., 2010).

![Glutamate receptors](Figure 1.2 Subtypes of glutamate receptors (Watkins & Jane, 2006))
1.1.6.3 Glutamate and Ischaemia

Excessive levels of glutamate in the extracellular space, e.g. following brain ischaemia, leads to over activation of GluRs and induces excitotoxicity which results in cell death (Benveniste, et al., 1984). Glial cells, especially astrocytes, express both iGluRs and mGluRs (Gallo & Ghiani, 2000) (Belachew & Gallo, 2004). Synthesis of glutamate occurs after glutamate uptake by astrocytes and then it is converted into glutamine via glutamine synthetase. This process occurs only in astrocytes and not in neurons, and then this glutamine will be freed from the astrocyte and taken up by the nearby neurons. In turn, glutamate operates as a glutamate precursor, which is converted by mitochondrial glutaminase and stored in vesicles until required (Chen & Swanson, 2003). Glutamate transporters are responsible for glutamate reuptake, and there are five types which have been cloned and known as EAAT1 (GLAST) and EAAT2 (GLT) which are glial transporters and EAAT3 (EAAC), EAAT4 and EAAT5 are neuronal transporters (Shigeri, et al., 2004) (Danbolt, 2001). Some knockdown studies, conducted in rats using glial glutamate transporters, revealed that high levels of extracellular glutamate causes excitotoxic neurodegeneration which highlights the importance of astrocytes in controlling glutamate uptake and maintaining a non-toxic concentration (Rothstein, et al., 1996)(Tanaka, et al., 1997) (Watase, et al., 1998). Also, neuronal cortical culture studies showed that cultures that lack astrocytes are 100 times more vulnerable to glutamate injury than cultures with adequate astrocytes (Rosenberg & Aizenman, 1989). During normoxic conditions, maintaining the non-toxic levels of glutamate and keeping it at less than 1μM, consumes very high energy which is thought to be the highest overall energy usage in the brain (Sibson, et al., 1998). The reason behind that is due to the high energy consumption by glutamate transporters which are ATP-dependent. However, during ischaemia, astrocytes lose the ability to regulate glutamate uptake and tend to release it via reversal of glutamate transporters, although astrocytes are responsible for the presence of glutamate in the extracellular space, it has been shown that larger amounts of glutamate are released from neurons (Rossi, et al., 2000).

1.1.6.4 Glutamate release following white matter injury

Recent studies have shown the importance of GluRs in mediating injury to white matter during ischaemia (Ziskin, et al., 2007) (Kukley, et al., 2007). Physiologically,
glutamate transporters are responsible for the up-take of glutamate from the extracellular space, which in turn prevents excessive accumulation of glutamate and over-activation of GluRs that would lead to excitotoxicity in white matter (Matute, 2011); (Rothstein, et al., 1996). However, during ischaemia, high levels of glutamate occurs as a result of a reversal of glutamate release via glutamate transporters. It has been shown that reversal of glutamate transporters occurs during ischaemia due to ATP depletion and increased extracellular Na\(^+\)/K\(^+\) levels (Longuemare & Swanson, 1995), (Szatkowski & Attwell, 1994). However, it’s thought that once the levels of Na\(^+\)/K\(^+\) are restored, the role of glutamate transporters is essential in order to limit the excitotoxicity (Sheldon & Robinson, 2007). Generally, the concentration of glutamate inside neurons is considerably higher than in astrocytes which suggest that glutamate-mediated excitotoxicity during ischaemia is purely neuronal (Rossi, et al., 2000) (Rossi, et al., 2007). However, astrocytes are responsible for the glutamate conversion process which consumes high levels of ATP, and during ischaemia reduction of ATP levels leads to accumulation of glutamate within astrocytes (Rossi, et al., 2007), (Rossi, et al., 2000). In addition, inhibition of glutamate uptake in oligodendrocyte cultures results in increased glutamate concentration then excitotoxicity, however, kainate and AMPA-R antagonists show a significant protective effect (Domercq, et al., 2005). Any change in the activity of glutamate-producing enzymes, e.g. glutaminase in microglia, and the decrease of glutamate transporter expression may result in producing high levels of glutamate that accumulate in the extracellular space and lead to glial cell death (Matute, 2011). Several studies have attempted to identify the cause of increased extracellular glutamate after ATP-depletion using glutamate transporter inhibitors. Katsumori and his group (1999) used trans-pyrrolidine-2,4-dicarboxylate, a pan glutamate transporter inhibitor on rat hippocampal slices, and found that pyrrolidine-2,4-dicarboxylate can block the Ca\(^{2+}\)-independent release of glutamate during K\(^+\) depolarization in neonatal rats but not in adult rats (Katsumori, et al., 1999).

Levels of EAAC1 are considerably higher in neonatal stages whereas GLAST and GLT-1 levels are relatively low during this period (Furuta, et al., 1997a), suggesting that this reversal occurs mainly through EAAC1, which is a neuronal glutamate transporter. The selective inhibitor of GLT-1 (dihydrokainate) increases the Ca\(^{2+}\) independent release of glutamate in hippocampal slices of adult rats, suggesting that in adult rats, the reversal of EAAC1 could be masked by glutamate uptake through
GLT-1 (Katsumori, et al., 1999). In contrast, during forebrain ischaemia, Seki and others (1999) have used microdialysis to measure the levels of glutamate and aspartate in the presence of dihydrokainate and found a significant decrease in glutamate and aspartate levels, suggesting that GLT-1 contributes to the accumulation of glutamate and aspartate in the extracellular space during ischaemia (Seki, et al., 1999). Actually reversal of the glutamate transporter is recognized to be one of the key factors that leads to glutamate release during ischaemia. However, in some cases, alterations in glutamate transporters leads to neurodegeneration, while in other conditions changes in glutamate transporters may occur as a consequence of neurodegenerative events.

GluRs are expressed in oligodendrocytes and play a key role in controlling Ca²⁺ influx (Quardouz, et al., 2009a) (Quardouz, et al., 2009b). Many studies have investigated the mechanism of ischaemic injury focusing on the role of glutamate receptors. A recent study showed that oxygen glucose deprivation (OGD) for 60 minutes caused significant injury to pre-myelinated axons, from postnatal day (P) 10 RON, but application of MK-801 (NMDA-R blocker) and NBQX (non-NMDA receptor blocker) were protective suggesting that NMDA-R activation is probably one of many factors mediating injury of pre-myelinated axons (Alix & Fern, 2009). The presence of NMDA-R at this stage in RON development (i.e. P10) was confirmed by Domingues et al., who showed, using PCR, the presence of NR1, NR2 and NR3 subunit mRNA (Domingues, et al., 2011). NMDA-Rs have also been localised using immuno-labelling and electron microscopy techniques on oligodendrocyte processes where they contact with pre-myelinated axons (Salter & Fern, 2005). Activation of NMDA-R in P10 mouse optic nerve (MON) has been shown to contribute to the damage of oligodendrocyte processes during ischaemic insult (Salter & Fern, 2005). Distribution of NMDA-Rs on oligodendrocyte processes which intertwining with myelinated axons may contribute in damaging the neighbouring axons e.g. death of astrocytes would affect glutamate uptake and affects neighbouring cells (Hertz, 2008).

Ischaemic injury in astrocytes evokes different mechanisms according to their stage of maturation. For instance VGCCs are the key mediator in ischaemic insult of P0-P2 RON astrocytes and several studies showed that increasing [Ca²⁺], in P0 RON astrocytes is through voltage gated calcium channels (VGCCs) but not via glutamate-mediated Ca²⁺ influx or intracellular stores (Fern, 1998). Whereas in P10 astrocytes ischaemic injury is largely mediated by Na⁺, K⁺,Cl⁻ co transporters (Fern, 1998)
Thus, increasing our understanding of central white matter injury mechanisms is essential in developing potential therapeutic approaches for various brain diseases as PVL and stroke. In fact it has been shown in many studies that ischaemic injury in pre-myelinated axons is $Ca^{2+}$ dependent whereas in the myelinated axons it is both $Na^+$ and $Ca^{2+}$ dependent (Stys, 1998) (Alix & Fern, 2009).

### 1.1.7 Voltage-Gated Calcium Channels

Calcium channels are multimeric proteins composed of a pore-forming $\alpha_1$ subunit and three accessory subunits ($\alpha_2\delta$, $\beta$ and $\gamma$). It is the pore forming $\alpha_1$ subunit which distinguishes the $Ca^{2+}$ channel subtypes and there are ten $\alpha_1$ subunit genes known (Catterall, 2000). The $\alpha_1$ subunit is responsible for the voltage dependent opening and closing of the channels and it also contains the binding sites for numerous pharmacological agents (and toxins). The amino acid sequence is arranged in four repeated domains (I-IV); each domain contains six membrane spanning regions (S1-6). The S4 segment within each domain contains positively charged Arginine and Lysine residues (Figure 1-5) (Catterall, 2000).

![Figure 1.1.3 Structure of voltage gated Ca$^{2+}$ channels (Catterall, 2000).](image)

### 1.1.8 VGCC subunits and the function of Ca$^{2+}$ channels

The $\alpha_1$ subunit is able to form a functional $Ca^{2+}$ channel without the auxiliary subunits. The other subunits play an effective role in channel modulation, gating, expression and
pharmacology (Bergsman, et al., 2000). Firstly, β subunits play a crucial role in targeting the complex of Ca^{2+} channel subunits, they also regulate protein kinases, and they act as modulators of the gating and pharmacological properties of α_{1} subunits (Bergsman, et al., 2000). Secondly, the α_{2}δ subunit is a dimer, comprising of glycosylated α_{2} and δ proteins connected by disulfide bonds. This pair of subunits showed an effective role in channel gating. The δ subunit is a transmembrane protein and α_{2} is entirely extracellular (Hofmann F, 1994). The diversity of the α_{2}δ genes has only recently begun to be characterized, and less is known about this subunit’s effect on channel properties than that of the β subunit (Bergsman, et al., 2000). Thirdly, γ subunit which is (25-38 kDa), and has four transmembrane domains. Similar to the α_{2}δ subunits, there is little known about its effect on channel properties, even though it has been shown to promote inactivation (Letts VA, 1998).

1.1.9 Molecular terminology of VGCCs

In mammalian VGCCs, the pore forming α is encoded by ten genes which are labelled α_{1}, for the initial skeletal muscle isoform, and then α_{1} A to I. More recently a new nomenclature of VGCCs was introduced which divide the channels into three structurally and functionally related families (Ertel E, 2000). The new system uses the chemical abbreviation of the ion, Ca, followed in subscript by V for voltage, the main physiological regulator of the channels. A number, currently 1 to 3, places the α subunits into genetic subfamilies (Cav1, Cav2, and Cav3). Lastly, the order of discovery within the family is then conveyed through n, for example, Cav1.1 is the first member of the Cav1 family which mediate currents initially termed L-type. Cav2.1 is the molecular name of P/Q-type VGCCs (Mori Y, 1991) and Cav2.2 referred to N-type currents (Dubel SJ, 1992) and cloned Cav2.3 corresponds to R-type currents. Finally, the Cav3 subfamily mediates the T-type currents (Perez-Reyes, 2003).
Table 1-1 Classification and pharmacology profiles of VGCCs. (Budde et al., 2002)

<table>
<thead>
<tr>
<th>Native current</th>
<th>α1-subunit subtype</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-type</td>
<td>α1C (Cav1.2)</td>
<td>Dihydropyridines.</td>
</tr>
<tr>
<td></td>
<td>α1D (Cav1.3)</td>
<td>Phenylalkylamines.</td>
</tr>
<tr>
<td></td>
<td>α1F (Cav1.4)</td>
<td>Benzothiazepnes.</td>
</tr>
<tr>
<td></td>
<td>α1S (Cav1.1)</td>
<td></td>
</tr>
<tr>
<td>P/Q-type</td>
<td>α1A (Cav2.1)</td>
<td>ω-agatoxin IVA.</td>
</tr>
<tr>
<td>N-type</td>
<td>α1B (Cav2.2)</td>
<td>ω-conotoxin GVIA.</td>
</tr>
<tr>
<td>R-type</td>
<td>α1E (Cav2.3)</td>
<td>SNX-482.</td>
</tr>
<tr>
<td>T-type</td>
<td>α1G (Cav3.1)</td>
<td>Ethosuximide.</td>
</tr>
<tr>
<td></td>
<td>α1H (Cav3.1)</td>
<td>Mibebradil.</td>
</tr>
<tr>
<td></td>
<td>α1I (Cav3.1)</td>
<td>Kurtoxin.</td>
</tr>
</tbody>
</table>

1.1.10 Expression of VGCCs in central axons

Optic nerve axons express VGCCs and mediate activity-dependent [Ca²⁺]ᵢ increase, which is required for exocytosis (Kukley, et al., 2007) (Alix, et al., 2008). Studies using electron microscopy have shown that vesicle-like structures are present in axons and glial cells of rat optic nerve and filled with glutamate (Back, et al., 2007). Astrocytes in P10 RON mediate ischaemic injury by Na⁺, K⁺ and Cl⁻ co transporters, while in P0–P2 RON, ischaemic astrocyte injury is mediated by VGCCs (Thomas, et al., 2004) (Fern, 1998). This is consistent with tissue print studies which are used to probe the presence of VGCC in RON astrocytes at P2-P10 and confirmed the presence of VGCCs type T and L in P2, while the presence of T-type was clearly reduced at P10 (Barres, et al., 1990a). Recently, clusters of P/Q- and L-type VGCC have been identified at early stages during the formation of the nodes of Ranvier which suggest the relationship between central white matter myelination and presence of P/Q-type (Alix, et al., 2012). A study by Alix and his group (2012) found that VGCCs are expressed in myelinated and non-myelinated axons; and act as the main source of Ca²⁺ influx during ischaemic injury in developing axons (Alix, et al., 2008) (Alix, et al., 2012).
Axonal calcium channels are possibly important in many pathological conditions such as ischaemia. The immature white matter axons represent the major part of injury in several immature white matter injuries such as PVL which is the main pathology associated with cerebral palsy (CP) (Back & Rivkees, 2004). As ischaemic axonal injury is mostly calcium mediated, calcium channels play a key role in calcium influx into immature white matter axons during ischaemia (Fern, et al., 1995) (Stys, et al., 1992).

Investigation of the VGCC’s properties and function led to the development of a new generation of drugs for the treatment of chronic pain. The relationship between VGCC activity alteration and development of pathological pain conditions produced a strong interest in further understanding of how VGCCs regulate neuronal processes, including nociception. Many studies in DRG neurons have showed that G-protein coupled receptor activation; led to the internalization of VGCC α1 subunits (Altier, et al., 2006) (Tombler, et al., 2006). However, recent findings by Alex et al, (2008) demonstrated that voltage-gated calcium channels (VGCCs) are expressed in central axons as they start to myelinated, and shown that VGCCs contribute to action potential conduction in central axons that are starting to myelinated. However, it’s not known yet if this going to be the same for peripheral axons or at the optic nerve head where axons become non-myelinated.

### 1.1.11 NMDA receptors - distribution and structure

NMDA receptors are one of the ionotropic glutamate receptor families which are expressed on some astrocyte populations (Muller, et al., 1993) and the processes of some oligodendrocytes. They have been shown to play an important role in mediating ischaemic injury (Salter & Fern, 2005). The NMDA receptor is a hetero-oligomeric receptor that can be assembled from four out of seven identified subunits NR1, NR2A-D and NR3A-B. Importantly, the type of glutamate receptor determines the pharmacological properties of the receptor, NMDARs have slower kinetics compared to AMPA and kainate receptors yet have a higher Ca²⁺ permeability than non-NMDA receptors (Lipton & Stys, 2007). Unlike AMPA and kainate receptors, which are activated only by glutamate, NMDARs must also bind glycine as a co-agonist (Furukawa, et al., 2005). The NMDAR is further modulated by a voltage-dependant block by Mg²⁺ - on release of Mg²⁺ the Ca²⁺ conductance is increased dramatically.
(Lipton & Stys, 2007). In 1993, Karp et al were first to clone the glycine-binding NR1 subunit, which is encoded by a single gene, with eight splice variants (a-h) (Cull-Candy, et al., 2001); NMDA receptors always contain an NR1 subunit along with a subsequent NR2 or NR3 subunit (Schuer, et al., 2008). NR2 subunits bind glutamate and are encoded by four genes (NR2A-D). Conventionally, NMDARs are composed of NR1 and NR2 subunits, as a dimer of dimers and must be activated by both glutamate and glycine. The third NMDAR subunit, NR3 was found to be commonly expressed on neurons in addition to glial cells (Cheng Wang, 1996). Two genes encode NR3 subunits, NR3A and NR3B. The NR3B subunit is expressed predominantly on motoneurons while NR3A expression in contrast was found to be more widely distributed on glial cells (Chatterton, 2002). NR3 subunits can form channels with NR1 subunit, as a dimer of dimers just like NR1/NR2 channels, which results in a glycine-activated NR1-NR3A NMDA-R (Wada, et al., 2006).

1.1.12 The role of NMDA-Rs in the CNS

NMDA-Rs play important roles in diverse processes in the CNS such as excitatory synaptic transmission, plasticity, learning and memory in addition to excitotoxicity. Other possible roles for NMDA-Rs may involve regulating oligodendrocyte development and myelination (Yuan, et al., 1998). Oligodendrocyte NMDA-Rs show a very weak block by Mg²⁺ at resting membrane potential and under ATP-depletion they regulate part of the inward current that is produced in oligodendrocytes in conditions such as stroke, PVL and spinal cord ischaemia (Ziak, et al., 1998). Recently some studies showed the presence of NMDA-Rs during the oligodendrocyte myelinating process. As the intracellular spaces in these processes are relatively small the receptor-mediated ion influx evokes high concentration of intracellular ions and subsequently could cause a disruption in the process of myelination (Dewar, et al., 2003). NMDA-Rs have a higher affinity to glutamate than AMPA/KA-Rs which makes them vulnerable to be activated in many neurodegenerative disorders that involve a sustained increase in the concentration of extracellular glutamate such as multiple sclerosis (MS) (Karadottir, et al., 2005). Therefore, the presence of NMDA-Rs on oligodendrocytes may contribute to damaging white matter when high levels of glutamate are increased in the extracellular space following spinal cord injury, PVL, stroke and MS (Dewar, et al., 2003) (Volpe, 2001). In fact, during ischaemia, NMDA-
R activation on oligodendrocyte processes in the optic nerve leads to glutamate release which eventually causes degeneration to those processes (Salter & Fern, 2005). Thus, it’s important to determine if there are differences in the vulnerability to injury between developing and adult white matter and whether these differences are due to any variation in the contribution of NMDAR activation to the injury produced following OGD.

1.1.13 NMDA-R antagonists – neuroprotective efficacy

NMDA receptor antagonists have been extensively studied for use in the treatment of diseases with excitotoxic pathology, such as stroke. Although using NMDA-R antagonists as a treatment of such disorders have been successful in animal models (Lipton & Rosenberg, 1994), clinical trials have failed largely due to the severe side effects that include memory impairment, nausea, vomiting and neuronal cell death which were all caused by complete blockage of the NMDA-Rs channel (Lipton & Rosenberg, 1994). However, to avoid these side effects, anti-excitotoxicity treatments should be designed to block the over activation of NMDA-Rs without interfering with the normal activity of the receptors. To date, competitive antagonists failed in clinical trials as they bind at the agonist binding sites where glycine or glutamate bind and block the normal function of NMDA-Rs (Lipton & Rosenberg, 1994); (Lipton, 1993); (Reisberg, et al., 2003); (Koroshetz & Moskowitz, 1996); (Palmer, 2001). Other types of antagonists are non-competitive which act on the allosteric site of the receptor whereas uncompetitive antagonist, which are also defined as an inhibitor, act after activation of NMDA-R by glycine or glutamate. This mechanism of action of uncompetitive antagonists may make them suitable as a treatment for neurodegenerative disorders such as ischaemia. Eventually, NMDA-R channel blockers, such as MK-801, were developed and widely used in animal models (Wong, et al., 1986); (Gill, et al., 1987). However, the high affinity channel blockers, including MK-801 and phencyclidine (PCP), have a slow on/off rate from the channel resulting in a longer dwell time which leads to the blocker (drug) accumulating in the channel and causing unacceptable side effects in animal models like memory and learning impairment, which prevent these blockers from progressing clinically (Parsons, et al., 1999). On the other hand, using blockers with low affinity, such as memantine, which display short dwell time (fast on/off rate), decreases the chance of such adverse effects (Parsons, et al., 1999). Memantine is the first acting oral medication to treat patients
with moderate to severe Alzheimer’s disease (AD) (Reisberg B, 2003). Memantine works and enters the NMDA-R channel only when the channel is opened by the agonist (Lipton, 2004). At a concentration which is prescribed to patients, memantine enters the channel when it is excessively activated e.g. by excessive release of glutamate (Lipton, 2004). In fact, it has been proved that memantine displays minimal side effects such as restlessness and dizziness while still providing neuroprotection (Lipton & Rosenberg, 1994); (Lipton, 1993). Although many years of continuous work in developing drugs that interact with NMDA-R complexes selectively has occurred, only memantine has progressed clinically. However, more research needs to be done in order to understand the pharmacology and function of the NMDA-R subunits which would lead to better selective antagonists and then possibly treat several CNS disorders in which NMDA-Rs are involved. A recent study by Trotman et al (2014) investigated the dose response effect of memantine following ischaemic stroke at doses relevant to those used clinically for the treatment of Alzheimer’s disease. They found low doses of memantine decreased the lesion volume while high doses increased it. Their result proposes that lower doses of memantine are protective clinically whereas the high concentrations produce toxicity which may explain why there was variability in the degree of protection in previous studies. The neuroprotective effect of low-dose memantine may represent a potential pharmacological intervention and suggests that prophylactic treatment for patients at risk of stroke is feasible (Trotman, et al., 2014).

1.1.14 NMDA-R antagonists and white matter injury

Recent research has demonstrated that oligodendrocytes express NMDA-Rs at different stages of development in addition to the myelination process (Karadottir, et al., 2005), (Micu, et al., 2006), (Salter & Fern, 2005). Also, activation of oligodendrocyte NMDA-Rs plays a key role in regulating Ca²⁺ influx in white matter injury (Stys & Lipton, 2007). Accumulation of glutamate in the extracellular space leads to activation of NMDA-Rs in several conditions such as MS, stroke and PVL (brain damage in premature infants leading to a permanent neurological disability) (Karadottir, et al., 2005); (Micu, et al., 2006); (Salter & Fern, 2005); (Werner, et al., 2001); (Back, et al., 2007), thereby damaging the oligodendrocytes (Micu, et al., 2006); (Salter & Fern, 2005). A study by Karadottir and colleagues (2005) demonstrated that oligodendrocyte NMDA-Rs contain at least NR1, NR2C and NR3 subunits. (Karadottir, et al., 2005).
NMDA-Rs located on oligodendrocytes are less vulnerable to Mg²⁺ block than neuronal NMDA-Rs, which allows a significant current to be generated even at the cell resting potential (Karadottir, et al., 2005). Mimicking ischaemia can generate inward currents which are partly glutamate-mediated at oligodendrocyte NMDA-Rs (Karadottir, et al., 2005). A number of studies attempted to block NMDA-Rs in order to treat white matter injury. Micu and colleagues (2006) investigated the role of NMDA-Rs in chemical ischaemia by using NaN3 (mitochondrial inhibitor) and omitting glucose, they established that NMDA-Rs are responsible for the Ca²⁺ influx in myelin during ischaemia. Also they identified NR1, NR2 and NR3 subunits in myelin sheath using both immunohistochemistry and immunoprecipitation on adult RONs and attempted to reduce myelin damage during ischaemia using an NMDA-R antagonist (MK-801). Their data showed that mature myelin responds independently to injury and found that Ca²⁺ increase is due to myelinic NMDA-R activation and this increase was completely eliminated after MK-801 application which significantly reduces myelin injury (Micu, et al., 2006). They also applied NBQX, an AMPA/KA-R antagonist, which significantly prevented Ca²⁺ influx within oligodendrocyte cell bodies and myelin sheath. These findings may indicate a new therapeutic target in conditions where demyelination is a main feature such as MS and brain ischaemia (Micu, et al., 2006). Bakiri and his group (2008) investigated the role of NMDA-Rs in ischaemic white matter and they used NBQX as a treatment but failed to show any recovery of compound action potentials (CAPs) post-ischaemia. However, using MK-801, in its own or with combination of NBQX and memantine, resulted in a significant CAPs recovery. They suggested that blocking the NMDA-Rs by memantine is more likely voltage-dependent than MK-801. Glutamate release during ischaemia activates oligodendrocyte AMPA/KA-Rs and subsequently leads to depolarisation that causes a decrease in the ability of memantine to block the NMDA-Rs whereas this has less effect on MK-801 (Bakiri, et al., 2008). As a result, using MK-801 alone or memantine with AMPA/KA-R blockers could be a valuable therapy for white matter disorders (Bakiri, et al., 2008) however, there is still a need to further understand the mechanisms of injury which may be specific to white matter during ischaemia. Thus, understanding any differences in white matter, due to development or sex for example, might be important.
1.1.15 White matter and sex differences

Sex differences can influence the structure of the brain. For example, females have a higher grey matter to white matter ratio than males (Allen, et al., 2003), and a larger number of unmyelinated fibers in the central white matter (Mack, et al., 1995). A study by Cerghet and colleagues (2006) has reported that oligodendrocyte density in the corpus callosum of female rats is lower than in males by 20 to 40%. In fact, this difference is possibly due to a developmental alteration in the rate of the addition of oligodendrocytes. However, this difference is not maintained in adulthood as Rivers and colleagues (2008) suggested that new oligodendrocytes additional rate in the corpus callosum of middle aged female mice is equivalent to the middle aged male mice. Remarkably, in the corpus callosum, twice the number of new oligodendrocytes are born in the young adult female mice compared to males, but the survival of these new born cells was equally decreased (Cerghet, et al., 2006). Additionally, this balance was found to be maintained after injury. Such as the efficiency of remyelination in central WM is equal for young adult male and female rats, although with ageing, remyelination happened more efficiently in adult female rats than in adult males (Li, et al., 2006). In the CNS, sex differences occur in the incidence rate of demyelinating disorders such as MS with females experiencing higher incidence rates and interestingly pregnant patients tend to relapse less frequently than non-pregnant females (Confavreux, et al., 1998). Patients who relapse less frequently are likely to be experiencing fewer new demyelinated lesions (van Walderveen, et al., 1994), although it may also be due to the high proliferation of the OPCs throughout pregnancy and postpartum, and a subsequent myelination increase. In fact it seems that these effects are regulated by prolactin which is one of the pregnancy hormones (Gregg, et al., 2007). Gregg and colleagues suggested that it might be useful to inject non-pregnant mice with prolactin in order to examine these effects and might be a potential target to treat WM injury (Gregg, et al., 2007).

1.1.16 Sex differences and white matter injury

Recently, cerebral ischaemia has been classified as a sexually dimorphic diseases during the course of the life span (Venna, et al., 2012), but the underlying mechanisms responsible for these distinctions may vary with age. Many studies suggested that ischaemic stroke occurs less often in females than males. When comparing both sexes,
an ischaemic sensitivity of a male phenotype was uncovered. A study by Yamori, et al (1976), using stroke-prone and genetically hypertensive rats showed that females have longer expectancy life than males and the presence of vascular lesions and cerebral haemorrhage was decreased in females compared to males (Yamori, et al., 1976). Importantly, the resulting damage following ischemic brain injury also appears to differ according to sex in many animal models. After a comparable insult from focal or global cerebral ischaemia, female rodents maintain less tissue damage and more improved functional outcomes compared to age-matched males. Comparable sex-specificity can be demonstrated in cell culture developed without background sex steroids. For example, female-derived neuronal cultures are less susceptible than male-derived cultures to pharmacological insults used to mimic brain injury, e.g., peroxynitrite or glutamate (Du L, et al., 2004). This difference might result from the male cells inability to sustain the intracellular glutathione levels after nitrosative stress (Du L, et al., 2004). Although these finding appear to be not limited to neurons, for example, after oxygen-glucose deprivation insult, female astrocytes (Liu, et al., 2007) and female hippocampal slices (Li H, et al., 2005) have less extensive cell death; these outcomes suggest that sex-related sensitivity to cerebral ischaemia is partially related to sex differences, i.e. chromosome complement of the cells. However, hormonal influences may also play an essential role after ischaemic brain injury cell death and recovery.

It has been established that women have less destructive CNS lesions than men (Marshall, 1995) and diffuse axonal loss is more predominant in men with MS (Cook SA, et al., 1999). A study by Jung-Testas et al (1991), showed that women have large number of brain lesions associated with lowest level of testosterone (Jung-Testas I, et al., 1991); other studies revealed that relapse rate and disability progression decreases during pregnancy (Quadros PS, et al., 2002). Sex difference in adult stroke has been to a great extent attributed to the protective impact of estrogens (Turtzi & McCullough, 2008) (Koellhoffer & McCullough, 2013). Neonatal male mammals such as rodents, horses and humans, experience a testosterone surge within 24h following conception, and after that the levels reduce and approach that of females during the rest of the neonatal period (Corbier P, et al., 1992).

Comprehensive understanding of presence sex differences at the cellular level of the CNS and how hormones can influence cell proliferation and cell death may suggest
that hormones not only regulate the immune system but also indicate how glial cells contribute to the differences in disease phenotype between male and female MS patients. This is very important to improve the ongoing clinical studies which use sex hormones to changing the course of the disease. Studies of sexual dimorphism in white matter are limited to gross anatomical differences and myelinated axonal number. Therefore it is important to study the sex differences in perspective of response to injury and how sex could affect response to treatment.
1.1.17 Objectives

Glutamate is the main excitatory neurotransmitter in the brain and may contribute to acute brain damage after cerebral ischaemia or traumatic brain injury. It may also contribute to chronic neurodegeneration disorders such as Alzheimer’s disease and MS. In animal models of cerebral ischaemia, NMDA-R antagonists protect against acute brain damage but clinical trials failed because of the unacceptable side-effects like hallucinations and memory deficit. This study aimed to examine whether the acute exposure of NMDA-R blockers such as MK-801 and memantine has an effect on the ultrastructure features of developing and adult central white matter. Postnatal day 0 (P0) rat optic nerve (RON) and adult RONs were used in this study.

Also this research has focused on determining if central white matter is sexually dimorphic by comparing the ultrastructure features of optic nerve between both sexes by looking at development and adulthood, and whether there is any sexual difference in the white matter in response to OGD and NMDA-R block injury. The second part of this research has focused on examining the expression of voltage-gated calcium channels in peripheral axons during development and adulthood.
Chapter 2 Materials and Methods

2.1 Animals

All animal procedures were conducted in accordance with local ethical review and conformed to UK Home Office/European Directive regulations, animals were culled by vertebral dislocation.

2.2 Animal models and dissection

2.2.1 Rat Optic Nerve (RON)

The optic nerve is a CNS white matter tract that contains central axons of retinal ganglion cells. Rat optic nerves (RON) were chosen to study the mechanisms of white matter injury since RONs are easily accessible and lack neuronal cell bodies and synaptic structures (Stys, 1998).

Optic nerves were dissected from Wistar rats of various ages and perfused with artificial cerebrospinal fluid (aCSF). Once the head was separated from the body fine-tipped scissors were used to free the optic nerves from the eyes, by making a cut behind the eye balls, through the orbit. Adult skull is very hard thus bone forceps were typically used to crush the skull whereas the neonate skull is softer. Then the upper part of the skull was removed and the brain removed and transferred into aCSF solution. Both optic nerves were observed and separated from the optic chiasma by using micro forceps. Each optic nerve was immediately fixed in 2% formaldehyde/4% glutaraldehyde buffer and stored at 4°C for the sex difference study using electron microscopy technique or embedded in Tissue Tek in dry ice bucket for immunohistochemistry experiments.

In order to determine which voltage gated calcium channels (VGCCs) are expressed in unmyelinated regions of myelinated axons generally the head region of the adult rat optic nerve was obtained by dissecting the whole adult rat eye and embedding in Tissue Tek and frozen in dried ice with hexane and then stored at -20 °C for cryostat sectioning.
2.2.2 Rat Sciatic Nerve (RSN)

The sciatic nerve is a very long and large nerve, which appears as a thick whiteish cord. It runs from the lower part of the spine through the thigh, which connects the muscles of the leg and foot with the spinal cord. The mid-thigh incision method was used to dissect and harvest the RSN. A small incision is made laterally by using large scissors and the skin is pulled back so the muscles of the posterior thigh were torn apart to expose the sciatic nerve. Continuing splitting the muscles until the approximate length of the nerve is exposed. Then the nerve was gently lifted using forceps and removed by cutting at the proximal and distal ends (Bala, et al., 2014). Sciatic nerves were dissected from Wistar rats of various ages and perfused with artificial cerebrospinal fluid (aCSF).

2.3 Solution preparation

2.3.1 Artificial cerebrospinal fluid (aCSF)

Artificial cerebrospinal fluid (aCSF) is the most appropriate physiological solution used to maintain ionic balance and is a source of energy to dissected nerves. aCSF composition in (mM) was: NaCl, 126; KCl, 3; NaH₂PO₄, 2; MgSO₄, 2; CaCl₂, 2; NaHCO₃, 26; glucose, 10; pH, 7.45, bubbled with 95% O₂, 5% CO₂ and maintained at 37°C. For oxygen glucose deprivation (OGD) experiments, aCSF was prepared without glucose and bubbled with 95% N₂, 5% CO₂ and maintained at 37°C. For glutamate receptor block experiments, nerves were immersed in aCSF (control), aCSF + memantine (1µM) and aCSF + MK-801(10µM) for 90 minutes at 37°C.

2.3.2 Phosphate Buffer Solution (0.1M PBS)

0.1 M PBS was made by adding 3.16 M KH₂PO₄, 292 M Na₂HPO₄, and 123 M NaCl, to 1000 ml of DD H₂O and the pH was adjusted to 7.4.

2.3.3 Phosphate Buffer Goat serum Triton (PBGST)

Immunohistochemistry experiments need a blocking agent to avoid non-specific binding of either the primary or secondary antibody. PBGST is composed of PBS, 10% of goat serum and 0.5% Triton X to make the PBGST solution.
2.3.4 Paraformaldehyde fixative solution (PFA)

Formaldehyde is a toxic fixative which must be handled with extra care and prepared inside a fume hood. To prepare 2% PFA 20g of paraformaldehyde was added to 400 ml of 0.2M Na$_2$HPO$_4$ and heated to maximum of 60°C in order to allow the paraformaldehyde to dissolve. The solution was cooled and 100 ml of 0.2M NaH$_2$PO$_4$ was added followed by 500 ml of distilled water (DDH$_2$O) to achieve a final volume of 1 L.

2.4 Electron microscopy

The imaging and embedding of the nerves procedures were carried out under the supervision of the electron microscopy technician (Natalie Allcock).

2.4.1 Embedding

RONs were embedded within resin to preserve them and for ease of sectioning ready for viewing under the microscope.

The RONs were embedded into resin using the following steps:

- 2 Propylene oxide: 1 modified Spurrs low viscosity resin (hard formula) for 90 minutes.
- 1 Propylene oxide: 1 modified Spurrs low viscosity resin for 60 minutes.
- 1 Propylene oxide: 2 modified Spurrs low viscosity resin for 60 minutes.
- 100% modified Spurrs low viscosity resin for 30 minutes.
- 100% modified Spurrs low viscosity resin for overnight.
- Fresh modified Spurrs low viscosity resin for 3 hours, two times.
- Embed and polymerise at 60 °C for 16 hrs.

2.4.2 Making glass knives and trimming the resin blocks

The glass knives were made using a Bromma LKB 7800 glass knife maker. It was important to use a new glass knife each time before sectioning because the knife-edge becomes blunt and chipped during use. Also, it was necessary to use clean and sharp knives to ensure that the resin was sectioned without score marks, which would disrupt the nerves.
The resin blocks were trimmed to obtain a small, trapezoid cutting face around the embedding nerve. This is important, as the small block face reduces the tension along the knife edge, for enhanced cutting. The trapezoid cutting face is especially useful in order to obtain resin slices, which would collect in a ribbon in the plastic boat attached to the glass knife (filled with distilled water); a ribbon of resin slices would therefore be easier to collect with copper grids.

2.4.3 Microtome sectioning

Resin sections were cut using a Reichert Ultra cut E microtome. Initially, thick slices were cut at 0.5 µm, collected onto glass slides, stained with toluidine blue for ~2-3 minutes and washed in distilled water. Thick samples were viewed under an Olympus BH2 light microscope. Collecting thick sections initially is important as it confirms the sectioning was taking place in the right area of the block face and confirms that the nerve was central in the cutting face and to ensure that structures were not cut off due to the trimming.

Thin slices were sectioned at about 90 nm (which appear silvery gold in the water boat), relaxed with dichloromethane-soaked cotton bud sticks and collected onto copper mesh grids. The grids were air dried for a few minutes, followed by examination via the electron microscope to collect high magnification images of axons and glial cells within the optic nerve.

2.4.4 Staining

The samples on the grids were counterstained by submerging each grid into a Petri dish containing a droplet of Reynolds’ lead citrate for 3-5 minutes, in a CO₂-free environment (nitrogen box) and then subsequently washed in a series of three small beakers containing fresh boiled-distilled water. Washing must be performed with care to ensure the sections are not lost in the water; however, it must be performed properly to remove the excess stain, which would appear on the TEM images, obstructing the view.
2.4.5 Transmission electron microscopy imaging

Stained grids were observed using a JEOL electron microscope (JEOL USA, Inc.) with an accelerating voltage of 80 kV. Scale bars were added to each image.

![Transmission Electron Microscopy](image)

**Figure 2-1** Transmission Electron Microscopy
2.4.6 Scoring system

- **Viability scoring system of axons**
  The scoring system was used depending on the following:

1. Presence of an intact axolemma.
2. Presence of microtubules.
3. Presence of a debris-free axoplasm.

Axons that showed all three features were given a score of 3 and axons with none of the features present were given a score of 0.

- **Injury scoring system of glial cells**
  The scoring system was used depending on the following:

2. Swollen mitochondria (with loss of normal internal structure).
3. Swollen ER and/or clear vacuoles

   A completely normal cell had a score of 0 and a dead cell 3 points.
2.5 Immunohistochemistry (IHC)

Rat sciatic nerves (P0, P10, P20 and adult) were dissected and transferred into Tissue Tek (Sakura Finetek U.S.A, Torrance, CA), and frozen in dry ice/hexan. Approximately 20 µm tissue sections were obtained using a Bright cryostat microtome (Bright Instrument Co, Ltd, Huntington, UK) and sections were mounted on super frost plus slides (Menzel-Glaser).

The sections were fixed in 2% paraformaldehyde for 30 minutes (neonatal) or 1 hour (adult), and then washed two times for 5 minutes each with 0.1 M PBS. After fixation, sections were pre-incubated for 2 hrs at room temperature with 0.1M PBS with 10% blocking goat serum and 0.5% Triton-X100 (Sigma; PBSGST).

The sections were incubated in primary antibodies (or negative control) plus PBSGST overnight at 4°C. Primary antibodies included:

- Polyclonal α₁a (Alomone; 1:200), α₁c, α₁d (Sigma; 1:200), and affinity-purified α₂δ-2 (1:200), antipan-Ca²⁺ channel (Sigma; 1:200).
- Monoclonal antipan-Na⁺ channel (Sigma; 1:200), anti-neurofilament light (Sigma; 1:200), anti-GFAP (Glial Fibrillary Acidic Protein) (Sigma; 1:500).

The sections were then washed with PBSGST three times for 5 minutes each. Sections were incubated for 1 hr at room temperature with secondary antibodies as given below:

- Alexa-488 Goat anti-mouse IgG (Molecular Probes, OR, USA) - dilution 1:1000.
- Alexa-568 Goat anti-rabbit IgG (Molecular Probes, OR, USA) - dilution 1:1000.
- Alexa-488 Goat anti-rabbit IgG (Molecular Probes, OR, USA) - dilution 1:1000.
- Alexa-568 Goat anti-mouse IgG (Molecular Probes, OR, USA) - dilution 1:1000.

After that sections were then washed twice for 5 minutes each in: PBSGST, 0.1 M PBS and then in 0.05 M PBS and sections were examined by scanning confocal microscopy using an Olympus FV1000 Fluoview confocal image system.
For triple labelling experiments:

- Polyclonal anti-pan Ca\(^{++}\) channel (Sigma; 1:200).
- Monoclonal anti-GFAP (Glial Fibrillary Acidic Protein) (Sigma; 1:500).
- Monoclonal anti-neurofilament light-(70K Da, NF-L) (Sigma; 1:200).

The sections were then washed with PBSGST three times for 5 minutes each. Sections were incubated for 1 hr at room temperature with secondary antibodies as given below:


After that sections were washed twice for 5 minutes each in: PBSGST, 0.1 M PBS and finally in 0.05 M PBS and sections were examined by scanning confocal microscopy using an Olympus FV300 Fluoview confocal image system.

The specificity of the secondary antibodies was established by omitting the primary antibody and incubating the sections for 1 hr at room temperature with only secondary antibodies. In addition, for the antipan-Ca\(^{++}\) channel staining, a preabsorption control experiment was also performed. The primary antibody was first incubated with the preabsorption control peptide, at a recommended ratio of 1µg peptide to 1µg antibody for 2 hours in PBSGST at the usual dilution 1:200. This mixture was then applied to the tissue and left overnight at 4°C and then incubated with secondary antibody as previously stated. Also all of the calcium channel and anti-neurofilaments-L (NF-L) antibodies that were used in this study had been used in previous studies within our laboratory (Alix, 2008; Alix and Fern 2009). The anti-glial fibrillary acidic protein (GFAP) antibody is commonly used for immunohistochemistry experiments and was used in many previous studies (Shannon et al., 2007).

### 2.6 Image collection

Sections were examined by scanning confocal microscopy using an Olympus FV1000 Fluoview confocal image system. For single labelling, the images were collected using a single channel, which excites at 488 nm. Double-labelled images were collected using excitation at 488 and 568 nm. The images were captured using a Kalman filter.
2.7 Statistics and data analysis

Results are reported as mean ± SEM. Data are shown for both the number of nerve sections exposed to each condition and the number of axons analysed for each condition (Huria, et al., 2014). SEM represents the standard error of the mean values. Statistical significance was determined by ANOVA with Turkey’s post-test or using unpaired t test with Welch's correction.

2.8 Magnetic resonance imaging (MRI)

MRI is an imaging technique which produces 3D detailed anatomical images without using the radiation. It is usually used for diagnosis and detection of diseases. MRI is a technology that detects the changes in the direction of the rotational axis of protons found in the water that makes up the living tissues.

2.8.1 MRI Protocol

In order to measure the corpus callosum, 6 brains from adult male and female rats were fixed in order to scan them. The protocol requires that the animals are first anaesthetised with sodium pentobarbital and then perfused sterile saline followed by 4% paraformaldehyde (in PBS). Brains were removed and left to post-fix in 4% paraformaldehyde (in PBS) overnight. On the day of scanning, the brains underwent
several washes in sterile saline before placing into a proton-free solution fomblin (Sigma) overnight prior to the scan.

- T2w slice (2D) – 0.07mm in-plane resolution, 0.8mm slice thickness.
- DTI acquisition (planned from T2w scan) – Agilent 14-direction DTI (dual(2)), b-value=1000 s/mm²
- Analysis performed in VnmrJ 4.0 (Agilent) and 3D Slicer.
- Corpus callosum masks generated from T2w data and applied to DTI parameter maps.

2.8.2 Corpus Callosum output measures

- Volume.
- Apparent Diffusion Coefficient (mm²/sec).
- Fractional Anisotropy (0 < FA < 1).
- Diffusion parameters (ADC and FA) are used to assess differences in white matter.
- ROIs are drawn on T2-weighted images (higher contrast) and applied to DTI data.

2.8.3 MRI Hardware

- Fixed brains placed in fomblin fluid (Sigma Aldrich) → No NMR signal (proton-free) and susceptibility matched to tissue.
- 9.4 Tesla / 310 ASR Agilent preclinical MRI scanner.
  - Actively decoupled volume transmit coil.
  - 2-channel phased array coil for mouse brain imaging.
- ASR = actively shielded resonator.
• Actively decoupled means the transmit RF coil is switched off during signal reception.
Figure 2-3 Pictures showing scanner and mouse brain coil.
Chapter 3: Determining the ultrastructural changes in white matter following OGD or NMDA-R block

3.1 Introduction

Some tissues and cells can live for a few minutes without any blood supply. However, cells in the brain and heart cannot live without a continuous blood supply and it only takes 4 minutes or so of ischaemia to permanently damage these tissues. Discontinued blood flow (ischaemia) to the brain, or part of the brain, can lead directly to apoptotic and/or necrotic cell death of neurons (axons and soma). Necrotic cell death is characterized by a severe and sudden injury associated with a subsequent inflammatory response. By contrast, apoptosis is more subtle, akin to fading away. Apoptotic cells shrink, whereas necrotic cells explode, and apoptosis requires protein synthesis and may take many hours-days to occur.

In early observations of the mechanisms of ischaemic injury, the focus was on simple biochemical and physiological changes which were known to result from the interrupted circulation of blood flow. Examples of these changes are: acidosis due to anaerobic generation of lactate, loss of high-energy compounds, and no reflow due to swelling of astrocytes with compression of brain capillaries (Darwin, 1995). Consequently, researchers have demonstrated that the damage produced following ischaemia is more complicated than was previously thought, involving the action and interaction of many factors.

To probe the mechanisms of ischemic injury in the CNS, neonatal rat optic nerve (RON) was used in this study because it is easily accessible and lacks neuronal cell bodies and synaptic structures. The optic nerve morphology has been largely studied at the ultrastructural level (Peters & Vaughn, 1967) (Vaughn, 1969) (Skoff, et al., 1980) (Waxman, et al., 1992) (Butt, et al., 1994c). In the early post-natal period, axons are unmyelinated, around 0.2 – 0.3 μm in diameter, and surrounded by glial cell processes (Peters & Vaughn, 1967). In the cross section of the optic nerve, the axon appears as cylinders that contain microtubules and neurofilaments as well as mitochondria (Peters & Vaughn, 1967). Glial cells in the neonatal optic nerve consist of astrocytes as well as glioblasts, microglia and mitotic cells (Skoff, et al., 1976a)(Vaughn, 1969). Astrocytes are characterised by their stellate shape, nuclei,
dense cytoplasm and endoplasmic reticulum (ER) (Vaughn, 1969). Oligodendrocytes appear at approximately P5 and start the onset of myelination by P8 (Skoff, et al., 1976a) (Vaughn, 1969). Development of the astrocyte processes appears to occur in two stages; the large processes divide the axons into large groups in the early stage and the finer processes extend to fill up small spaces between axons following myelination from around P12 onward. Oligodendroglial cells differentiate at a slightly later stage and their precursor cells do not appear until ~P4 onwards (Morcos & Chanling, 1997).

Ischaemic injury affects optic nerve morphology which has been illustrated at the ultrastructural level in several studies, which demonstrated that axons of diameter less than 0.4 μm are protected by blocking Glu-receptors, whereas a combination of blocking Glu-receptors and VGCCs is protective in axons of diameter more than 0.4 μm which indicates the important role of VGCC and Glu-receptors in OGD-inducing ischaemia (Alix, et al., 2012) (Alix & Fern, 2009) (Back, et al., 2007) (Waxman, et al., 1992) (Thomas, et al., 2004) (Wilke, et al., 2004). It has been stated that ischaemia mediates extensive injury to RON astrocytes (Wilke, et al., 2004) (Thomas, et al., 2004). Following ischaemia, astrocytes exhibit swollen intracellular organelles such as ER and mitochondria (Wilke, et al., 2004) (Thomas, et al., 2004) (Back, et al., 2007) (Waxman, et al., 1992). Also after ischaemic exposure many vacuoles appear in the glial and axonal cytoplasm (Wilke, et al., 2004) (Back, et al., 2007). However, the contribution of NMDAR activation to optic nerve damage following ischaemia is still unknown and whether the contribution of NMDARs varies according to the stage of development.

Acute cerebral ischaemia leads to rapid and extensive necrosis due to large activation of Ca²⁺ activated cell death cascades. During ischaemia, depolarisation leads to increase glutamate in the extracellular space through synaptic release. It has been thought that failure of glutamate re-uptake mechanisms is the main cause that leads to cell death; however, this is not the case. The high levels of glutamate in the extracellular space results in excitotoxic activation of glutamate receptors e.g. NMDA-R, resulting in rapid alterations of ionic homeostasis which activate ischaemic cascades.
NMDA receptor antagonists have been extensively studied for use in the treatment of diseases with excitotoxic components in their pathology, such as stroke. Memantine is a first acting oral medication for the treatment of Alzheimer’s disease (AD) (Reisberg B, 2003). It is thought that by blocking the NMDA receptor and the effects of glutamate, memantine may protect nerve cells from excess stimulation by glutamate. Several studies confirmed the presence of NMDA-Rs in the axon cylinder however their function is not known yet (Ehlers, et al., 1998) (Wang, et al., 2011). NMDA-R expression in axons is very high during early white matter development, where the axons are not myelinated (Aoki, et al., 1994) (Dean, et al., 2005). NMDA-R expression on oligodendrocyte processes increases the sensitivity of these structures to ischaemia (Salter & Fern, 2005) (Karadottir, et al., 2005). Excitotoxicity has been investigated in immature central axons that have started to myelinate (Alix, et al., 2012) (Alix & Fern, 2009). However, the vulnerability of premyelinated axons to excitotoxic injury in the period before the consolidation of synapses has not been examined. A recent study by Huria et al (2014) examined the mechanism of injury in these structures and confirmed NMDA-R expression on mid-gestation of human periventricular white matter axons. It has been proposed that both pre-myelinated and myelinated white matter is vulnerable to over-activation of NMDA-Rs that leads to excitotoxic injury. Also, energy deprivation in pre-myelinated white matter results in damage to axons and glial cells due to the disruption in ion homeostasis after ATP reduction. Subsequent glutamate release into the extracellular space causes over-activation of axonal NMDA-Rs and increases injury to glial cells. Also due to the high levels of glutamate during ischaemia, GABA and glycine are increased and leads to over-activation of Gly-Rs and GABA-Rs resulting in further excitotoxic damage (Huria, et al., 2014).

In this study, transmission electron microscopy (TEM) was used to determine the ultra-structural changes of axons and glial cells after exposure to OGD. In addition, the role of NMDA-Rs in mediating the ischaemic response was investigated in developing and mature white matter through the application of NMDA-R blockers. The data were collected (blinded to age and treatment) from cross-section analysis of ultra-micrographs. The morphological features of OGD-induced injury and NMDA-R block were scored using a previously published system (Constantinou & Fern, 2009). In addition, immuno-staining was used to determine the expression of the NR1
and NR2A subunits of NMDA-Rs in neonatal and adult white matter. In this study, expression of NR1 and NR2A subunits were found in developing white matter using immuno-staining techniques, which is an indication that acute injury of pre-myelinated optic nerve axons during 60 minutes OGD results from activation of NMDA-Rs.
3.2 Results

3.2.1 The ultrastructural features of neonatal RON and the effect of OGD

In electron micrographs the cross section of the P0 rat optic nerve (figure 3-1) shows normal axons with a round appearance or occasionally oval or elongated and which contain microtubules, occasional mitochondria, and a small amount of smooth endoplasmic reticulum. The interaxonal space appears to be filled with a certain thin fluid but no cellular components are present. Astrocyte processes contain dense bundles of filaments and ribosomal clusters. Occasionally degenerating axons, probably by apoptosis, can be observed. The P0 rat optic nerve was exposed to 60 minutes of OGD and the effect on the ultrastructure examined. As figure 3-2 shows axolemma degradation occurred along with the presence of numerous vacuoles and some axons had lost their microtubules.

Under control conditions (figure 3-3), astrocytes appear ultra-structurally normal with a nucleus, a well-developed Golgi apparatus, rich in ribosomes, endoplasmic reticulum and numerous mitochondria. Ultrastructure examination showed that post-ischemic (i.e. exposure to 60 minutes OGD) astrocytes had lost their processes and frequently were necrotic. As a result of injury of glial cells, the cell body often contained large vacuoles and swollen organelles such as mitochondria and endoplasmic reticulum. In addition, a breakdown in the cell membrane was frequently seen and in some of the electron micrographs, glial cells were found that appeared to be completely dead (figure 3-4).
3.2.2 Comparison between ultrastructure of neonatal RON - control axons and OGD

In electron micrographs the cross section of the P0 rat optic nerve (figure 3-1) shows normal axons with a round appearance or occasionally oval or elongated and which contain microtubules, occasional mitochondria, and a small amount of smooth endoplasmic reticulum. The interaxonal space appears to be filled with a certain thin fluid but no cellular components are present. Astrocytes processes contain dense bundles of filaments and ribosomal clusters.

Following 60 minutes of OGD, electron micrographs (figure 3-2) show axolemma degradation, numerous vacuoles and some axons had lost their microtubules.
Figure 3-1 Electron micrographs showing normal cross sections of P0 rat optic nerve axons.

The axons have a round appearance; also note the presence of microtubules (MT), extracellular space (ES) between the axons and glial process. The white arrow is pointing to smooth endoplasmic reticulum (SER) which is apparent at the edges of the process. The red tracings indicate how the axons area has been measured. *Some axons are starting to die because of the natural process of apoptosis which eliminates unwanted neurons

(Scale bar = 1 μm).
Figure 3-2 Cross sections of P0 RONs show the abnormal appearance of axons after OGD. It can be clearly seen that there are many swollen axons (*) which lost their intact axolemma and other axons lost their microtubules. (Scale bar = 1 μm.)
**Figure 3-3** Electron micrographs show typical glial cell from P0 rat optic nerves.

Nucleus (N), Cisternae of the Golgi apparatus (G), Mitochondria (Mit) and Endoplasmic Reticulum (ER) are the prevalent cytoplasm organelles.

(Scale bar = 2 μm).
Figure 3-4 Electron micrographs showing the effect of OGD exposure on the ultrastructural features of glial cells.

The red arrow shows the breakdown of cell membrane, (*) swollen mitochondria (Mit), and swollen Endoplasmic Reticulum (ER).

(Scale bar = 1 μm).
3.2.3 Quantifying the effect of OGD on neonatal RON

The number of identifiable axons was counted and measured in the same field of view (area) in each electron micrograph from control and test nerves. The axon area and diameter were outlined by hand on the screen and measured using NIH Image J 1.45 software. The mean for each condition (control and injury) was calculated using Excel and all analyses were done with the analyzer being blinded to the condition. For each condition 2 nerves from 2 separate animals were used and a number of sections prepared from each nerve. This resulted in 5 nerve sections being exposed to the control condition and 4 nerve sections being exposed to the OGD condition. In terms of number of axons counted there were 636 in the control condition and 324 in the OGD nerves.

As already demonstrated in figure 3-1 optic nerve axons of varying sizes group together into bundles and are separated by glial processes and connective tissue. Moreover, for estimating the total number of axons accurately, numbering the axons was done by hand for calculation and the axon area and diameter were measured using NIH Image J 1.45 software. The axon density was calculated using the following equation:

$$\text{Density} = \frac{\text{Total (n) of axons in graph}}{\text{Total area within the graph}}$$

Firstly, the axons in the optic nerve at P0 were compared to the number of axons present under OGD conditions – this resulted in a slight decrease in the number of axons counted under OGD conditions because of damage to the axolemma (figure 3-4). Data are shown for both number of nerve sections exposed to each condition (figure 3-5, A-D) and number of axons analysed for each condition (figure 3-5, E-H). Under OGD condition axons were exposed to 60 minutes OGD; there were significant differences in the area and diameter distributions of the axons compared to the control. Figure 3-4, shows some axons with swelling axolemma and mitochondria. There was a significant decrease in axons number following OGD (mean= 0.16 ± 0.023) (figure 3-5, B) when compared to the control nerves (0.11µm ± 0.002). Axons density following OGD was (mean= 2.287 ± 0.194 axons/µm²; n=324 axons/4 sections in 2 nerves whereas in control nerves is (4.60 ± 1.11 axons/µm²; n=636 axons / 5 sections,
2 nerves); (figure 3-5, D). Blinded axons viability scores were considerably lower in axons following OGD (2.08 ± 0.035 compared to control condition where most of the axons have a score “3”, which relates to no pathology (2.80 ± 0.049; n=636/5 sections); (figure 3-5, C).
(A) Axon area (µm²)

(B) Axon diameter (µm)

(C) Axon density (axons/µm²)

(D) Axon viability scoring
Figure 3-5 Examining the effect of OGD on axons taken from neonatal optic nerve.

Graphs show the difference between control and OGD P0 RON in terms of axon area (A), axon diameter (B), axon viability (C) and axon density (D). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections exposed to each condition – control n = 5, OGD n = 4; number of axons analysed – control n = 636, OGD n = 324.
3.2.4 Examining the ultrastructural features of adult RON and the effect of OGD

The cross section of adult RON electron micrograph (figure 3-6) shows normal axons which contain microtubules, occasional mitochondria, and a small amount of smooth endoplasmic reticulum. Oligodendrocyte processes contain ribosomal clusters. The adult RON was exposed to 60 minutes OGD and examined at the ultrastructure level. Figure 3-8 shows the presence of numerous vacuoles and some axons had lost their microtubules.

Under control conditions (figure 3-7), oligodendrocytes appear normal with a nucleus, Golgi apparatus, endoplasmic reticulum and mitochondria. Ultrastructure examination showed that post-ischemic oligodendrocytes had lost their processes and the cell body in some graphs has large vacuoles and swollen mitochondria and breakdown in the cell membrane. Also, some of the glial cells were found to be completely dead (figure 3-9).
Figure 3-6 Electron micrographs showing normal adult RON (control axons). Scale bar = 2 μm
Figure 3-7 Cross sections of adult RONs show the abnormal appearance of axons after OGD. (*) indicates swollen axons.

Scale bar = 1 μm.
**Figure 3-8** Electron micrographs show typical glial cell from adult rat optic nerves.

Nucleus (N), Cisternae of the Golgi apparatus (G), Mitochondria (Mit) and Endoplasmic Reticulum (ER) are the prevalent cytoplasm organelles.

(Scale bar = 2 μm and 5 μm in B).
**Figure 3-9** Electron micrographs showing the effect of OGD exposure on the ultrastructural features of glial cells.

The black arrow shows vacuoles and swollen mitochondria.

Scale bar = 2 μm
3.2.5 Quantifying the effect of OGD on adult RONs

The number of axons in aCSF-control was compared to the number of axons present in OGD condition. The data are shown for both number of nerve sections exposed to each condition (figure 3-10, A-D) and number of axons analysed for each condition (figure 3-10, E-H). Axons diameter analysis showed a significant decrease in axons number following OGD (mean= 0.80 ± 0.0096 n= 1154 /10 sections) (figure 3-10, F). Axon viability scores showed that most of the axons have a score 2, (mean= 2.42 ± 0.016; n= 1292 /13 sections) (figure 3-10, G) whereas the viability scores were considerably lower following OGD (1.39 ± 0.018; n= 1154 /10 sections) (figure 3-10, G). Axon density following OGD was (mean= 3.26 ± 0.031 axons/μm²; n= 1154 axons/10 sections in 3 nerves; (figure 3-10, H) compared to axon density in the control nerves (mean= 3.8 ± 0.02 axons/mμ²; n= 1292 axons/13 sections, 3 nerves) (figure 3-10, H).
Figure 3-10 Examining the effect of OGD on axons taken from adult optic nerve.

Graphs show the difference between control and OGD adult RON in terms of axon area (A), axon diameter (B), axon viability (C) and axon density (D). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections exposed to each condition – control n = 13, OGD n = 10; number of axons analysed – control n = 1292, OGD n = 1154.
3.2.6 Determining whether blocking NMDARs affects the development of RONs

In order to examine the effects of NMDAR block in the developing RONS they were exposed to the NMDAR blockers memantine and MK-801. Memantine and MK-801 are both uncompetitive antagonists at the NMDA receptor. MK801 has shown effectiveness in protecting neurons in cell culture and animal models of excitotoxic neurodegeneration. Memantine blocks over-activation of NMDARs when they are involved in pathological conditions but it does not interfere with the physiological roles of NMDA-R.

The ultrastructural features of P0 and adult RONs were compared following exposure to aCSF, 1 µM memantine dissolved in aCSF; (see methods Section 2.3.1) or 10 µM MK-801 dissolved in aCSF; (see methods Section 2.3.1).
3.2.7 Examining the effect of NMDA-R blockers on the ultra-structural features of neonatal RONs

Cross-sections of P0 RONs controls - aCSF were analysed and axons were identified by their cylindrical structure, presence of intact axolemma and microtubules. Axons were normally found in clusters (figure 3-11) and surrounded by glial filaments, known as astrocyte processes (figure 3-12). Normal astrocytes contain endoplasmic reticulum, mitochondria and Golgi apparatus (figure 3-12).

Axonal response following exposure to (1μM) memantine for 90 minutes revealed some axons with swelling in the mitochondria, loss of microtubules, presence of debris and loss of the intact axolemma, (figure 3-13). Memantine also caused significant damage to glial cells of P0 rat optic nerve. For example, Figure 3-14 indicates the swollen mitochondria and abnormal distribution of endoplasmic reticulum also the presence of clear vacuoles.

Axons in nerves exposed to (10μM) MK-801 for 90 minutes also showed a swelling in the mitochondria, loss of microtubules and loss of the intact axolemma (figure 3-15). The effect of MK-801 on glial cells was also significant with presence of swollen mitochondria, vacuoles and abnormal distribution of endoplasmic reticulum (for figure 3-16).
Figure 3-11 Electron micrographs showing control-aCSF P0 RON (control axons). Scale bar = 2 μm
**Figure 3-12** Electron micrographs showing glial cells of P0 RON (control glial cells).

*Scale bar = 2 μm*
**Figure 3-13** Electron micrographs showing the effect of memantine on P0 rat optic nerve axons.

The arrows indicate the breakdown of some axons and the asterisk indicate the vacuoles that caused by the effects of memantine.

(B) is a higher magnification of the box in the image (A). Scale bar = 1µm except (D) = 2 µm.
Figure 3-14 Electron micrograph shows the effect of memantine on glial cell of P0 rat optic nerve.

The arrow indicates the swollen mitochondria, and (*) indicate the abnormal distribution of endoplasmic reticulum also we can clearly see many vacuoles within the micrograph. Scale bar = 1µm.
**Figure 3-15** Electron micrograph shows the damage that MK-801 caused to axons of P0 rat optic nerve. The arrows indicate the breakdown of some axons.

Scale bar = 1µm.
Figure 3-16 Electron micrograph shows the effect of MK-801 on glial cell of P0 rat optic nerve.
The black arrows indicate swollen mitochondria, (*) indicates the abnormal distribution of endoplasmic reticulum. Scale bar = 2μm.
3.2.8 Quantifying the effect of NMDA-R block on neonatal RON

The results seen in figure 3-17 show a significant difference in axonal area after exposure to memantine (mean= 0.121 µm² ± 0.003; n=1628 axons / 12 sections - 3 nerves) or MK-801 (mean= 0.109 µm² ± 0.0013; n=1745 axons / 12 sections - 3 nerves) when compared to exposure to control conditions (mean = 0.097 µm² ± 0.0011, n = 2054/12 sections). Blind counting of axonal density showed a significant difference in the number of identifiable axons following exposure to memantine (mean= 3.95 ± 0.018 axons/µ²; n=1628 axons/12 sections - 3 nerves; P < 0.001 vs control perfused RONs) (figure 3-17, C) and MK-801 (mean= 4.099 ± 0.021 axons/µ²; n=1745 axons / 12 sections - 3 nerves; P < 0.001 vs control perfused RONs) (figure 3-17, C), when compared to exposure to control conditions (mean= 4.84 axons/µ² ± 0.027; n= 2054/12 sections - 3 nerves). Also injury scoring analysis revealed that there was a significant difference in the axon viability scores between these three conditions, whereas after exposure to memantine the viability scores of axons was significantly lower (mean= 1.65 ± 0.041; P < 0.001 vs control perfused RONs) (figure 3-17, D) and MK-801 (mean= 1.92 ± 0.0125; P<0.001 vs control perfused RONs) (figure 3-17, D) when compared to the control data where most of the axons have a score 2, (mean= 2.2 ± 0.013; n=2054/12 sections) (figure 3-17, D).

Injury scoring of glial cells showed that almost all glial cells had a score of 0 which correlates to no pathology under control-aCSF perfusion (mean= 0 ± 0; n=12/12 sections) (figure 3-18). Glia injury after 90 minutes exposure to memantine resulted in a lower score in many cells (mean= 1.5 ± 0.398; n=12/12 sections, 3 nerves; P < 0.001 vs control perfused RONs) (figure 3-18) and MK-801 perfused nerves were also significantly affected, where the cells appeared swollen and showed severely disrupted processes and the membrane integrity was lost (mean= 0.42 ± 0.288; n=12/12 sections, 3 nerves; P < 0.001 vs control perfused RONs) (figure 3-18).
*** = p < 0.001 (vs. control).
Figure 3-17 Comparison study of neonatal rat optic nerve following exposure to control (i.e. control aCSF, MK-801 or memantine).

Measures taken were A. axon area; B. axon diameter; C. axon viability scores; D. axon density. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections exposed to each condition – control n = 12, MK-801 n = 12, memantine n = 12; number of axons analysed – control n = 2054, MK-801 n = 1745, memantine n = 1628. Data are presented as mean ± SEM. Significance was determined by one-way analysis of variance (ANOVA) using Tukey’s post-hoc test. ***= p<0.001(vs. control), ###: p<0.001 (vs. MK-801).
Figure 3.18 Comparison study between the control and the effect of MK-801 and memantine on glial cell of neonatal rat optic nerve.

Data are presented as mean ± SEM, control n = 12 sections, MK-801 n = 12 sections, memantine n = 12 sections. Significance was determined by one-way analysis of variance (ANOVA) and post-hoc test. *** = P <0.001 (vs. control).
**Table 1:** Calculating Chi-Square ($X^2$) between the control and the effect of NMDA-r antagonists on glial cell of neonatal rat optic nerve:

$$X^2 = \sum \frac{(\text{Observed frequency} - \text{Expected frequency})^2}{\text{Expected frequency}}$$

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Memantine</th>
<th>MK-801</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed (o)</td>
<td>0</td>
<td>1.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Expected (e)</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Deviation (o - e)</td>
<td>-3</td>
<td>-1.5</td>
<td>-2.6</td>
</tr>
<tr>
<td>Deviation^2 (o - e)^2</td>
<td>9</td>
<td>2.25</td>
<td>6.76</td>
</tr>
<tr>
<td>(o - e)^2/e</td>
<td>3</td>
<td>0.75</td>
<td>2.25</td>
</tr>
<tr>
<td>$X^2 = \sum (o - e)^2/e = $</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Degrees of freedom - (number of levels - 1) = 3-1 = 2

$X^2 = 6$

DF = 2

$\alpha = 0.05$

Critical value = 5.99

$X^2 (2) = 6, P \leq 0.05$
3.2.9 Examining the effect of NMDA-R blockers on the ultra-structural features of adult RONs

Adult RONs were isolated and exposed to control-aCSF, MK-801 (10µM) or memantine (1µM) for 90 minutes. The morphometric analysis of the ultra-micrograph of adult RONs (figure 3-19) revealed that after acute exposure to memantine and MK-801 axons still maintained normal appearance i.e. rounded or occasionally oval or elongated and containing few microtubules in addition to the occasional presence of mitochondria and a small number of smooth endoplasmic reticulum. In addition, the glial cell micrographs (figure 3-20) show normal oligodendrocyte processes containing dense bundles of filaments and ribosomal clusters and presence of healthy looking mitochondria.
Figure 3-19 Electron micrographs showed that there was not any effect from memantine (1μM) (B), and MK-801(10μM) (C), on axons of adult rat optic nerve comparing to the control condition (A).

Representative ultra-micrographs of healthy looking axons (rounded, oval or elongated) under each condition showing normal distribution of microtubules (red arrows) and myelin sheath (MS) and mitochondria (Mit) (black arrows)

Scale bar = 2μm.
Figure 3-20 Electron micrographs showed no effect of memantine (B) and MK-801(C) on glial cells of adult rat optic nerve compared to the control condition (A).

Representative ultra-micrographs of normal glial cell under each condition showing normal nucleus (N), presence of cisternae of the Golgi apparatus (G), also presence of healthy mitochondria (Mit) and endoplasmic reticulum (ER)

Scale bar= 2µm.
3.2.10 Quantifying the effect of NMDA-R block on adult RONs

These results (*figure 3-21*) showed no significant difference in axonal area after exposure to memantine (mean = 0.88 µm² ± 0.037, n = 475 axons) or MK-801 (mean = 0.85 µm² ± 0.036, n= 683 axons) when compared to exposure to control conditions (mean = 0.83 µm² ± 0.04, n = 596). Blind counting of axonal density showed no significant difference in the number of identifiable axons following exposure to memantine (mean= 3.36 axons/µm² ± 0.005) and MK-801 (mean=3.45 axons/µm² ± 0.014) vs control conditions (3.23 axons/µm² ± 0.04). Also the injury scoring analysis revealed that there were not any significant differences in the axon viability scores between these three conditions.
Figure 3-21 Comparison study between control and exposure to MK-801 or memantine on axons of adult RONs.

Data is shown for axon area (A), axon diameter (B), axon viability (C) and axon density (D) for each treatment group. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections exposed to each condition – control n = 4, MK-801 n = 4, memantine n = 4; number of axons analysed – control n = 596, MK-801 n = 683, memantine n = 475.

Results showed no significance differences in the mean of either axon diameter or axons density. Also there is no significant difference in the axons viability scoring. Data collected are presented as mean ± standard error and the significance was determined using ANOVA using Tukey’s post-hoc test.
Figure 3-22 Comparison study between the control and the effect of MK-801 and memantine on glial cell of adult rat optic nerve.

Data are presented as mean ± SEM. Control (n=6 sections), memantine (n=7 sections) and MK-801 (n=7 sections). These results showed no significance differences were found and the significance was determined using one-way ANOVA using Tukey’s post-hoc test.
**Table 2:** Calculating Chi-Square ($X^2$) between the control and the effect of NMDA-r antagonists on glial cell of adult rat optic nerve:

$$X^2 = \sum \frac{(\text{Observed frequency} - \text{Expected frequency})^2}{\text{Expected frequency}}$$

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>memantine</th>
<th>MK-801</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed (o)</td>
<td>0</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>Expected (e)</td>
<td>1.75</td>
<td>1.75</td>
<td>1.75</td>
</tr>
<tr>
<td>Deviation (o - e)</td>
<td>-1.75</td>
<td>-1.61</td>
<td>-1.61</td>
</tr>
<tr>
<td>Deviation$^2$ (o - e)$^2$</td>
<td>3.1</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>$(o - e)^2/e$</td>
<td>1.77</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>$X^2 = \sum (o - e)^2/e$</td>
<td>4.77</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Degrees of freedom - (number of levels - 1) = 3 - 1 = 2

$X^2$ = 4.77

DF=2

$\alpha = 0.05$

critical value= 5.99

$X^2 (2) = 4.77$, $P > 0.05$
3.2.11 Comparison study between OGD injury and the effect of NMDA-R blockers on axons and glial cells of neonatal rat optic nerve

In order to investigate whether OGD causes a similar amount of damage as blocking NMDARs to the central white matter the data obtained from examining the effect of OGD on neonatal RON were compared with the effect of NMDA-R block on neonatal RON data.

Figure 3-23 shows a significant reduction in the viability of axons, axon density and under OGD induced injury compared to NMDA-R antagonists in the developing rat optic nerves and the significance was determined using two ways ANOVA using Tukey’s post-test. Also, an injury scoring system was used to determine damage to glia cells damage and completely normal cell scored 0 and a dead cell scored 3. OGD mediated injury to developing glial cells caused greater damage than the toxicity that was produced after NMDA-R block (figure 3-24).
Axon density (axons/µm²)

- Control
- Memantine
- MK-801
- OGD

Axon diameter (µm)

- Control
- Memantine
- MK-801
- OGD

Axon viability scoring

- Control
- Memantine
- MK-801
- OGD

- N/S
- *
- ***

Axon density (axons/µm²)

- Control
- Memantine
- MK-801
- OGD

- ***
- ***
- ***
Figure 3-23 Comparison studies looking at the effects of MK-801, Memantine and OGD on the ultrastructure of neonatal rat optic nerve.

Measures taken were A. axon diameter; B. axon viability scores; C. axon density. Data are shown for both number of nerve sections exposed to each condition (A-C) and number of axons analysed for each condition (D-F). Number of nerve sections exposed to each condition – control n=12, MK-801 n = 12, memantine n = 12, OGD n = 4; number of axons analysed – control n = 2054, MK-801 n = 1745, memantine n = 1628, OGD n = 324. Data are presented as mean ± SEM. The significance was determined using one way ANOVA using Tukey’s post-test.

*= p<0.0379 (vs. OGD), ###= p 0.0002 (vs. OGD), ***= p 0.0003 (vs. OGD), ****= p<0.0001(vs. OGD).
Figure 3-24 Comparison study between the control and the effect of MK-801 and Memantine and OGD on glial cell injury in neonatal RON.

No glial injury being seen under control conditions with the largest amount of injury being seen under OGD conditions. Results are presented as mean ± SEM. The significance was determined using one-way ANOVA using Sidak’s multiple comparisons test-prism software. (n=6 per group).
Table 3: Calculating Chi-Square ($X^2$) between the control and the effect of NMDA-competitive antagonists and OGD injury on glial cell of neonatal rat optic nerve:

$$X^2 = \sum \frac{(\text{Observed frequency} - \text{Expected frequency})^2}{\text{Expected frequency}}$$

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>memantine</th>
<th>MK-801</th>
<th>OGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed (o)</td>
<td>0</td>
<td>0.42</td>
<td>1.5</td>
<td>2.75</td>
</tr>
<tr>
<td>Expected (e)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Deviation (o - e)</td>
<td>-1.5</td>
<td>-1.08</td>
<td>0</td>
<td>1.25</td>
</tr>
<tr>
<td>Deviation$^2$ (o - e)$^2$</td>
<td>2.25</td>
<td>1.17</td>
<td>0</td>
<td>1.56</td>
</tr>
<tr>
<td>$(o - e)^2/e$</td>
<td>1.5</td>
<td>0.78</td>
<td>0</td>
<td>1.04</td>
</tr>
<tr>
<td>$X^2 = \sum (o - e)^2/e = 3.32$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Degrees of freedom - (number of levels - 1) = 4-1=3

$X^2= 3.32$

DF=3

$\alpha= 0.05$

Critical value= 7.815

$X^2 (2) =3.32, P < 0.05$
3.2.12 The localisation of NMDA-Rs subunits (NR1 and NR2A) in the RONs comparing neonatal and adulthood

Immuno-staining of rabbit monoclonal NR1 antibody and rabbit polyclonal NR2A showed the expression of NR1 in P0, P4, P20 and adult RONs (*see* *figure* 3-25). The NR2A subunit is also expressed in P0, P4, P20 and adult RONs (*see* *figure* 3-26). Controls showed the absence of immuno-staining when the NR1 and NR2A primary antibodies were omitted from the protocol.
Figure 3-25 localisations of NMDA-Rs subunit expression (NR1) in the RONs.

- **A1-3**, P0 RON labelled with NR1 (green).
- **B1-3**, P4 RON labelled with NR1 (green).
- **C1-3**, P20 RON labelled with NR1 (green).
- **D1-3**, adult RON labelled with NR1 (green).
- **A4, B4, C4, D4**, no immunoreactivity is observed following omission of the NR1.

Scale bar = 2 μm.
Figure 3-26 localisations of NMDA-Rs subunit expression (NR2A) in the RONs.

- A1-3, P0 RON labelled with NR2A (green).
- B1-3, P4 RON labelled with NR2A (green).
- C1-3, P20 RON labelled with NR2A (green).
- D1-3, adult RON labelled with NR2A (green).
- A4, B4, C4, D4, no immunoreactivity is observed following omission of the NR2A.

Scale bar = 2 μm.
3.3 Discussion

This chapter has demonstrated that OGD has an effect on both neonatal and adult RONs whereas blocking NMDA-R has a severe effect on neonatal white matter but no effect during adulthood. There is increasing recognition of the vulnerability of white matter, like grey matter, to the damaging consequences of ischaemia. White matter occupies around 10% of the rodent brain volume, while in human brains it reaches approximately 50% thus, white matter forms a critical role in normal brain functioning and susceptibility to injury (Baltan, 2009). Many studies have shown the complexity of the mechanisms of white matter injury and how this varies according to stage of development (Alix & Fern, 2009) (Salter & Fern, 2005) (Fern, 1998) (Alix, et al., 2012) (Wilke, et al., 2004) (Thomas, et al., 2004) (Shannon, et al., 2007) (Stys, 1998) (Stys, 2005). However, the novelty of this study is that age is very important factor in white matter injury. For instance, central white matter goes through some molecular architecture changes due to the aging processes which play a key role in ischaemic injury (Ay, et al., 2005) (Schaller, 2007). A study by Baltan et al. 2008 using mouse optic nerve (MON) as a model for ischaemic injury showed that older adults were more vulnerable to ischaemic injury than younger adults. Also, they showed that the degree of damage during ischaemia was greater in older adults than that in younger adults and they confirmed that the mechanism of injury in older adults is mainly glutamate-dependent (Baltan, et al., 2008). There are several other age-related changes that could increase the vulnerability of white matter to ischaemia such as the alteration of Na⁺ concentration and the reduction in Na⁺-ATPase performance (Scavone, et al., 2005). Furthermore, any mitochondrial dysfunction which leads to ATP depletion, formation of free radicals, oxidative stress and glutamate transporters might contribute to glutamate toxicity in developed white matter (Schaller, 2007). These results highlight the possibility that treatment strategies derived from experimental work on neonatal tissue could be ineffective in treating adults. Therefore, an improved understanding of the mechanism of injury of mature and immature white matter is important to develop a potential treatment for many brain disorders such as PVL and stroke.

Within this study electron microscopy examination of the ultrastructure of axons revealed that exposure to OGD and/or NMDA receptor antagonists mediated significant damage to developing white matter. This was characterised by neonatal
RON axons, exposed to OGD, showing numerous vacuoles and axolemma degradation, while glial cells displayed swollen organelles also large vacuoles within the cell process and some micrographs showed completely dead cells. In the presence of NMDA-R antagonists similar morphological features in both axons and glial cells from neonatal RONs were demonstrated. Although after exposure to NMDA-R antagonists there tended to be less dead glial cells present and glial cells tended to show more damage limited to the organelles rather than the whole cell bodies. Both OGD and NMDA-R antagonists significantly reduced the viability of axons and axon density along with significantly injured glial cells. Although both exposure to OGD and NMDA receptors caused similar damage of developing white matter it would appear that exposure to OGD caused more severe level of damage compared to NMDA-R antagonists. However, in the adult RON exposure to NMDA-R antagonists did not have any impact on the white matter and the data showed no significant differences in the viability of axons, axon density and did not affect the glial cells.

It has been well documented that activation of NMDA-Rs plays a vital role in the pathological injury that occurs after an ischaemic insult. However, still many of these studies have failed clinically, basically due to the severe side effects which were not expected from the experimental studies. Using memantine to treat patients with AD may suggest that it is appropriate for consideration for treatment of other neurodegenerative diseases, for instance stroke, specifically if low doses are able to be applied to be effective whilst reducing the occurrence of side effects. Several studies have found that treatment with memantine is protective in vivo models of stroke. However, this study aims to investigate the impact of using memantine and MK-801 at low concentrations. This study has showed the toxic effects of memantine and MK-801 on developing central white matter. Memantine is a licensed treatment of AD at low systemic concentrations, which produces few side effects (Reisberg B, 2003).

During depolarisation, NMDA-R channels open and allow memantine to bind internally within the receptor. Memantine displays a low-affinity for binding the receptor and rapid dissociation kinetics, which allows it to dissociate quickly from the pore before becoming trapped during the resting membrane (Blanpied, et al., 1997) (Rogawski & Wenk, 2003). Whereas MK-801 is a non-competitive NMDA-R antagonist which blocks the receptor by binding within the receptor binding site same
as memantine (Lord, B, et al., 2013). However, MK-801 does not dissociate from the receptor as fast as memantine do during the channels open confirmation. Hence, MK-801 remains trapped within the channel which leads it to increase and accumulate around the receptor causing to a complete inhibition of the NMDA-R (Rogawski & Wenk, 2003).

In order to test NMDA-R block toxicity, MK-801 and memantine were applied during aCSF conditions. Testing MK-801 at 10μM and 1μM memantine to block the NMDA-R which could indicate whether blocking the receptor was the cause for the toxicity or not. To investigate glial cell death during memantine and MK-801 antagonist applications across 90 minutes’ experimental period, memantine data was compared to aCSF-control and MK-801 data. Application of memantine during aCSF conditions at low concentrations showed significant glial cell death and low axon density compared to MK-801 and aCSF-only control conditions. These data also demonstrated that developing white matter is more vulnerable to excitotoxicity during NMDA-R block than mature white matter.

This study has also demonstrated the expression of NMDA-Rs (NR1 and NR2A) at various times of central white matter development using immuno-staining technique. Several studies have focused on later developmental age when axons started to myelinate, and they found that NMDA-R are mostly located at the oligodendrocyte processes (Karadottir et al., 2005; Salter and Fern, 2005; Micu et al., 2006). Many studies showed that NR1 subunit is expressed on astrocytes, microglia and sub-plate neurons (Gottlieb and Matute, 1997; Schipke et al., 2001). Some studies showed that glutamate receptors can be expressed on both oligodendroglia and astrocytes (Gallo and Ghiani, 2000) (Fern and Moller, 2000). The final data presented in this chapter is the expression of NMDA-Rs in immature and mature RONs using antibodies against NR1 and NR2A which are both obligatory. High levels of NR1 and NR2A expression are found in developing and mature RONs. These findings suggest that NMDA-Rs may play a key role in regulating central white matter development and injury. These results suggest that developing white matter injury may NMDA-R dependent processes. The presence of the NR1 and NR2A subunits in rat optic nerves may be suggests that axonal damage and excitotoxicity occurs directly because of the over-activation of NMDA-Rs.
In summary, the work stated in this chapter has presented new data extending our knowledge of the contribution of NMDA-Rs to developing central white matter injury. In the immature white matter application of NMDA receptors antagonists produced toxicity which was not seen in adult tissue and may have implications for the use of NMDA-R targets to treat developmental injuries.
Chapter 4 Investigating the sexual dimorphism of white matter and its response to injury.

4.1 Introduction

As the previous chapter demonstrated OGD has an effect on both neonatal and adult RONs whereas blocking NMDA-Rs has a severe effect on neonatal white matter but has no effect during adulthood. In the current chapter a similar experimental paradigm was applied but this chapter aimed to investigate whether any sex differences occurred in the response to OGD-injury or NMDA R block.

Sex differences have been established anatomically in many regions of the grey matter within the CNS. In contrast, reports of sexual dimorphism in white matter are limited to gross anatomical differences and numbers of myelinated axons. Nottebohm and Arnold were the first who demonstrated sexual dimorphism in the brain in 1976. This study led to many other studies in rodents and humans which have focused on sex differences mainly in the hypothalamus and hippocampus (Zaidel, et al., 1994) (Mong, et al., 1999) (Barrera, et al., 2001). The hypothalamus and frontomedial cortex in men have larger volumes relative to the cerebral volume (Goldstein, et al., 2001). Most of the studies examining morphological differences using MRI techniques, have focused on grey matter (Filipek, et al., 1994) (Gur, et al., 1999), for instance, human females have larger areas related to language functions (Schlaepfer, et al., 1995) (Harasty, et al., 1997). On the other hand a few reports have assessed the sex differences in white matter which show that men have larger overall volume of white matter compared to women (Goldstein, et al., 2001) (Filipek, et al., 1994). However, other studies showed that women have larger white matter volumes involved in interhemisphere connectivity (Highley, et al., 1999) (Nopoulos, et al., 2000). Studies of post-mortem sexual differences demonstrated that men have larger genu of the corpus callosum (Witelson, 1989) than women suggesting that males have larger fibers than in females.

Normal CNS white matter comprises glial cells and both myelinated and unmyelinated axons. A few studies examined the presence of sexual dimorphism in white matter tracts, and demonstrated that white matter volume in areas such as the corpus callosum (Gur, et al., 1999) (Fitch, et al., 1990) is increased in males compared to females. Microscopically, the number of myelinated axons in the splenium and genu regions of
the corpus callosum is larger in male rats than female rats (Kim & Juraska, 1997) (Nunez, et al., 2000) (Mack, et al., 1995). Several electron microscopy studies reported that the corpus callosum size and myelination rate is increased between middle-age and adult rats (Yates & Juraska, 2007). Another study by Yang et al, 2008, confirmed that the volume of white matter myelinated axons and myelin sheaths is significantly larger in young male rats than young female however in middle-aged rats the volume is decreased in the males and increased in the females. Several studies attempted to explain the sex difference in animals by the effect of the sex hormones on brain development. The sex hormones, testosterone and estrogen, are involved in sexual differentiation. A study by Mac Lusky NJ et al (1987) demonstrated that sex steroid receptors are present in very high levels in cortical regions including the corpus callosum during the early postnatal period.

Sex hormones in developing animals might play a key role in the differences of the myelination rate as the steroid hormones in the CNS are responsible for increasing the expression of myelin proteins (Nunez, et al., 2000) (Jung-Testas & Baulieu, 1998). Also some studies reported the protective effect of sex steroids on the myelin of developing white matter and injured white matter, such as estrogen plays a protective role against hyperoxia-induced cell death in developing white matter (Gerstner, et al., 2007). In a spinal cord injury model, progesterone showed an increase in oligodendrocytes progenitor density and restored myelination rate (De Nicola, et al., 2006), also in experimental autoimmune encephalitis (EAE), which is model of multiple sclerosis (MS); a combination of progesterone with estradiol showed a restoration of myelin protein expression (Garay, et al., 2008). Some studies showed that females have less myelin volume and fewer oligodendrocytes relative to males. A histological study of neuroglia in the spinal cord has doubted the differences of neuroglia number between females and males (Knapp, et al., 1996) nevertheless they never attempted to quantify these outcomes.

This study was aimed at investigating whether any differences, due to sex, occurred in normal developing white matter and in response to injury. It is important to consider whether sex affects response to injury and to date, only two reports have compared differences according to sex in the cognitive deficiencies occur following vascular dementia, and both studies showed sexual dimorphism in behavioural symptoms.
associated with vascular dementia (Bckwalter et al., 1996; Xing et al., 2012). Yet, there are hardly any pathological studies that examine the response to injury according to sex. Thus this study aimed to determine whether sex influences the changes of white matter integrity which occur as outcomes of ischaemic injury and also when blocking the NMDA receptor which might reveal pathological and mechanistic differences after ischaemic injury.
4.2 Results

4.2.1 Determining the n value for comparison

In this study, the number of identifiable axons was counted and measured in the same field of view (area) in each electron micrograph from control and test nerves. NIH Image J 1.45 software was used to measure the axon area and diameter. The mean for each condition (control and injury) was calculated and all analyses were done with the analyzer being blinded to the condition. Thus, with regards to n values there could be some disagreement regarding what the n value should be i.e. the n value could be number of animals, number of nerve sections or number of axons analysed. In this chapter, the data are presented as both the number of axons analysed and number of nerve sections. In terms of testing for significant differences only differences that are significant when comparing number of nerve sections are commented on (although all data shown for completeness). In the neonatal experiments, each condition has 6 nerves separated from 3 animals. The number of nerve sections taken from the control female rats n = 16 and control males is 17; and the number of axons analysed from the female rats are 3449, male rats are 3707. The nerve sections being exposed to the OGD condition from female rats are 17, males are 18. In terms of number of axons counted there were 2139 in the female and 2230 in the males. The nerve sections being exposed to memantine taken from female rats and males was 18; number of axons analysed from female 6105 and male 3988. Also number of nerve sections taken from female and males that being exposed to MK-801 was 18 and the number of axons analysed from female was 4085 and male 5337.

In adult experiments, each condition has 6 nerves separated from 3 animals. The number of nerve sections taken from the control female rats n = 18 and control males is 18; and the number of axons analysed from the female rats are 2001, male rats are 2031. The nerve sections being exposed to the OGD condition from female rats are 12, males are 18. In terms of number of axons counted there were 1282 in the female and 2261 in the males. The nerve sections being exposed to memantine taken from female rats 26 and from males was 20; number of axons analysed from female 1110 and male 1826. Also the number of nerve sections were taken from the female
rats 28 and the males was 24 that being exposed to MK-801 and the number of axons analysed from female was 1105 and male 1476.
4.2.2 To determine the sex differences in control-aCSF P0 RONs

The mean axon area in neonatal female rats was 0.085 µm² (± 0.0011, n = 3449 axons, 16 sections) and in neonatal male rats it was 0.091 µm² (± 0.0008, n = 3707 axons, 17 sections) (figure 4-1, D). Axonal diameter analysis showed that in the female rats the mean was 0.31 µm (± 0.0018, n = 3449 axons, 17 sections) and in male rats the mean was 0.33 µm (± 0.0014, n = 3707 axons, 17 sections) (figure 4-1, E). There was no significance difference in axon density between female and male rats (females = 6.28 axons/µm² ± 0.025, n = 3449 axons, 16 sections; males = 6.20 axons/µm² ± 0.033, n = 3707 axons, 17 sections), when comparing either the number of nerves or number of axons analysed (figure 4-1, F).

<table>
<thead>
<tr>
<th>Axon area</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.085244</td>
<td>0.001137</td>
<td>3449</td>
<td>&lt; 0.0001</td>
<td>16</td>
<td>0.0167</td>
<td>0.7927</td>
</tr>
<tr>
<td>Male</td>
<td>0.090679</td>
<td>0.000826</td>
<td>3707</td>
<td>17</td>
<td>0.012198</td>
<td>0.0167</td>
<td>0.7927</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon diameter</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.313687</td>
<td>0.001777</td>
<td>3449</td>
<td>&lt; 0.0001</td>
<td>16</td>
<td>0.026089</td>
<td>0.6145</td>
</tr>
<tr>
<td>Male</td>
<td>0.330426</td>
<td>0.001382</td>
<td>3707</td>
<td>17</td>
<td>0.020402</td>
<td>0.6145</td>
<td>0.6145</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon density</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>6.281413</td>
<td>0.025925</td>
<td>3449</td>
<td>0.0582</td>
<td>16</td>
<td>0.380626</td>
<td>0.8975</td>
</tr>
<tr>
<td>Male</td>
<td>6.200905</td>
<td>0.033137</td>
<td>3707</td>
<td>17</td>
<td>0.489328</td>
<td>0.8975</td>
<td>0.8975</td>
</tr>
</tbody>
</table>

Table 4-1 The mean of axon area, diameter and density of P0 male and female RON.
Figure 4-1 Sex differences of neonatal rat optic nerve.

Graphs show the difference between males and females of P0 RON in terms of axon area (A), axon diameter (B) and axon density (C). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-C) and number of axons analysed for each condition (D-F). Number of nerve sections taken from female rats n = 16, males n = 17; number of axons analysed – female n = 3449, male n = 3707. The significance was determined using unpaired t test with Welch’s correction.

*** = $P < 0.0001$. 
4.2.3 Determining sex differences in normal adult RONs

The mean axon area in adult female rats was 0.398 µm² (± 0.0098, n = 2001 axons, 18 sections) and in adult male rats was 0.352 µm² (± 0.0088, n = 2131 axons, 18 sections) (figure 4-2, D). Axonal diameter analysis showed that in the female rats the mean axon diameter was 0.649 µm² (± 0.0067, n = 2001 axons, 18 sections) and in the male rats the mean axon diameter was 0.612 µm² (± 0.00597, n = 2131 axons, 18 sections) (figure 4-2, E). The axon density was not reduced in females rats (mean= 3.11 axons/µm² ± 0.025, n = 2001 axons, 18 sections) compared to male rats (mean= 3.66 axons/µm² ± 0.021, n = 2131 axons, 18 sections) when comparing the number of nerve sections (figure 4-2, F).

<table>
<thead>
<tr>
<th>Axon area</th>
<th>Mean</th>
<th>SEM</th>
<th>(n) of axons</th>
<th>P</th>
<th>(n) of sections</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.398000</td>
<td>0.009788</td>
<td>2001</td>
<td>0.0004</td>
<td>18</td>
<td>0.1032</td>
<td>0.7440</td>
</tr>
<tr>
<td>Male</td>
<td>0.351600</td>
<td>0.008817</td>
<td>2131</td>
<td></td>
<td>18</td>
<td>0.095935</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon diameter</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.649225</td>
<td>0.006670</td>
<td>2001</td>
<td>&lt; 0.0001</td>
<td>18</td>
<td>0.070326</td>
<td>0.7016</td>
</tr>
<tr>
<td>Male</td>
<td>0.612235</td>
<td>0.005970</td>
<td>2131</td>
<td></td>
<td>18</td>
<td>0.064958</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon density</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>3.113470</td>
<td>0.025288</td>
<td>2001</td>
<td>&lt; 0.0001</td>
<td>18</td>
<td>0.266624</td>
<td>0.1254</td>
</tr>
<tr>
<td>Male</td>
<td>3.656566</td>
<td>0.020659</td>
<td>2131</td>
<td></td>
<td>18</td>
<td>0.219441</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-2 The mean of axon area, diameter and density of adult male and female RONs
Figure 4-2 Sex differences of adult rat optic nerve.

Graphs show the difference between males and females of adult RON in terms of axon area (A), axon diameter (B) and axon density (C). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-C) and number of axons analysed for each condition (D-F). Number of nerve sections taken from female rats n =18, males n = 18; number of axons analysed – female n = 2001, male n = 2031. The significance was determined using unpaired t test with Welch's correction.

*** = P = 0.0004, **** = P < 0.0001.
4.2.4 Determining the sex differences of P0 RONs following OGD-induced injury

Following exposure to OGD the mean axon area in neonatal females was 0.10 µm² (± 0.00121, n = 2139 axons, 17 sections) and in neonatal male rats it was 0.11 µm² (± 0.00198, n = 2230 axons, 18 sections) (figure 4-3, E). Axon diameter analysis showed that in the female rats the mean was 0.35 µm (± 0.0021, n = 2139 axons, 17 section) and in the male rats the mean was 0.37 µm (± 0.0020, n = 2230 axons, 18 sections) (figure 4-3, F). In terms of axonal viability there was no significant difference following OGD between females (mean= 1.16 ± 0.0079; n= 2139 /17 sections) and males (1.14 ± 0.0077; n= 2230 /18 sections) (figure 4-3, G). There was not any significance difference in the axon density between female and male rats (mean= 3.55 axons/µm² ± 0.025, n = 2139 axons, 17 sections), (mean= 3.50 axons/µm² ± 0.013, n = 2230 axons, 18 sections), respectively (figure 4-3, H).
<table>
<thead>
<tr>
<th>Axon area</th>
<th>Mean</th>
<th>SEM</th>
<th>(n) of axons</th>
<th>P</th>
<th>(n) of sections</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.10</td>
<td>0.00121</td>
<td>2139</td>
<td>&lt; 0.0001</td>
<td>17</td>
<td>0.014</td>
<td>0.7042</td>
</tr>
<tr>
<td>Male</td>
<td>0.11</td>
<td>0.00198</td>
<td>2230</td>
<td></td>
<td>18</td>
<td>0.022</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon diameter</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.35</td>
<td>0.0021</td>
<td>2139</td>
<td>&lt; 0.0001</td>
<td>17</td>
<td>0.024</td>
<td>0.5836</td>
</tr>
<tr>
<td>Male</td>
<td>0.37</td>
<td>0.0020</td>
<td>2230</td>
<td></td>
<td>18</td>
<td>0.027</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon viability scores</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>1.16</td>
<td>0.0079</td>
<td>2139</td>
<td>0.0699</td>
<td>17</td>
<td>0.088</td>
<td>0.8719</td>
</tr>
<tr>
<td>Male</td>
<td>1.14</td>
<td>0.0077</td>
<td>2230</td>
<td></td>
<td>18</td>
<td>0.086</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon density</th>
<th>Mean</th>
<th>SEM</th>
<th>N</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>3.55</td>
<td>0.025</td>
<td>2139</td>
<td>0.0761</td>
<td>17</td>
<td>0.280</td>
<td>0.9525</td>
</tr>
<tr>
<td>Male</td>
<td>3.50</td>
<td>0.013</td>
<td>2230</td>
<td></td>
<td>18</td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-3 The mean of axon area, diameter and density of P0 male and female RONs after exposure to OGD.
Figure 4-3 Comparison study between neonatal male and female RONs after 60 minutes OGD-induced axonal injury.

Graphs show the difference between males and females of P0 RON in terms of axon area (A), axon diameter (B), axon viability scores (C) and axon density (D). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections taken from female rats n = 17, males n = 18; number of axons analysed – female n = 2139, male n = 2230. The significance was determined using unpaired t test with Welch's correction. **** = \( P < 0.0001 \).
4.2.5 Determining the sex differences of adult RONs following OGD-induced injury of adult RONs

Following OGD the mean axon area in female rats was 0.36 µm² (± 0.013, n = 1282 axons, 12 sections) and in adult male rats it was 0.36 µm² (± 0.012, n = 2261 axons, 18 sections) (figure 4-4, E). Axon diameter analysis showed that in the female rats the mean value was 0.596 µm (± 0.0091, n = 1282 axons, 12 section) and in male rats the mean value was 0.595 µm (± 0.0069, n = 2261 axons, 18 sections) (figure 4-4, F). Axon viability scores showed no significance difference between females (mean= 1.37 ± 0.015; n= 1282 /12 sections) and males (mean = 1.37 ± 0.013; n= 2261 /18 sections) (figure 4-4, G). Following OGD there was no difference in axon density between females (mean= 3.08 axons/µm² ± 0.025, n = 1282 axons, 12 sections) and males (mean= 3.55 axons/µm² ± 0.019, n = 2261 axons, 18 sections), respectively (figure 4-4, H).
<table>
<thead>
<tr>
<th>Axon area</th>
<th>Mean</th>
<th>SEM</th>
<th>(n) of axons</th>
<th>P</th>
<th>(n) of sections</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.3594</td>
<td>0.012686</td>
<td>1282</td>
<td>0.9954</td>
<td>12</td>
<td>0.131123</td>
<td>0.9996</td>
</tr>
<tr>
<td>Male</td>
<td>0.3595</td>
<td>0.011986</td>
<td>2261</td>
<td></td>
<td>18</td>
<td>0.13434</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon diameter</th>
<th>Mean</th>
<th>SEM</th>
<th>N</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.5957</td>
<td>0.00909</td>
<td>1282</td>
<td>0.9650</td>
<td>12</td>
<td>0.09396</td>
<td>0.9967</td>
</tr>
<tr>
<td>Male</td>
<td>0.5952</td>
<td>0.00687</td>
<td>2261</td>
<td></td>
<td>18</td>
<td>0.07695</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon viability scores</th>
<th>Mean</th>
<th>SEM</th>
<th>N</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>1.36693</td>
<td>0.0147</td>
<td>1282</td>
<td>0.9979</td>
<td>12</td>
<td>0.1529</td>
<td>0.9998</td>
</tr>
<tr>
<td>Male</td>
<td>1.36698</td>
<td>0.0125</td>
<td>2261</td>
<td></td>
<td>18</td>
<td>0.1398</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon density</th>
<th>Mean</th>
<th>SEM</th>
<th>N</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>3.0779</td>
<td>0.0245</td>
<td>1282</td>
<td>&lt; 0.0001</td>
<td>12</td>
<td>0.2531</td>
<td>0.1681</td>
</tr>
<tr>
<td>Male</td>
<td>3.5521</td>
<td>0.0194</td>
<td>2261</td>
<td>***</td>
<td>18</td>
<td>0.2178</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-4 The mean of axon area, diameter and density of adult male and female RONs after exposure to OGD.
**Figure 4** Comparison study between adult male and female RONs after 60 minutes of OGD-induced axonal injury.

Graphs show the difference between males and females of adult RON in terms of axon area (A), axon diameter (B), axon viability scores (C) and axon density (D). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections taken from female rats n = 12, males n = 18; number of axons analysed – female n = 1282, male n = 2261. The significance was determined using unpaired t test with Welch’s correction. *** = P < 0.0001.
Thus, to summarise this section so far sex did not affect axonal area, diameter or density in either P0 or adult nerves. In addition, following exposure to OGD there were no sex differences in axonal area, diameter, density or viability. Although some statistically significant differences were seen when comparing the number of axons analysed these differences did not remain when the data was analysed according to number of nerve sections. The next step now is to determine if the response to NMDAR block (either through application of memantine or MK-801) differed between the two sexes at P0 or in adults.
4.2.6 Determining the sex differences of P0 RONs after exposure to memantine

Following exposure to memantine there was no significant difference in axon area between females (mean = 0.17µm² ± 0.007, n = 6105 axons, 18 sections) and males (mean = 0.18 µm² ± 0.003, n = 3988 axons, 18 sections) (figure 5-5, E). In addition there was no significant difference in axonal diameter (females = 0.4 µm ± 0.003, n = 6105 axons/18 sections; males = 0.5 µm ± 0.003, n = 3988 axons/18 sections, (figure 5-5, F). Axon viability scores showed a significant difference, in the female rats (mean= 1.28 ± 0.006; n= 6105 /18 sections) whereas the viability scores in the males were (1.31 ± 0.008; n= 3988 /18 sections) (figure 5-5, G). The axon density between female and male rats were (mean= 9.6 axons/µm² ± 0.07, n = 6105 axons, 18 sections), (mean= 6.3 axons/µm² ± 0.04, n = 3988 axons, 18 sections), respectively (figure 5-5, H).
<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>(n) of axons</th>
<th>P</th>
<th>(n) of sections</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Axon area</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.167887</td>
<td>0.006609</td>
<td>6105</td>
<td>0.0217</td>
<td>18</td>
<td>0.121708</td>
<td>0.8991</td>
</tr>
<tr>
<td>Male</td>
<td>0.184442</td>
<td>0.002882</td>
<td>3988</td>
<td>18</td>
<td>0.042891</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Axon diameter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.400177</td>
<td>0.003004</td>
<td>6105</td>
<td>&lt; 0.0001</td>
<td>18</td>
<td>0.0553</td>
<td>0.3746</td>
</tr>
<tr>
<td>Male</td>
<td>0.460201</td>
<td>0.002488</td>
<td>3988</td>
<td>18</td>
<td>0.0370</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Axon viability scores</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.276003</td>
<td>0.006063</td>
<td>6105</td>
<td>0.0032</td>
<td>18</td>
<td>0.1117</td>
<td>0.8578</td>
</tr>
<tr>
<td>Male</td>
<td>1.304915</td>
<td>0.00771</td>
<td>3988</td>
<td>18</td>
<td>0.11477</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Axon density</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9.624438</td>
<td>0.071571</td>
<td>6105</td>
<td>&lt; 0.0001</td>
<td>18</td>
<td>1.31808</td>
<td>0.0318</td>
</tr>
<tr>
<td>Male</td>
<td>6.281434</td>
<td>0.04355</td>
<td>3988</td>
<td>18</td>
<td>0.648237</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4-5 The mean of axon area, diameter and density of P0 male and female RONs after exposure to memantime
Figure 4-5 Comparison study between P0 male and female RONs after exposure to memantine.

Graphs show the difference between males and females of P0 RON in terms of axon area (A), axon diameter (B), axon viability scores (C) and axon density (D). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections taken from female rats n = 18, males n = 18; number of axons analysed – female n = 6105, male n = 3988. The significance was determined using unpaired t test with Welch's correction. * = P = 0.217, ** = P = 0.0032, *** = P < 0.0001.
4.2.7 Determining the sex differences of adult RONs after exposure to memantine

Following exposure of adult RONs to memantine there were no significant differences according to sex in axon area (females = 0.80 µm² ± 0.023, n = 1110 axons, 26 sections; males = 0.60 µm² ± 0.014, n = 1826 axons, 20 sections, figure 4-6, E) axon diameter (females = 0.95 µm ± 0.011, n = 1110 axons/26 sections; males = 0.81 µm ± 0.008, n = 1826 axons, 20 sections, figure 4-6, F) or Axon viability scores (females = 2.19 ± 0.012; n= 1110 /26 sections; males = 2.25 ± 0.010; n= 1826 /20 sections, figure 4-6, G). However, there was a statistically significant difference in axon density between females (mean= 1.19 axons/µm² ± 0.005, n = 1110 axons, 26 sections) and males (mean= 2.58 axons/µm² ± 0.055, n = 1826 axons, 20 sections) whether comparing the number of axons analysed or number of nerve sections (figure 4-6, H).
<table>
<thead>
<tr>
<th>Axon area</th>
<th>Mean</th>
<th>SEM</th>
<th>(n) of axons</th>
<th>P</th>
<th>(n) of sections</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.801575</td>
<td>0.023288</td>
<td>1110</td>
<td>&lt; 0.0001</td>
<td>26</td>
<td>0.15216</td>
<td>0.3272</td>
</tr>
<tr>
<td>Male</td>
<td>0.597927</td>
<td>0.01444</td>
<td>1826</td>
<td></td>
<td>20</td>
<td>0.137975</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon diameter</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.944563</td>
<td>0.011052</td>
<td>1110</td>
<td>&lt; 0.0001</td>
<td>26</td>
<td>0.072213</td>
<td>0.2021</td>
</tr>
<tr>
<td>Male</td>
<td>0.810583</td>
<td>0.007747</td>
<td>1826</td>
<td></td>
<td>20</td>
<td>0.074023</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon viability scores</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>2.19009</td>
<td>0.012322</td>
<td>1110</td>
<td>&lt; 0.0001</td>
<td>26</td>
<td>0.080509</td>
<td>0.6180</td>
</tr>
<tr>
<td>Male</td>
<td>2.25356</td>
<td>0.010184</td>
<td>1826</td>
<td></td>
<td>20</td>
<td>0.097306</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon density</th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>1.190091</td>
<td>0.005254</td>
<td>1110</td>
<td>&lt; 0.0001</td>
<td>26</td>
<td>0.034331</td>
<td>0.0163</td>
</tr>
<tr>
<td>Male</td>
<td>2.580425</td>
<td>0.05513</td>
<td>1826</td>
<td></td>
<td>20</td>
<td>0.526777</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4-6** The mean of axon area, diameter and density of adult male and female RONs after exposure to memantine.
Figure 4-6 Comparison study between adult male and female RONs after exposure to memantine.

Graphs show the difference between males and females of adult RON in terms of axon area (A), axon diameter (B), axon viability scores (C) and axon density (D). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections taken from female rats n = 26, males n = 20; number of axons analysed – female n = 1110, male n = 1826. The significance was determined using unpaired t test with Welch's correction. * = P = 0.0163, *** = P < 0.0001.
4.2.8 Determining the sex differences of P0 RONs after exposure to MK-801

Following exposure to MK-801 there were no statistically significant differences in axon area (females = 0.33 µm² ± 0.006, n = 4085 axons, 18 sections; males = 0.14 µm² ± 0.002, n = 5337 axons, 18 sections, figure 4-7, E) or axon viability (females = 1.64 ± 0.011; n= 4085 /18 sections; males = 1.64 ± 0.009; n= 5337 /18 sections, figure 4-7, G). There was a significant difference, according to sex, in axon diameter (females = 0.59 µm ± 0.004, n = 4085 axons/18 section; males = 0.41 µm ± 0.002, n = 5337 axons, 18 sections, figure 4-7, F) and axon density (females = 6.44 axons/µm² ± 0.005, n = 4085 axons, 18 sections; males = 8.41 axons/µm² ± 0.055, n = 5337 axons, 18 sections, figure 4-7, H).
### Axon area

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>(n) of axons</th>
<th>P</th>
<th>(n) of sections</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.325832</td>
<td>0.006227</td>
<td>4085</td>
<td>&lt; 0.0001</td>
<td>18</td>
<td>0.09381</td>
<td>0.0703</td>
</tr>
<tr>
<td>Male</td>
<td>0.139591</td>
<td>0.001492</td>
<td>5337</td>
<td></td>
<td>18</td>
<td>0.025696</td>
<td></td>
</tr>
</tbody>
</table>

### Axon diameter

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>N</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.593587</td>
<td>0.004001</td>
<td>4085</td>
<td>&lt; 0.0001</td>
<td>18</td>
<td>0.060279</td>
<td>0.0096</td>
</tr>
<tr>
<td>Male</td>
<td>0.40552</td>
<td>0.001649</td>
<td>5337</td>
<td></td>
<td>18</td>
<td>0.028401</td>
<td></td>
</tr>
</tbody>
</table>

### Axon viability scores

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>N</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>1.639168</td>
<td>0.011009</td>
<td>4085</td>
<td>0.7730</td>
<td>18</td>
<td>0.165854</td>
<td>0.9843</td>
</tr>
<tr>
<td>Male</td>
<td>1.635052</td>
<td>0.009075</td>
<td>5337</td>
<td></td>
<td>18</td>
<td>0.123621</td>
<td></td>
</tr>
</tbody>
</table>

### Axon density

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>6.440569</td>
<td>0.024312</td>
<td>4085</td>
<td>&lt; 0.0001</td>
<td>18</td>
<td>0.36626</td>
<td>0.0193</td>
</tr>
<tr>
<td>Male</td>
<td>8.410938</td>
<td>0.040599</td>
<td>5337</td>
<td></td>
<td>18</td>
<td>0.699081</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-7 The mean of axon area, diameter and density of P0 male and female RONs after exposure to MK-801
Figure 4-7 Comparison study between P0 male and female RONs after exposure to MK-801.

Graphs show the difference between males and females of P0 RON in terms of axon area (A), axon diameter (B), axon viability scores (C) and axon density (D). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections taken from female rats n = 18, males n = 18; number of axons analysed – female n = 4085, male n = 5337. The significance was determined using unpaired t test with Welch's correction. * = P = 0.0193, ** = P = 0.0096 *** = P < 0.0001.
4.2.9 Determining the sex differences of adult RONs after exposure to MK-801

Following exposure to MK-801 there were no statistically significant differences, according to sex, in axon area (females = 0.88 µm² ± 0.022, n = 1105 axons, 28 sections; males = 0.62 µm² ± 0.015, n = 1476 axons/24 sections, figure 4-8, E), axon diameter (females = 0.997 µm ± 0.011, n = 1105 axons/28 section; males = 0.84 µm ± 0.00798, n = 1476 axons/24 sections, figure 4-8, F) or axon viability scores (females = 2.19 ± 0.014; n= 1105/28 sections; males = 2.28 ± 0.012; n= 1476/24 sections, figure 4-8, G). However, there was a significant difference, according to sex, in axon density (females = 1.11 axons/µm² ± 0.044, n = 1105 axons/28 sections; males = 1.73 axons/µm² ± 0.056, n = 1476 axons/24 sections, figure 4-8, H) regardless of whether the number of nerve sections or number of axons analysed were compared.
Table 4-8 The mean of axon area, diameter and density of adult male and female RONs after exposure to MK-801

<table>
<thead>
<tr>
<th>Axon area</th>
<th>Mean ± SEM</th>
<th>(n) of axons</th>
<th>P</th>
<th>(n) of sections</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.8794 ± 0.0218</td>
<td>1105</td>
<td>&lt; 0.0001</td>
<td>28</td>
<td>0.1368 ± 0.0215</td>
<td>0.1524</td>
</tr>
<tr>
<td>Male</td>
<td>0.6204 ± 0.0146</td>
<td>1476</td>
<td>&lt; 0.0001</td>
<td>24</td>
<td>0.1142 ± 0.0146</td>
<td>0.0922</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon diameter</th>
<th>Mean ± SEM</th>
<th>n</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.9973 ± 0.0109</td>
<td>1105</td>
<td>&lt; 0.0001</td>
<td>28</td>
<td>0.0689</td>
<td>0.0922</td>
</tr>
<tr>
<td>Male</td>
<td>0.8476 ± 0.0079</td>
<td>1476</td>
<td>&lt; 0.0001</td>
<td>24</td>
<td>0.0626</td>
<td>0.0922</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon viability scores</th>
<th>Mean ± SEM</th>
<th>N</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>2.1910 ± 0.0138</td>
<td>1105</td>
<td>&lt; 0.0001</td>
<td>28</td>
<td>0.0868</td>
<td>0.4905</td>
</tr>
<tr>
<td>Male</td>
<td>2.2786 ± 0.0117</td>
<td>1476</td>
<td>&lt; 0.0001</td>
<td>24</td>
<td>0.0915</td>
<td>0.4905</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Axon density</th>
<th>Mean ± SEM</th>
<th>N</th>
<th>P</th>
<th>n</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>1.1109 ± 0.0435</td>
<td>1105</td>
<td>&lt; 0.0001</td>
<td>28</td>
<td>0.0435</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Male</td>
<td>1.7292 ± 0.0560</td>
<td>1476</td>
<td>&lt; 0.0001</td>
<td>24</td>
<td>0.0560</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>
Figure 4-8 Comparison study between adult male and female RONs after exposure to MK-801.

Graphs show the difference between males and females of adult RON in terms of axon area (A), axon diameter (B), axon viability scores (C) and axon density (D). Data are presented as mean ± SEM. Data are shown for both number of nerve sections exposed to each condition (A-D) and number of axons analysed for each condition (E-H). Number of nerve sections taken from female rats n = 28, males n = 24; number of axons analysed – female n = 1105, male n = 1476. The significance was determined using unpaired t test with Welch’s correction. *** = P < 0.0001.
Thus, to summarise sex did not affect axonal area, diameter or viability in either P0 or adult nerves following exposure to either MK-801 or memantine. However, sex did affect axon density in both P0 and adult nerves following exposure to either MK-801 or memantine.
4.2.10 Determining sex differences in central white matter of adult rat corpus callosum using magnetic resonance imaging

In order to determine whether any sex differences exist in the volume of central white matter, we undertook some preliminary magnetic resonance imaging (MRI) analysis of the adult rat corpus callosum. The main purpose of these experiments was to determine if MRI was a sensitive method for making suitable measurements of the central white matter. Many MRI studies use the tractography technique based on the diffusion tensor imaging (DTI) to create white matter maps (Fig 4-9,10,11). DTI measures water molecule diffusion in different directions, and the diffusion of water molecules is highest along the direction of axons which determines the normal orientation of the fibres and therefore creates the connectivity maps. However, DTI-based connectivity maps are calculated mathematically and they do not always represent true point-to-point connectivity (axon-based) (Alexander, et al., 2007) (Nucifora, et al., 2007). In this study, DTI maps produced using diffusion analysis in Agilent software (VnmrJ 4.0). Maps reveal white-matter/grey matter structure (e.g. high FA around brainstem = white matter).

The parameters ADC and FA (apparent diffusion coefficient and fractional anisotropy) were quantified from the diffusion tensor imaging (DTI) data. DTI is a widely accepted MRI method for assessing white matter (WM) integrity and therefore it was the best method to use to look for differences in corpus callosum structure. It has been used to study changes in white matter due to ageing, multiple sclerosis, stroke, WM development in children, etc. This is why we thought these DTI parameters were relevant for corpus callosum study and ultimately why we used them to explore potential white matter changes due to sex. ADC is a measure of the rate of microscopic water motion within tissue. Typically, it is low in white matter as the diffusion is very preferential in one direction (i.e. along the white matter fibres), higher in grey matter and of course highest in CSF within the ventricles where water can diffuse freely. FA is a measure of anisotropy, i.e. the tendency for water to diffuse in one direction as opposed to randomly. This tends to be higher (closer to 1) in white matter and again, this is because water tends to move preferentially along the fibres.
So, the two parameters are quite similar in what they describe but they are independent. Not much is known about how a change in one of the parameters correlates with a change in the other. Our results did not show any clear sex-related differences (Fig 4-10,1-11) and see (Table 4-9). But, these data did reveal some potentially interesting relationships between the parameters. ADC and FA were (possibly) positively correlated (not strong, r-squared = 0.1468). The strongest correlation was between volume and FA. Volume was measured from the T2-weighted structural scan and compared to FA for the same region. This implies that as corpus callosum volume gets bigger, FA becomes higher.
Figure 4.9 Corpus Callosum Masks. Agilent software (VnmrJ 4.0) was used to apply these masks to all the samples.
Figure 4-10 Fractional anisotropy (FA) maps derived from (A) adult male rat corpus callosum and (B) adult female rat corpus callosum.

Imaging acquired with T2w slice (2D) – 0.07mm in-plane resolution, 0.8mm slice thickness DTI acquisition (planned from T2w scan) – Agilent 14-direction DTI (dual(2)), b-value=1000 s/mm² and analysis performed in VnmrJ 4.0 (Agilent) and 3D Slicer.
Figure 4-11 Apparent Diffusion Coefficient (ADC) maps derived from (A) adult male rat and (B) adult female rat.

Imaging acquired with T2w slice (2D) – 0.07mm in-plane resolution, 0.8mm slice thickness DTI acquisition (planned from T2w scan) – Agilent 14-direction DTI (dual(2)), b-value=1000 s/mm² and analysis performed in VnmrJ 4.0 (Agilent) and 3D Slicer.
<table>
<thead>
<tr>
<th>Brain</th>
<th>Volume (mm$^3$)</th>
<th>FA</th>
<th>ADC (x10$^{-6}$ mm$^2$/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female 1</td>
<td>123.44</td>
<td>0.69</td>
<td>774.79</td>
</tr>
<tr>
<td>Female 2</td>
<td>135.71</td>
<td>0.52</td>
<td>705.82</td>
</tr>
<tr>
<td>Female 3</td>
<td>123.04</td>
<td>0.56</td>
<td>691.37</td>
</tr>
<tr>
<td>Female 4</td>
<td>108.74</td>
<td>0.39</td>
<td>617.88</td>
</tr>
<tr>
<td>Female 5</td>
<td>101.66</td>
<td>0.43</td>
<td>714.97</td>
</tr>
<tr>
<td>Male 1</td>
<td>136.97</td>
<td>0.64</td>
<td>506/41</td>
</tr>
<tr>
<td>Male 2</td>
<td>101.47</td>
<td>0.36</td>
<td>447.48</td>
</tr>
<tr>
<td>Male 3</td>
<td>119.38</td>
<td>0.53</td>
<td>745.44</td>
</tr>
<tr>
<td>Male 4</td>
<td>127.49</td>
<td>0.42</td>
<td>687.28</td>
</tr>
<tr>
<td>Male 5</td>
<td>118.35</td>
<td>0.41</td>
<td>744.66</td>
</tr>
</tbody>
</table>

**Table 4.9** Table shows the results for volume, FA and ADC.

There does not appear to be much in terms of significant differences between males and females here but possibly some trends (e.g. volume may be weakly correlated with FA and ADC (see male 2 results, FA and ADC are weakly correlated).
$R^2 = 0.5964$

$R^2 = 0.0267$
Figure 4-12 Shows the relationship between volume, FA and ADC.
4.3 Discussion

The previous chapter aimed to explore the effect of age on injury to white matter induced by either exposure to OGD or NMDA-R block. However, this chapter aimed to determine whether sex affected the response to either OGD or NMDA-R block examining the effect at a developmental (i.e. P0) stage and adulthood. In addition, some preliminary experiments were conducted to examine the feasibility of using MRI to examine possible sex differences in the central white matter. The data from this chapter observed that sex did not affect the white matter (in terms of axon diameter, density, area) at either P0 or adulthood and in addition no sex differences were seen in response to OGD-injury. However, there were some differences in the response to NMDA-R block. Specifically, sex affected axonal density following exposure to MK-801 or memantine – such sex differences were seen at either P0 or adulthood.

Several studies showed how sex hormones influence brain structure and also brain function for instance mood and performance on motor tasks and cognitive abilities. Suggesting that the difference in the sex hormones between males and females may play role in the differences in the function and structure of their brain (Fink, et al., 1996) (Maki, et al., 2002). Also sex differences are recognised in relation to stroke. Several studies reported that females showed higher rate of stroke relapse compared with males (reviewed by (Gibson, 2013)) (Wang, et al., 2013), on the other hand, other studies showed no sex differences in stroke when comparing age and other related risk factors (Fukuda, et al., 2009). In the current study we didn’t observe any sex differences in response to OGD injury in neonatal and adult rats. Although sex differences might impact the mechanisms of injury and the consequences after ischaemia (Gibson, 2013) it wasn’t evident in this particular model and most of previous studies have focused on grey matter rather than white.

An interesting finding of this chapter were the sex differences found in axonal density following exposure to either Mk-801 or memantine treatment. Such sex differences were found at both P0 and adulthood. In fact density is one of the most important parameters used in determining the significance of white matter injury, for instance axonal lose following ischaemia. Also density can play a key role in determining some sex differences in white matter. A study by Cerghet and his group (2006) showed that oligodendrocyte density was on average of 30% greater in males than in females in
three different white matter regions; corpus callosum, spinal cord and fornix (Cerghet et al., 2006).

Although the exact mechanism of the sex differences in response to NMDAR block were not determined, the present outcomes could be a cautionary note regarding the choices of dosage and drug according to sex. There is emerging evidence that sex may effect treatment effectiveness across a range of conditions and it may be that NMDAR activity is regulated by adrenal and gonadal steroids (Weiland, 1992) (Akinci & Johnston, 1993).

This study also went on to examine the central white matter for possible sex differences. Some DTI studies showed a significant sex difference (Hsu, et al., 2008) (Sullivan, et al., 2008) (Huster, et al., 2009) (Sullivan, et al., 2001), while other studies showed no sex difference (Kennedy & Raz, 2009) (Kennedy & Raz, 2009) (Lee, et al., 2009) (Ota, et al., 2006). However, one study by Hsu JL et al, 2008 investigated the sex effects on white matter integrity through the brain, significant sex differences in FA were found in the right deep temporal region (Hsu, et al., 2008). Several DTI studies have examined age and sex interaction in diffusion characteristics (Hsu, et al., 2008). (Sullivan, et al., 2001) (Sullivan, et al., 2008) (Kennedy & Raz, 2009) (Schneiderman, et al., 2007). Some of these studies demonstrated that there was no significant difference between age and sex interaction, (Hsu, et al., 2008) (Sullivan, et al., 2001) (Kennedy & Raz, 2009), and others reported significant interactions of diffusion properties between sex and age (Sullivan, et al., 2008) (Abe, et al., 2010) (Kochunov, et al., 2010). Together sex differences and sex-age interactions of white matter integrity need more evaluation with a large sample size. The current study established that MRI could be used to make relevant measurements of central white matter and further studies should be expanded to include higher n values and also samples from different developmental stages.

To date, investigators have observed corpus callosum volume in both children and adolescents and they detected inconsistent results (Giedd, et al., 1999) (Lenroot, et al., 2007) (Rauch, et al., 1994). MRI studies to date demonstrated a significant difference in relative volume of the corpus callosum which is larger in females than males; and the differences were found mostly in the anterior, central and posterior segment of corpus callosum. In addition, these sex differences are examined specifically during
the adolescence period, rather than adolescence and childhood (Giedd, et al., 1999) (Lenroot, et al., 2007). The results of the studies that investigate the sexual dimorphism in the total relative volume of the corpus callosum, during childhood and adolescents were varied. Some investigators did not report any sex differences in the callosum area (Rauch, et al., 1994), while others found differences (Giedd, et al., 1999) (Lenroot, et al., 2007). Many studies observed some evidence of sex differences and/or sex dimorphic age-related growth of the corpus callosum volume throughout the adolescence (Giedd, et al., 1999) (Lenroot, et al., 2007) (De Bellis, et al., 2001). These sex differences were limited to the anterior and central parts of the corpus callosum (Giedd, et al., 1999). Thus, it would be interesting for us to conduct an analysis of adolescent-relevant ages in order to see if any sex differences can be observed in the RON.

Sex differences occur almost in every brain region, such as, amygdala, neocortex and hippocampus (Juraska, 1991). Sex difference studies have focused on investigating the anatomical structure differences between the males and females e.g., areas of the cortical mantle are significantly thicker in females than in males (Luders & et al, 2005), and the ratios of grey to white matter also differ significantly between the sex in various areas of the human cortex (Allen, et al., 2003). However, in several cases, the differences are not apparent in the anatomical structure, but it might be due a functional aspect. Thus, from our current approach we cannot conclude that sex differences were not present (for example in axonal function which can be measured by using electrophysiology technique) but were only absent in those measures we used.

However, studies investigating potential sexual dimorphisms in both normal structure/function and response to injury are important as they may help increase understanding and treating some CNS-related disorders which show sex differences in their incidence and nature. Such diseases include; Alzheimer’s disease (AD), post-traumatic stress disorder (PTSD), stroke, MS, schizophrenia and autism (Hines, 2004), (Shors, 2002), (Klein & Corwin, 2002). The existence of sex differences in any disorder requires examining the sex influences in the clinical research to fully understand and treat the disorder.
**Chapter 5: Investigation of the expression of voltage-gated calcium channels in peripheral nervous system during myelination.**

### 5.1 Introduction

The previous two chapters have demonstrated that OGD has an effect on both neonatal and adult RONs whereas blocking NMDA-R has a severe effect on neonatal white matter but no effect during adulthood. Also these chapters investigated whether the sex is affected following injury by either OGD or NMDA-R block during development and adulthood. However, data showed that no sex differences were observed at either P0 or adulthood and also no sex differences were seen in the response to OGD-injury.

Many studies have focused on investigating the characterization and distribution of the Na\(^+\) and K\(^+\) channels in the axolemma. However, Ca\(^{2+}\) channels remain relatively under studied. In mammals, axons possess many proteins that are involved in Ca\(^{2+}\) homeostasis but studies did not show any evidence of presence of Ca\(^{2+}\) channels or their function, possibly due to the small size of the axons and the fact they are difficult to access for electrophysiological techniques especially unmyelinated axons (Jackson et al., 2001). Ca\(^{2+}\) imaging techniques have been very effective in studying the presence of Ca\(^{2+}\) channels on the axolemma. Lev-Ram and Grinvald (1987) were the first who provided the evidence of dependent Ca\(^{2+}\) transient activity in central axons using Ca\(^{2+}\) indicator (FURA-2) (Foster et al., 1982; Lev-Ram and Grinvald, 1987).

Several studies have provided evidence of Ca\(^{2+}\) influx into unmyelinated axons, for example: Scholfield, 1988; Quasthoff et al., 1995; Callewaert et al., 1996; Luscher et al., 1996; Wachtler et al., 1998; Forti et al., 2000; Jackson et al., 2001. The first study was by Scholfield (1988) who described the effect of blocking Ca\(^{2+}\) channels on action potential which was recorded from olfactory cortex unmyelinated axons, after exposure to the K\(^+\) channel blockers (TEA and 4-AP). Another study by Grafe and colleagues (1995) reported Ca\(^{2+}\) potential in the unmyelinated C fibres of human sural nerve. Also Grafe and his group used confocal microscopy and showed Ca\(^{2+}\) entry into unmyelinated axons using rat vagus nerves during stimulation (Wachtler et al., 1998). The group established that the activity induced Ca\(^{2+}\) transients were voltage-gated calcium channel (VGCC)-mediated and gave the ability of Ca\(^{2+}\) to block the rise of [Ca\(^{2+}\)]. Also, other studies have shown axonal Ca\(^{2+}\) transients are VGCC-mediated.
Using confocal microscopy, Callewaert et al (1996) has demonstrated fast Ca\textsuperscript{2+} transients in axons of Purkinje neurons in cerebellar slices from neonatal rats. Axonal transients were determined after single action potential and increased when the action potential frequency increased. These transients were totally eliminated by a VGCC blocker (oi-agatoxin IVA) and they were involved in regulating the firing rate of action potentials. The researchers argued that this happened due to the activation of Ca\textsuperscript{2+} activated K\textsuperscript{+} conductance and therefore the channels possibly play an important role as determinant in discharge frequency of neurons. A role in the modulation of axonal excitability was also recognized when Ca\textsuperscript{2+} transients were recorded from cultured dorsal root ganglion cells using unmyelinated axons (Luscher et al., 1996). Also, it has been demonstrated that when Sr\textsuperscript{2+} was substituted for Ca\textsuperscript{2+} this prevented failure of the action potential (Luscher et al., 1994) and the authors demonstrated that Ca\textsuperscript{2+} accumulation during trains of action potentials may modulate excitability because of the inactivation of Ca\textsuperscript{2+} components of the action potentials. There was not enough evidence of Ca\textsuperscript{2+} influx into myelinated axons, this might be due to the difficulty of determining this signal from the axons only but not from the myelin sheath. The results from the Lev-Ram and Grinvald study were initially supported by the indirect indications of the presence the VGCCs on myelinated axons and concluded from the protective action of L-type and N-type of VGCC blockers following anoxia in the adult RON (Fern et al., 1995). Other studies have also reported the presence of L-type VGCCs on mature axons and showed that L-type VGCC block has a protective effect during anoxia (Brown et al 2003) (Sun and Chiu 1999). A study by Chiu and his group used myelinated axons of MONs and they showed a uniform Ca\textsuperscript{2+} influx during action potential (Zhang et al., 2006).

Abnormal calcium regulation has been involved in retinal ganglion cells (RGCs) degeneration in many injury models of optic neuropathy. As calcium has roles in both triggering apoptotic pathways in injured cells and maintaining homeostasis in healthy cells, investigation of the regulation of VGCCs as a potential approach to decrease the loss of RGCs is necessary. The easy access to the retina and their structure provides advantages for the investigation of the mechanisms of calcium signalling in both the somata of ganglion cells as well as in their unmyelinated axons. The second aim of the present study was to localize the distribution of VGCC subtypes in the axons of ganglion cells in the normal retina. This study showed the expression of the L-type
VGCC subunit in rat RGC axons. On the other hand there was no expression of the P/Q-type VGCC subunit in unmyelinated axons of RGC. Ca$^{2+}$ imaging studies of whole mounted retinas revealed that selective Ca$^{2+}$ channel antagonists decreased calcium depolarization-evoked signals mediated by L-, N-, P/Q- and T-type VGCC in the RGC cell bodies however only the L-type VGCC is located in the axons (Sargoy et al., 2014). The contribution of VGCC subtypes to Ca$^{2+}$ signals in RGC bodies and axons may give an opportunity to develop specific approaches to protect RGCs and their axons after injury.
5.2 Results

5.2.1 Expression of voltage-gated calcium channels in the peripheral nervous system (rat sciatic nerve) during development

Using antibodies against L-type, P/Q-type and Na channels, double labelling studies were carried out across a variety of early postnatal ages in preparations from the rat sciatic nerve (RSN). The P/Q-type and L-type of VGCCs were present at low levels at P0 (Figure 5-2, Figure 5-3). However, P10 showed an intense staining and co-expression of P/Q-type of VGCCs with the axonal marker neurofilament light (70DK, NF-L), and began to decrease to be almost absent by P20 (Figure 5-2). At P10, Na+ channel expression was evident, although not as much as the P/Q-type but started to increase by P20 and adult (Figure 5-4). Following on the co-labelling experiments were carried out using GFAP as a marker to stain Schwann cells and revealed a co-localisation of Schwann cells with Ca2+ pan by P10 and continued till adulthood (Figure 5-5).
Figure 5-1 Ca²⁺ Pan VGCC staining with axonal marker neurofilament light (70K Da, NF-L).

- **A1-3**, P0 RSN co-labelled for Ca²⁺ Pan (green, A2) and the neurofilament marker (NF-L, red, A1) followed by overlap image (A3). Subunits clusters are found on NF-L+ axons.
- **B1-3**, P10 RSN co-labelled for Ca²⁺ Pan (green, B2) and the neurofilament marker (NF-L, red, B1), followed by overlap image (B3). Subunits clusters are found on NF-L.
- **C1-3**, P20 RSN double labelled for Ca²⁺ Pan (green, C2) and NF-L (red, C1), some subunits overlap with NF-L and some do not.
- **D1-3**, Adult RSN double labelled for Ca²⁺ Pan (green, D2) and NF-L (red, D1), some subunits overlap with NF-L and some do not.
- **E1-3**, no immunoreactivity is observed following omission of the Ca²⁺ Pan and NF-L primary antibody.

**Scale bar = 2 μm.**
Figure 5-2 P/Q-type VGCC clusters (anti-α1A), and neurofilament marker (70K Da, NF-L).

- **A1-3**, P0 RSN co-labelled for α1A (green, A2) and the neurofilament marker (NF-L, red, A1) followed by overlap image (A3). Subunits clusters are found on NF-L+ axons.
- **B1-3**, P10 RSN co-labelled for α1A (green, B2) and the neurofilament marker (NF-L, red, B1), followed by overlap image (B3). Subunits clusters are found on NF-L.
- **C1-3**, P20 RSN double labelled for α1A (green, C2) and NF-L (red, C1), some subunits overlap with NF-L and some do not.
- **D1-3**, Adult RSN double labelled for α1A (green, D2) and NF-L (red, D1), some subunits overlap with NF-L and some do not.
- **E1-3**, no immunoreactivity is observed following omission of the α1A and NF-L primary antibody.

**Scale bar = 2 μm**
Figure 5-3 L-type VGCCs labelling (anti-α1D subunits) with neurofilament light (70K Da, NF-L).

- **A1-3**, P0 RSN. No α1D (green, A2) VGCC immunoreactivity is observed.
- **B1-3**, P10 RSN, a little of α1D subunits are apparent (green, B2), and the neurofilament marker (NF-L, red, B1). The merged image is shown in B3.
- **C1-3**, At P20 there are still some α1D clusters are observed (green, C2).
- **D1-3**, Adult RSN again no α1D clusters were observed (green, C2).
- **E1-3**, a primary omission control experiment for the anti-α1D antibody.

Scale bar = 2 μm.
Figure 5-4 Ca²⁺ pan labelling with Na⁺ pan antibodies.

- **A1-3**, A P0 RSN. No Na⁺ pan (red, A2) immunoreactivity is observed.
- **B1-3**, P10 RSN, again no Na⁺ pan subunits are apparent (red, B2), and Ca²⁺ pan (green, B1). The merged image is shown in B3.
- **C1-3**, At P20 Na⁺ pan clusters are now present (red, C2).
- **D1-3**, Adult RSN, the number of Na⁺ pan clusters has increased (red, C2).
- **E1-3**, a primary omission control experiment.

*Scale bar = 2 μm.*
Figure 5-5 Ca** Pan VGCC staining with Schwan cell marker (GFAP).

- **A1-3**, P0 RSN co-labelled for Ca** Pan (green, A2) and GFAP (red, A1) followed by overlap image (A3). Subunits clusters did not found on Schwan cells.
- **B1-3**, P10 RSN co-labelled for Ca** Pan (green, B2) and GFAP (red, B1), followed by overlap image (B3). Subunits clusters are found on NF-L.
- **C1-3**, P20 RSN double labelled for Ca** Pan (green, C2) and GFAP (red, C1), some subunits overlapped with GFAP and some do not.
- **D1-3**, Adult RSN double labelled for Ca** Pan (green, D2) and GFAP (red, D1), some subunits overlapped with GFAP and some do not.
- **E1-3**, no immunoreactivity is observed following omission of the Ca** Pan and GFAP primary antibody.

Scale bar = 2 μm.
5.2.2 Triple labelling IHC experiment

This experiment was conducted to assure that axonal and glial staining. Adult (figure 5-6) and P10 RONs were chosen in this experiment as they both represent a crucial stage of development. However, there was some inconsistency in the results obtained from the P10 sections in terms of the intensity of staining.
Figure 5-6 Triple labelling staining of adult RSN with Ca**anti-Pan(green) and Schwan cell marker GFAP (red) and neurofilament light (blue) - (70K Da, NF-L).

- **A4** overlap image of adult RSN co-labelled for Ca** Pan (green, A2) and GFAP (red, A3) and NF (blue, A1). Subunits of VGCC clusters were found on Schwan cells and axons (red arrow).
- **B3** overlap image of adult RSN co-labelled for Ca** Pan (green, B2) and NF (blue, B1). Subunits of VGCC clusters were found on axons (green arrow).
- **C3** overlap image of adult RSN co-labelled for GFAP (red, C2) and NF (blue, C1).
- **D3** overlap image of adult RSN co-labelled for Ca** Pan (green, D1) and GFAP (red, D2). Subunits of VGCC clusters were found on Schwan cells (black arrow).
- **A5** no immunoreactivity is observed following omission of the Ca** Pan and GFAP and NF-L primary antibody.

*Scale bar = 2 μm.*
5.2.3 Are voltage gated Ca\(^{2+}\) channels expressed in unmyelinated central axons as well as non-myelinated central axons?

The existence of VGCCs in neuronal cell bodies is well established. However, there is fairly little information with regard to the expression of VGCCs on axons. Using the isolated head region of the adult rat optic nerve, the expression of VGCCs on retinal ganglion cell axons (RGC) were investigated. Normally, within the retina and in the first region of the optic nerve, RGC axons remain unmyelinated but once they exit the eye through the lamina cribrosa they become myelinated (see figure 5-7). L-type of VGCCs immunoreactivity was expressed along the unmyelinated RGC axons in the lamina cribrosa in the initial portion of the optic nerve head (figure 5-10, C2). In contrast, P/Q-type of VGCCs did not show any immunoreactivity (figure 5-10, B2). Finally, glial cells also were stained with GFAP and Ca\(^{2+}\) pan and showed expression along the unmyelinated RGC axons in the lamina cribrosa and the retina. As with all antibodies used here, staining was eliminated by pre-incubation with blocking peptide.
Figure 5-7 Immunohistochemistry graph shows the head region of P0 rat optic nerve.

- **(A)**, A P0 RON Single stained for the NF-L (red).
- **(B)**, A P0 RON Single stained for the Ca^{2+} Pan (green). The merged image is shown in **(C)**.

Scale bar = 2 μm
Both primary omission control and cross reactivity control experiment yielded no immunoreactivity.

**Figure 5-8** Cross reactivity control of P0RON using Ca pan and NF-L.

*Scale bar = 2 μm.*
Figure 5-9 Preadsorption control of P0 RON using Ca pan and NF-L.

Scale bar = 2 μm
Figure 5-10 Different area of head region of P0 RON showing different type of VGCCs.

- **A1, B1, C1, D1, E1**, IHC images show the retina co-stained with different VGCCs subtypes and NF-I and also Ca pan antibody and GFAP.

- **A2, B2, C2, D2, E2**, IHC images show the head of the rat optic nerve co-stained with different VGCCs subtypes and NF-I and also Ca pan antibody and GFAP.

- **A3, B3, C3, D3, E3**, IHC images show the main part of the rat optic nerve co-stained with different VGCCs subtypes and NF-I and also Ca pan antibody and GFAP.

- **A4, B4, C4, D4, E4**, A primary omission control experiment.

*Scale bar = 2 μm*
5.3 Discussion

Numerous studies have described the localization of specific Ca$^{2+}$ channel types. Ca$^{2+}$ channels are found to be expressed in the soma, dendrites and the nerve terminals. L-type channels are mostly found in cardiac tissue; but some isoforms are found in the soma of neurons and the proximal dendrites (Hell, et al., 1993). P/Q-type, N-type and R-type channels are found in somas, dendrites and presynaptic terminals and they contribute to the induction of LTP at mossy fibre synapses and are involved in controlling neurotransmitter release (Catterall, et al., 2005), (Westenbroek, et al., 1992), (Dietrich, et al., 2003). Also some T-type isoforms are expressed at dendrites (Catterall, et al., 2005).

In this chapter VGCCs were found to be present in early postnatal development of RSN. As immuno-histochemical data indicated both L-type and P/Q-type channel subunits were at low expression at P0 and started to increase by P10 and then began to decline to be almost absent by P20. Na$^+$ channel expression was evident at P10, and started to increase by P20. These data contrast with the study published by Brown et al. (2001) using adult RON as they showed that L-type expression is along the length of axons. However, the result is consistent with other studies which report increases in Ca$^{2+}$ dependent fluorescence localisation for example, Forti et al. (2000), Llano et al. (1997) and Mackenzie et al. (1996). This study also showed that VGCCs are expressed in adult Schwan cells of RSN. In addition, immunohistochemistry staining of the head region of adult RON showed expression of L-type but not P/Q-type channels at the nerve head where the axons are unmyelinated. A study by Sargoy et al., (2014) demonstrated that retinal ganglion cells (RGC) somata express the L-, P/Q-, N-, and T-type of VGCCs whereas RGC axons express mainly L-type VGCCs and all these channels subtypes contribute in strong calcium signalling. They also reported that T-type localisation in the axons of RGC was not established, as the T-type VGCC does not have a distinct role of calcium signalling in these structures (Sargoy et al., 2014).

It is already known that Ca$^{2+}$ contributes to the process of axon outgrowth (Henley & Poo, 2004), and dendrite formation (Konur & Ghosh, 2005). The main increases in [Ca$^{2+}$]i that are brought about by axonal VGCCs might be involved in the developmental processes with which their expression coincides. The $\alpha_{1A}$ subunits transient expression is particularly interesting as it happens just prior to the early
formation of the node of Ranvier and myelination. There is a close association with α_{1A} and α_{1C} subunits as they were both observed at P12 at which associated with Na^{+} channel clusters (Rasband et al., 1999b). VGCC clusters appear before Na^{+} channel clustering by a few days and particularly P/Q-type VGCCs drops as Na^{+} channel clusters appear. The finding of P/Q-type clusters near to glial processes and vesiculotubular complexes using the gold labelling technique adds additional evidence for a role of VGCCs in axon development and it may be that these vesicle components are present at the axolemma to mediate the transport of axolemmal proteins (Alex et al., 2008). A study by Hildebrand and Waxman (1984) also detected these clusters of vesiculotubular profiles in unmyelinated axons and proposed that they could play a role in axon development by fusing with the axolemma (Hildebrand and Waxman, 1984).

Recently, the T-type of VGCCs have been involved in the regulation of different pain states (Todorovic and Jevtovic-Todorovic, 2011). These channels are found in neuronal and non-neuronal cells and involved in cellular excitability (Kim et al., 2003). The P/Q-type and N-type VGCCs are expressed mainly in the presynaptic nerve terminals and are also associated with neurotransmitter release (French and Zamponi, 2005). The N-type of VGCCs is concentrated in the synaptic terminals within the spinal cord dorsal horn and in the dorsal root ganglia cell bodies and they play an important role in pain sensitivity (Snutch, 2005). Also the N-type mediated Ca^{2+} influx is responsible for neurotransmitter release, such as glutamate and substance P, which act postsynaptically (Snutch, 2005; Winquist et al., 2005; Zamponi et al., 2009b; Park & Luo, 2010).

In summary, this chapter describes the developmentally regulated expression of VGCCs on developing peripheral axons. The results presented provide evidence that axonal calcium channels may play a key role in myelination. The expression of P/Q-type channels increased around the onset of myelination and node of Ranvier formation which may play an important role in the signalling pathways that are involved in axons development and modulation. In addition, the present findings suggest that VGCCs are expressed on peripheral unmyelinated axons as well as central non-myelinated axons of RGC unmyelinated axons. These results may provide a potential therapeutic approach for protecting the RGCs and their axons after injury.
Chapter 6: Conclusions and future work

The effect of acute cerebral ischaemia leads to extensive necrosis due to the Ca\textsuperscript{2+} activated cell death pathways. During the early stages of ischaemia, the depolarisation leads to increased extracellular glutamate through synaptic release. One of the main pathophysiological events that causes cell death is thought to be largely due to the failure and reversal of glutamate re-uptake mechanisms. The high levels of extracellular glutamate lead to excitotoxic activation of the cell surface receptors such as the NMDARs, causing rapid changes in the ionic homeostasis that activates ischaemic pathways.

The association between brain ischaemia and glutamate was predicted over 50 years ago by Van Harreveld (1959). The extracellular glutamate neurotoxic effect in the retina following ischaemia was documented slightly earlier by Lucas and Newhouse (1957), and then in the brain (Olney & Sharpe, 1969). In fact, the term ‘excitotoxicity’ is used instead of ‘neurotoxicity’ and became a common expression for neurotransmitters excitatory in central neurons (Olney & De Gubareff, 1978). Glutamate activates nonNMDA-Rs as well as NMDA-Rs which are known to mediate glutamate-induced excitotoxicity in the brain (Choi & Rothman, 1990). However, excitotoxicity of glutamate is not the only reason for the brain damage that occurs during ischaemia as neurotransmitters other than glutamate can also mediate excitotoxicity in the brain such as high levels of dopamine which form reactive oxygen species and lead to mitochondrial dysfunction that is involved in neurodegenerative diseases e.g. Parkinson’s disease (Berman & Hastings, 1999).

Several studies have showed that inhibition of NMDARs reduces the injury following toxicity and ischaemia in both in vivo and in vitro models (Bakiri et al., 2009, Ahlgren et al., 2011, Gorgulu et al., 2000, Bonde et al., 2005, Culmsee et al., 2004). On the other hand, NMDA-R antagonists have failed within clinical trials for conditions such as ischaemic stroke. Previous studies have showed serious complications when using antagonists that targeted the NMDA-Rs (Ikonomidou and Turski, 2002). Most of these side effects were due to the abundant and identical antagonisms of glutamate receptors, with no ability to distinguish between normal physiological signalling and over-activation, therefore causing harmful side effects or may be due to differing effects they have on grey and white matter. It’s still accepted that over-activation of NMDA-
R in the acute phase of ischaemia, plays an important role in the injury that is caused following an ischaemic insult.

The initial experiments presented in this thesis revealed a toxic effect of NMDA-R antagonists such as MK-801 and memantine which is a clinically available treatment, at concentrations that have been used within other previous studies, where positive outcomes within ischaemia models were reported. The significance of blocking NMDA-Rs that are expressed in the developing white matter has not been examined before, the functions of which are not known yet. NMDA-R antagonists - memantine and MK-801- did not show any damage to the mature white matter as the data obtained here showed no significant differences in the viability of axons, axon density and no effect on glial cells. This thesis also investigated the effect of OGD on neonatal and mature white matter using RONs and the axonal and glial injury was assessed by electron microscopy. OGD-mediated injury and NMDA-R block yielded significant damage to developing white matter. In addition, the results of this thesis showed that acute exposure to OGD could cause significant damage to the axons and glial cells compared to that caused by the toxicity that is produced by blocking NMDA-Rs. This study aimed to investigate the impact of using memantine and MK-801 at low concentrations, and has showed the toxic effects of memantine and MK-801 on developing central white matter. These findings suggest that NMDA-Rs may play a key role in regulating central white matter development and injury. These results suggest that developing white matter injury may NMDA-R dependent processes.

This prominent role of glutamate excitotoxicity mediated by NMDA-Rs subject to the extracellular Ca²⁺ (MacDermott, et al., 1986). However, additional Ca²⁺ entry probably occurs through the Na⁺/Ca²⁺ exchanger and voltage gated Ca²⁺ channels (Choi, 1988). This study focused on investigating the effect of injury on white matter structure and future work could include looking at the effects of injury on the function of the white matter, further examining NMDA-R subunit expression and exploring changes that may occur in the functionality of NMDARs during development.

In this thesis the expression of voltage-gated calcium channels (VGCCs) in peripheral axons has been also investigated using the rat sciatic nerve (RSN) preparation. These channels have been identified in central axons as they begin myelination and VGCCs contribute to action potential conduction in central axons during this onset of
myelination (Alix et al, 2008). However, it is unknown whether this is unique to central axons or the same pattern occurs in peripheral axons or unmyelinated axons at the optic nerve head. The data presented here show that L-type and P/Q-type channel subunits were present at low levels at P0 and increased by P10 after which they declined by P20. Both double and triple labelling IHC experiments demonstrated that Schwann cells express VGCCs during the myelin formation process which starts from around P10. The expression of P/Q-type channels increased around the onset of myelination and node of Ranvier formation thus, may play an important role in the signalling pathways that are involved during axonal development and modulation. Other future plans could explore several questions such as; do VGCCs play a role in nerve conduction in peripheral axons which are actively myelinating? What about in non-myelinated peripheral axons during development and in the adult? Do VGCCs play a role in nerve conduction in axons that are demyelinated or are re-myelinating in disorders such as in multiple sclerosis (MS)? Do VGCCs contribute to the action potential in retinal axons that lose their myelin sheath as they enter the retina?

Another important issue that has been investigated within this thesis is whether the morphology of white matter is sexually dimorphic and the possibility of the presence any sex differences in terms of response to injury by exposing white matter to OGD and NMDA-R block. It should be stressed that most of the previous in vivo studies were done in male animals; however, some studies on pathological changes in the retrosplenic/cingulate cortex showed that females have higher sensitivity on average up to 10 fold (Fix, et al., 1995). Sex differences are also found to be in many brain areas structurally such as cortical mantle are thicker in female than in males (Luders & et al, 2005) and functionally in aspects of neurotransmitter function. Sex differences even occur in many CNS related disorders e.g. schizophrenia, stroke, multiple sclerosis and AD (Hines, 2004) (Shors, 2002). However, the current study focused on determining whether sex affected the response to either OGD or NMDA-R block. Also, some preliminary experiments were conducted to examine the feasibility of using MRI to examine possible sex differences in the central white matter. The data from this study observed that sex did not affect the white matter (in terms of axon diameter, density, area) at either P0 or adulthood and in addition, no sex differences were seen in response to OGD-injury. Though there were some differences in the response to NMDA-R block. Specifically, sex affected axonal density following
exposure to MK-801 or memantine – such sex differences were seen at both P0 or adulthood. The reason why female rats showed more sensitivity to the neurotoxic effects of NMDA-R antagonists is not clear yet, however, pharmacokinetic aspects may possibly play a role. Indeed, the present study indicates that using the same doses that have been used within previous literature for both memantine and MK-801 resulted in severe axonal damage in female rats when compared to the males. Several studies showed that MK-801 have a different effect in female and male rats e.g. long-lasting hypothermia, and an increase in polymorphonuclear phagocytes in the blood has been reported in female but not male rats (Nietosampedro, et al., 1991), also behavioural response is stronger in female than male rats (Wintrip, et al., 1998). In turn, this may indicate that for evaluating the safety margin of NMDA-R antagonists it is necessary to examine both sexes.

In summary, this thesis has demonstrated that pre-myelinated white matter is susceptible to injury under conditions of energy deprivation, as modelled by OGD. Such injury to developing white matter is probably mediated by the over-activation of NMDA-Rs due to a disruption of the ionic homeostasis following ATP reduction and a subsequent release of glutamate into the extracellular space. Previous research has demonstrated the protective potential of NMDAR antagonists during ischaemic conductions but these effects have largely been explored in the grey matter and in mature animals with little, or no, attention paid to effects on the white matter. Evidence presented here suggests that exposure of developing white matter to NMDAR antagonists resulted in damage to both axons and glia. NMDAR antagonists, such as memantine, are in current clinical use for disorders such as Alzheimer’s disease, thus it is important to understand whether such compounds have differing effects on white and grey matter. It would appear though that this detrimental effect of NMDAR antagonists is specific to developing, i.e. immature, white matter as the same effects were not seen following exposure of adult, i.e. myelinated, white matter. Thus, it is important to consider the mechanisms by which NMDARs may differ between myelinated and unmyelinated white matter. Important changes which occur as axons being to myelinate include changes in the expression of voltage gated calcium channels. In particular, as demonstrated here, the expression of P/Q-type channels increased around the onset of myelination and node of Ranvier formation suggesting may play an important role in the signalling pathways that are involved during axonal
development and modulating the response to ischaemic injury and glutamate signalling. As ischaemic injury can be considered to be sexually dimorphic in that males and females differ in terms of injury amount and incidence it was thought relevant to consider whether any sex differences occurred in response to NMDAR antagonists. Although sex did not affect the response to OGD-induced injury it did appear that females were more sensitive to the neurotoxic effects of the NMDAR antagonists compared to males. This has important implications as traditionally in vivo (and in vitro preparations) tend to be largely male-based yet drugs tested in clinical trial are often given to both sexes. Uncovering and understanding further any specificity to NMDAR activity could be crucial in developing sex-specific therapies for disorders such as ischaemia which affects both sexes.
Bibliography


Richardson, W., Young, K., Tripathi, R. & McKenzie, I., 2011. NG2-glia as multipotent neural stem cells: fact or fantasy?. *Neuron*, Volume 70, p. 661–673.


Tripathi, R. et al., 2010. NG2 glia generate new oligodendrocytes but few astrocytes in a murine experimental autoimmune encephalomyelitis model of demyelinating disease. J Neurosci, Volume 30, p. 16383–16390..


