Interaction of selective cellular elements in white matter injury: the role of astrocytes

By:

Hewa Hama Ameen

A thesis submitted as partial fulfillment of the requirement for the degree of

PhD in neuroscience

at the

University of Leicester

Department of Neuroscience, Psychology and Behaviour

2015
# Contents

Abstract .................................................................................................................. x

Chapter 1 ................................................................................................................... 1

1.1 Introduction ....................................................................................................... 1

1.2 White matter .................................................................................................... 3

1.3 Periventricular leukomalacia ........................................................................... 5

1.4 Stroke .................................................................................................................. 7

1.5 Multiple sclerosis ............................................................................................ 9

1.6 Glial cells .......................................................................................................... 11

1.7 Astrocytes ......................................................................................................... 13

1.8 Potassium regulations by astrocytes ............................................................... 14

1.9 Glial fibrillary acid proteins (GFAP) ............................................................. 16

1.10 Reactive astrocystosis ..................................................................................... 18

1.11 Problems of astrocyte identity ...................................................................... 21

1.12 NG2 cells ......................................................................................................... 24

1.13 Microglia .......................................................................................................... 25

1.14 Fluorocitric acid ............................................................................................ 27

1.14.1 Mechanism of action and consequences of FC treatment ..................... 29

1.14.2 Use of fluorotoxins in the study of brain metabolism ......................... 33

1.14.3 Support for glial specificity of fluorotoxins ........................................... 35

1.15 Glutamate ....................................................................................................... 37

1.16 Roles of astrocytes in glutamate handling ..................................................... 38

1.16.1 Glutamate synthesis and metabolism .................................................... 38

1.16.2 Glutamate release and reuptake ............................................................. 40

1.17 Glycogen ......................................................................................................... 42

1.18 Glycogen in brain ........................................................................................... 43

1.19 Role of lactate in brain metabolism ............................................................... 44

1.20 Astrocyte-neuron lactate shuttle ................................................................. 46

1.21 Rat Optic Nerve ............................................................................................. 48

1.22 Summary, Aims and Hypotheses .................................................................... 49

Chapter 2 Establishing the role of FC ................................................................... 51

2.1 Introduction .................................................................................................... 51

2.2 Materials and Methods .................................................................................. 53
Acknowledgement

I would like to thank Prof Bob Fern for his help and advice during the initial stages of my work. I would also like to express my sincerest appreciation to Dr. Andrew Young for his continuous help and dedication throughout the course of this study. Without his generous efforts, everything would have been a lot harder. He not only provided continuous scientific advice on every aspect of the thesis, but also was a great personal mentor and moral advisor. I am also indebted to Dr. Claire Gibson and Dr. Martine Hamann for their generous suggestions, encouragement and insightful comments. I am also grateful for the Department of Neuroscience, Psychology and Behaviour doctors, staff and students who have in different ways been helpful during my study, and especially Matthew Barker who, on several occasions, gave me invaluable advice on confocal microscopy. Finally, I would like to express my gratitude to Miss Natalie Allcock, who through her skill, patience, and continuous advice guided me to overcome many hurdles in electron microscopy.
List of Figures:

Figure 1-1: A brief diagram showing the action of FC ................................................................. 29
Figure 2-1: a brief over-view of the steps of immunohistochemistry protocols............................56
Figure 2-2: samples of GFAP scoring system .............................................................................59
Figure 2-3: samples of PI scoring system .................................................................................. 60
Figure 2-4: images of the first protocol .....................................................................................62
Figure 2-5: images of the second protocol ............................................................................... 63
Figure 2-6: oligodendrocyte staining ....................................................................................... 64
Figure 2-7: intensity measurement using ImageJ software ....................................................... 66
Figure 2-8: concentration of FC increased to 0.5 mM ............................................................... 67
Figure 2-9: substitution of glucose for L-lactate ....................................................................... 68
Figure 2-10: ImageJ analysis ...................................................................................................... 69
Figure 2-11: GFAP and PI co-staining in P0 RONs .................................................................. 71
Figure 2-12: visual analysis of GFAP changes in P0 RONs ........................................................ 72
Figure 2-13: cell death count in P0 RONs detected by PI staining ........................................... 72
Figure 2-14: NF and PI co-staining in PO RONs ........................................................................ 74
Figure 2-15: cell death count in P0 RONs detected by PI staining in conjunction with NF staining ...............................................................................................................................75
Figure 2-16: GFAP and PI staining in P10 RONs ..................................................................... 77
Figure 2-17: visual analysis of GFAP changes in P10 RONs ....................................................... 78
Figure 2-18: cell death count in P10 RONs detected by PI staining ........................................... 78
Figure 2-19: NF and PI staining in P10 RONs .......................................................................... 80
Figure 2-20: cell death count in P10 RONs detected by PI staining in conjunction with NF staining ...............................................................................................................................81
Figure 2-21: GFAP and PI staining in adult RONs .................................................................... 83
Figure 2-22: visual analysis of GFAP changes in adult RONs .................................................... 84
Figure 2-23: cell death count in adult RONs detected by PI staining ........................................ 84
Figure 2-24: NF and PI staining in adult RONs ........................................................................ 86
Figure 2-25: cell death count in adult RONs detected by PI staining in conjunction with NF staining ........................................................................................................................................87
Figure 3-1: some features of electrophysiological recording of CAPs ........................................ 98
Figure 3-2: CAP recording under control condition using glucose ...........................................101
Figure 3-3: CAP recording under control condition using L-lactate ........................................ 102
Figure 3-4: CAPs were preserved by both glucose and L-lactate ................................................103
Figure 3-5: FC treatment for 300 minutes induced severe CAP decline in all age groups ........ 105
Figure 3-6: FC treatment for 100 minutes revealed a unique form of injury in P0 RONs ...........107
Figure 3-7: CAP recording in P0 RONs for 300 minutes under different experimental conditions ........................................................................................................................................ 109
Figure 3-8: CAP recording in P10 RONs for 300 minutes under different experimental conditions ........................................................................................................................................110
Figure 3-9: CAP recording in adult RONs for 300 minutes under different experimental conditions ........................................................................................................................................111
Figure 3-10: CAP recording in P0 RONs for 100 minutes under different experimental conditions ........................................................................................................................................ 113
Figure 3-11: CAP recording in P10 RONs for 100 minutes under different experimental conditions ........................................................................................................................................ 114
Figure 3-12: CAP recording in adult RONs for 100 minutes under different experimental conditions ........................................................................................................................................ 115
Figure 3-13: effects of glutamate receptor blockers on P0 RONs treated with FC. ................................................................. 117
Figure 3-14: effects of glutamate receptor blockers on P10 RONs treated with FC. ................................................................. 119
Figure 3-15: effects of glutamate receptor blockers on adult RONs treated with FC. ................................................................. 121
Figure 3-16: change in the peaks of adult APs.......................................................................................................................... 123
Figure 4-1: a brief over-view of the steps of preparing samples for electron microscopy .................................................. 138
Figure 4-2: example of axon viability scoring system .................................................................................................................. 140
Figure 4-3: examples of glial viability scoring system .................................................................................................................. 142
Figure 4-4: normal features of axons in P0 RON ......................................................................................................................... 144
Figure 4-5: normal features of axons in P10 RON ......................................................................................................................... 145
Figure 4-6: normal features of axons in adult RON ....................................................................................................................... 147
Figure 4-7: normal features of astrocytes .................................................................................................................................. 150
Figure 4-8: normal features of oligodendrocytes .......................................................................................................................... 151
Figure 4-9: oligodendrocytes vs. astrocytes ................................................................................................................................. 152
Figure 4-10: astrocyte injury in P0 RON before stimulation ....................................................................................................... 155
Figure 4-11: astrocyte injury in P0 RON after stimulation ........................................................................................................ 157
Figure 4-12: astrocyte injury in P10 RON before stimulation ....................................................................................................... 159
Figure 4-13: features of necrosis ................................................................................................................................................ 160
Figure 4-14: astrocyte injury in P10 RON after stimulation ........................................................................................................ 161
Figure 4-15: astrocyte injury in adult RON before stimulation .................................................................................................... 163
Figure 4-16: astrocyte injury in adult RON after stimulation ...................................................................................................... 164
Figure 4-17: oligodendrocyte injury in P10 RON before stimulation .......................................................................................... 166
Figure 4-18: oligodendrocyte injury in P10 RON after stimulation ............................................................................................. 168
Figure 4-19: oligodendrocyte injury in adult RON before stimulation .......................................................................................... 169
Figure 4-20: oligodendrocyte injury in adult RON after stimulation .......................................................................................... 170
Figure 4-21: effects of FC on P0 axons before stimulation .......................................................................................................... 171
Figure 4-22: effects of FC on P0 axons after stimulation ........................................................................................................... 172
Figure 4-23: effects of FC on P10 axons before stimulation ........................................................................................................ 173
Figure 4-24: effects of FC on P10 axons after stimulation ........................................................................................................... 174
Figure 4-25: effects of FC on adult axons before stimulation ...................................................................................................... 177
Figure 4-26: myelin changes before and after stimulation in adult RONs .................................................................................... 178
Figure 4-27: effects of FC on adult axons after stimulation .......................................................................................................... 179
Figure 4-28: astrocyte score in all ages ....................................................................................................................................... 181
Figure 4-29: astrocyte process number ....................................................................................................................................... 182
Figure 4-30: astrocyte process length ........................................................................................................................................ 184
Figure 4-31: axon score in all ages ............................................................................................................................................ 185
Figure 4-32: detailed analysis of axon injury ............................................................................................................................... 186
Figure 4-33: microtubules numbers in axons .............................................................................................................................. 188
Figure 4-34: axon areas in all ages .............................................................................................................................................. 189
Figure 4-35: axon density in all ages ........................................................................................................................................... 191
Figures:
- Figure 4-36: axon diameters in all ages .......................................................... 193
- Figure 4-37: oligodendrocyte score ................................................................. 195
- Figure 4-38: rate of CAP decline in P0 RONs .............................................. 197
- Figure 4-39: rate of CAP decline in P10 RONs ............................................. 197
- Figure 4-40: rate of CAP decline in adult RONs .......................................... 198

List of tables
- Table 1: the percentage of CAPs at different time points after FC treatment ........................................... 106
- Table 2: rates of CAP decline in different ages at different frequencies ...................... 196

List of abbreviations:
- aCSF: artificial cerebro-spinal fluid
- AD: Alzheimer’s disease
- AMPA: α-amino 3-hydroxy 5-methyl 4-isoxazole propionic acid
- ANLS: astrocyte-neuron lactate shuttle
- AP: action potential
- ATP: adenosine tri-phosphate
- BBB: blood brain barrier
- Ca\(^{2+}\): Calcium
- cAMP: cyclic adenosine monophosphate
- CAP: compound action potential
- CNS: central nervous system
- CoA: co-enzyme A
- CP: cerebral palsy
- CVA: cerebro-vascular accident
- DTI: diffusion tensor imaging
- EAAT: excitatory amino acid transporters
- FA: Fluoroacetate
- FC: fluorocitric acid
- GFAP: glial fibrillary acidic protein
- GLAST: glutamate-aspartate transporter
- GluRB: glutamate receptor blocker
- GS: glutamine synthetase
- IF: intermediate filaments
- IL: Interleukins
- \(K^+\): Potassium
- Kir4.1: inward rectifying potassium channel, type 4.1
- LDH: lactate dehydrogenase
- MCT: mono-carboxylate transporters
- MONs: mouse optic nerves
- MPI: mean pixel intensity
- MRI: magnetic resonance imaging
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofilaments</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>Ammonia</td>
</tr>
<tr>
<td>NoR</td>
<td>nodes of Ranvier</td>
</tr>
<tr>
<td>OPC</td>
<td>oligodendrocyte precursor cells</td>
</tr>
<tr>
<td>PBS</td>
<td>physiological buffer solution</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PVL</td>
<td>periventricular leukomalacia</td>
</tr>
<tr>
<td>RA</td>
<td>reactive astrocytosis</td>
</tr>
<tr>
<td>RON</td>
<td>rat optic nerve</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>standard errors of mean</td>
</tr>
<tr>
<td>stim/min</td>
<td>stimulation per minute</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>WM</td>
<td>white matter</td>
</tr>
</tbody>
</table>

White matter injury is common in neurological conditions including stroke and cerebral palsy. However, how different cellular elements of white matter interact during injury remains largely unknown. This study examined the effects of astrocyte damage on neighbouring axons and oligodendrocytes. Astrocytes were metabolically inactivated by an astrocyte specific toxin, fluoroctinic acid in rat optic nerves from three different developmental ages, and the effects assessed by immunohistochemical staining. Subsequently, functionality of the nerves was assessed using electrophysiological recording of compound action potentials, and the role of glutamate was examined using glutamate receptor antagonists. Ultrastructural analysis of different cellular elements of white matter was performed using electron microscopy. Lastly, nerves were stimulated at different frequencies; compound action potential measurements and electron microscopic analysis were performed.

The nerves exhibited stable compound action potentials and normal ultrastructural features in the presence of glucose or lactate showing that lactate can be as effective as glucose in supporting axonal functions. Fluoroctinic acid treatment produced age-dependent structural injury in astrocytes, axons and oligodendrocytes, with compound action potentials decline in all ages. Adult nerves were most sensitive to fluoroctinic acid-induced injury and perinatal (P0) nerves behaved uniquely. Although the axons stained normally, they did not conduct action potentials. Glutamate receptor blockers only partially recovered compound action potentials.

These results show that rat optic nerves provide a stable model to study white matter physiology and pharmacology over several hours. Although axons stained normally, they did not conduct action potentials: therefore, immunohistochemical staining alone can be misleading in assessing cell viability and functionality. Metabolic inhibition of astrocytes led to age-dependant injury in all ages, with adult cells most sensitive to injury. Adult myelin sheaths showed features of injury common in aging suggesting that astrocyte malfunction produces a microenvironment around axons similar to that in normally aging brains. Lastly, higher frequency activation of nerves improved the neurological outcome when astrocytes were damaged, perhaps reflecting better utilization of the lactate by the stimulated nerves.
Chapter 1
1.1 Introduction

Prematurity and low birth weights are associated with neurological problems including epilepsy, cognitive and behavioural problems and cerebral palsy (CP) (Domowicz et al., 2011, McCarran and Goldberg, 2007). Cerebral palsy, which is a severe static debilitating neurological disease, is the most common type of brain disorder in neonates which affects 2:1000 live births (Kuban and Leviton, 1994). It is usually associated with white matter (WM) damage that may occur either in mid-gestation or during the perinatal period (Back et al., 2007a). Later in life, WM injury is also implicated in a number of serious brain disorders including stroke. Thus, injury to WM in stroke can be as clinically significant as injury to grey matter (Bei and Smith, 2012). Ischemia can produce substantial amount of brain WM damage (Fern et al., 1998), which is a major event in many pathological conditions (Thomas et al., 2004). During intra-uterine life, the human brain is prone to ischemic injury between 24-32 weeks of gestation and around the time of birth, which are times directly preceding the myelination of axons and during the start of myelination, respectively (Back et al., 2001, Sun et al., 2010, Back et al., 2007b). Brain resistance to ischemic injury decreases as myelination begins (Back et al., 2001). This period coincides with maturation of oligodendrocytes (Domowicz et al., 2011, Back et al., 2007a). As brain development at corresponding points of age is slower in rodents, the corresponding periods of high susceptibility to periventricular leukomalacia (PVL) (the main mid-gestation injury) is around postnatal day 0-2 (P0-2) and to hypoxic-ischemic injury (the main birth injury) is P8-12.

Different cellular elements of WM are affected by ischemia in diverse ways and the underlying mechanisms change with maturation (Tekkok et al., 2007). In addition, each element can affect the health of neighbouring elements. For instance, death of astrocytes is associated with the release of toxic substances such as tissue necrosis factor (TNF) that can injure neighbouring oligodendrocytes (Fern, 1998). Likewise, intracellular potassium ($K^+$) release from injured oligodendrocytes can result in axonal...
depolarization with subsequent cell swelling and axolemmal disruption and ultimately axonal death (Alix and Fern, 2009). In addition axon damage can be induced by loss of trophic support, release of free radicals or disturbed glial homeostatic functions (Back, 2006). Thus, the pathophysiology of WM injury is likely to be highly intricate involving multiple cell-cell interactions (Baltan et al., 2008, Tekkok et al., 2007). Because of the close physical proximity of these cellular elements within WM, interaction between them during the formation of a lesion seems inevitable. Nonetheless, how this interaction occurs and the extent to which it affects outcome of a pathological condition remains unknown.

This study investigated the effects of astrocyte damage on neighbouring axons and oligodendrocytes by inhibiting the astrocytes’ Kreb’s cycle through the chemical actions of a well-known astrocyte specific toxin called fluorocitric acid (FC). To do so, rat optic nerves (RONs) have been used as a model and different age points have been tested: P0-2 (P0, premyelinated); P8-12 (P10, actively myelinating) and adult (fully myelinated).

Initially, immunohistochemistry was used to recognise the selective damage of astrocytes and any possible effects on the axons. This was followed by examining the ability of the axons to conduct action potentials when the astrocytes were metabolically inactivated. Furthermore, the effects of glutamate receptor blockers on the whole preparation were examined. Finally, ultrastructural changes in astrocytes, axons and oligodendrocytes were investigated using electron microscopy.
1.2 White matter

White matter is the tissue that conveys signals between different regions of grey matter within central nervous system (CNS) or from the CNS to the periphery. WM is essentially made up of unmyelinated and myelinated axons and associated glial cells; it is white because the myelin layers that surround the axons are high in lipid. Broadly speaking, WM can be divided into three major categories; (1) projection tracts that extend vertically and connect cerebrum to the rest of the body, (2) commissural tracts that connect cerebral hemispheres to each other usually, although not exclusively, through the corpus callosum and (3) associated tracts that connect different centres of the same hemisphere of the brain to each other. As WM contains mainly axons and glial cells with few neuronal cell bodies, historically it has been viewed purely as signal transmitting wires with no particular involvement in information processing: nor was it thought to be affected by such information processing. A growing body of data has challenged this long held view recently. Scholz et al. (2009) showed that learning a new visio-motor skill (juggling) in adult humans induced alterations in WM structure in addition to grey matter changes; specifically, diffusion tensor imaging (DTI) showed a localized increase in fractional anisotropy, a measure of the microstructure of the WM. Similarly, Schlegel et al. (2012) detected structural changes in WM in adults who tried to learn modern standard Chinese as a second language for 9 months. Individuals were tested monthly for the duration of the study and compared to control individuals who did not study language. The DTI test revealed not only changes in the WM of the traditional left hemisphere language area, but also in the right hemisphere counterparts. DTI also revealed significant differences in the microstructure of WM’s of black belt karate (experts) individuals compared to beginners (Roberts et al., 2013). It is important, however, to notice that the biological basis for such observations remain largely unknown, although the authors argue that the myelin is the most likely structure to have undergone structural alteration. It seems, therefore, that the myelin of the brain may possess some kinds of plasticity similar to what has been detected and studied in grey matter previously.
Understandably, most of the studies of age related changes in CNS were focused on the changes that accompany Alzheimer’s disease (AD). This is not surprising considering the huge impact of AD on patients’ life style and its burden on their caregivers. In 2006, it was estimated that around 26.6 million patients were suffering from the disease worldwide (Brookmeyer et al., 2007). The number of patients suffering from AD is estimated to be quadrupled by 2050, by which time it is predicted that one in 85 people will experience the condition. As a result of high prevalence of AD, researchers were focused predominantly on the changes happening in grey matter. In fact, very early studies on age related changes in CNS showed as much as 50% decrease in human cortical cells in elderly people (Brody, 1955). However, recent evidence suggests that although some neuronal death may in fact happen inside old brains, it is nowhere near the previous estimation (reviewed in Peters et al. (1998)). Lack of conclusive results to support the view that the decrease in neuronal number is responsible for cognitive decline in old age led researchers to hunt for other possible abnormalities and investigate different components of CNS. Although Double et al. (1996) observed a 2ml per year decrease in total brain volume in elderly people compared to normal middle age; they concluded that the decrease was in fact totally confined to WM, not grey matter. Similar result were obtained by Tang et al. (1997) in which they showed a decrease in total WM volume in human cerebral hemisphere as well as 27% reduction in the total length of myelinated fibres within WM. Results from magnetic resonance imaging (MRI) scans have revealed age related changes in WM consistent with the decrease in WM volume within ageing brain of humans (O'Sullivan et al., 2001, Albert, 1993, Lee et al., 2009) and other mammals such as monkeys (Herndon et al., 1998, Wisco et al., 2008). As WM provides the connecting wires between the different centres of the brain, the authors suggest that such findings support a cortical disconnection hypothesis and may provide an explanation of cognitive decline in elderly people. What brings about the reduction in the total volume of WM is not fully understood yet, but reduction of the total numbers of WM fibres has been suggested as a possible reason. For example, Sandell and Peters (2001) detected an average of $1.6 \times 10^6$ nerve fibres within optic nerves of young monkeys (4-10 years) compared to as low as $9 \times 10^5$ in older monkeys (27-33 years).
Furthermore, WM can get injured in different ways which leads to significant behavioural and cognitive disturbances. In particular, three examples, PVL, stroke and multiple sclerosis (MS), are pertinent to the theme of this thesis, and are discussed briefly below.

1.3 Periventricular leukomalacia

PVL is a general term refers to injury of WM surrounding cerebral ventricles. Most often this lesion is associated with axonal and/or neuronal abnormalities. The term encephalopathy of prematurity is coined by Dr. Volpe recently to include both entities (Volpe, 2009a). PVL is also the most common lesion associated with CP; a severe chronic debilitating disease affecting nearly 2 in 1000 live births (Kuban and Leviton, 1994). PVL most commonly occurs as a result of hypoxia-ischemia during intrauterine life. However, it can happen during or after birth as well. PVL’s correlation with premature and low birth weight babies renders it an extraordinarily important health problem in neonates. In United States of America (USA) alone, approximately 63000 infants of the yearly 4 000 000 live births (accounting for around 1.5%) are born with very low birth weights (less than 1500 grams) (Hack and Costello, 2007), and similar figures have been obtained continually with no significant changes over several years (Mathews et al., 2011). The importance of subsequent brain injury in this group is determined by the fact that around 25-50% of these low birth weight infants suffer later in life from some kinds of cognitive, behavioural, attention or social deficits, with major motor dysfunction (cerebral palsy) in 5-10% (Bayless and Stevenson, 2007). Recent advances in neonatal intensive care unit have escalated the number of survivals of extremely low birth weight (less than 1000 grams) to 70% (Volpe, 2009b), but more than 50% exhibit motor deficits later in life.

PVL lesions can be either focal or diffuse in nature (Volpe, 2001). Characteristically, focal lesion leads to necrosis of most of the cellular components of WM with subsequent cyst formation and ventriculomegaly (Volpe, 2001). Focal necrotic lesions may be either microscopic or macroscopic. Conversely, diffuse lesion
is a much milder form of lesion and mostly happens superficially in the brain tissues. Necrotic lesion has been known for a long time to be associated with axon injury: for instance, beta amyloid precursor protein immunoreactivity was demonstrated with axonal swelling (spheroids) in PVL lesion in human (Arai et al., 1995). However, axonal injury with the milder form of diffuse lesion was not so clear. Recently, however, similar immunoreactivity has been shown in diffuse component of PVL and the results were also further confirmed by detecting an apoptotic marker called fractin (Haynes et al., 2008). This view of axonal injury concomitant with damage to glial compartment function in diffuse lesion has gained further support from DTI technique that showed findings consistent with axonal injury (Marlow et al., 2007). It is particularly important, however, to notice that whether axon injury occurs primarily or secondary to glial injury and possibly loss of trophic support remains to be fully elucidated.

The human brain is particularly susceptible to injury between 24-32 weeks of gestation (Back et al., 2001, Back et al., 2007b). Multiple factors interact to produce such vulnerability. Anatomically, brain WM gets its blood supply from long and short penetrating arteries. The ends of these vessels are not fully developed in premature infants. Thus any decrease in blood flow may result in severe decline of blood supply with subsequent injury (Volpe, 2001). In addition, preterm and even high-risk term infants have an impaired cerebral vascular autoregulation or pressure passive cerebral circulation, in which cerebral blood pressure mirrors arterial blood pressure (Boylan et al., 2000). In intact brain, cerebral blood flow remains largely stable during changes in arterial blood pressure, thus, ensuring an appropriate blood flow rate and preventing over and under perfusion (Budohoski et al., 2013). An additional crucial element in creating susceptibility of preterm infants to PVL is the presence of preoligodendrocytes which are highly ischemic vulnerable cells (Back et al., 2001). Preoligodendrocytes are intrinsically highly susceptible to hypoxia-ischemia (Back et al., 2001). An amalgam of these entities would obviously increase the risk of ischemia greatly. The pathophysiology of PVL is very complex process that involves at least two major upstream and two downstream mechanisms (reviewed in detail in Khwaja and Volpe (2008)). The two upstream mechanisms are ischemia and /or inflammation. Most of the times, these two mechanisms can co-exist and potentiate each other.
They can also trigger downstream mechanisms which are excitotoxicity and free radical production (reactive oxygen and reactive nitrogen species).

Despite being the most common form of brain injury in infants, currently there is no treatment prescribed for PVL. Treatments are usually aimed at secondary pathologies that accompany PVL. Studies that provide a potential route to prevention would be highly beneficial to the management of this condition.

1.4 Stroke

Stroke or cerebrovascular accident (CVA) is a loss of brain function due to loss of its blood supply. The consequences can be severe on patient’s mental and physical wellbeing. In United Kingdom (UK) alone, the burden of stroke patients on society was estimated to be around 9 billion pounds annually (Saka et al., 2009). One of the most important predisposing factors to stroke is old age, and as the average age of population is generally rising, it is expected that stroke will increase and will need even more resources for its treatment and prevention, thus placing an even increasing cost on societies’ shoulder. In 2009, stroke was reported to be the second most common cause of death, only after ischemic heart attack (Mathers et al., 2009). Stroke is divided into two major types, haemorrhagic and ischaemic. Haemorrhagic stroke, which is a less common type, accounts for around 20% of the stroke cases, and is caused by bleeding from a weakened blood vessel within brain parenchyma, this can be either primarily (spontaneous bleeding) or secondarily to other causes such as vascular malformation, neoplasia, coagulopathy or use of thrombolysis during ischemic stroke treatment (Wang, 2010). Ischemic stroke is by far the most common type accounting for around 80% of the cases (Zhang et al., 2013). Traditionally, it is thought that for a given ischemic insult, grey matter shows more vulnerability to injury compared to WM (Marcoux et al., 1982). Nonetheless, clinical data have shown that WM involvement in ischemic stroke is ubiquitous, which in turn, is a major cause of functional disability in CVA (Goldberg and Ransom, 2003). The exact mechanism of WM injury in CVA is not clear, but it seems complex and happens through multiple
processes. Axons, myelin sheath and their parent cells (oligodendrocytes), astrocytes and activated microglia all seem to contribute to how a lesion is formed and evolved inside WM after injury. Axons, like other parts of the neuron, are critically dependant on a continuous supply of energy to preserve their trans-membrane ion gradient and excitability. Ischemic insult typically compromises abnormal trans-membrane ion gradients as a result of Na\(^+\)-K\(^+\) ATPase failure, with subsequent intra-axonal sodium (Na\(^+\)) accumulation. This in turn activates different routes of calcium (Ca\(^{2+}\)) entry into the intracellular compartment from either extracellular space or as a result of release from intracellular storage (Ouardouz et al., 2006). The final common pathway of Ca\(^{2+}\) overload is the activation of Ca\(^{2+}\) dependent enzymes (such as calpains, phospholipases and other enzymes), culminating in cytoskeletal degradation and irreversible cell damage (Stys, 2004). Glutamate induced excitotoxicity also plays an important role in inducing injury to axons and glia (Stys, 2004).

Surprisingly, ischemic injury to WM in older animals proceeds differently from that in young animals. For instance, removal of extracellular Ca\(^{2+}\) or blockade of Na\(^+\)-Ca\(^{2+}\) exchanger did not protect WM from ischemic insult in old animals, as was the case with young mice (Baltan et al., 2008). In mice models, WM axons show an intrinsic increased vulnerability to ischemic injury as a result of enhanced excitotoxicity and decreased anaerobic function as a result of aging (Hamner et al., 2011, Baltan et al., 2008). These findings are important in understanding the possible mechanisms of injury in stroke patients as they clearly indicate that the mechanisms of ischemic damage to WM may vary greatly between young and old WM. In brief, a comprehensive understanding of the mechanisms of injury in WM of different ages may have a profound effect on producing age specific treatment for stroke. Specifically, it is not known whether the age-dependence of WM injury is due to changes in susceptibility to damage of the cellular components of WM, related to their developmental stage. One aim of the present research is to ascertain whether there is a difference in this susceptibility to cell damage across different ages, and if so, to investigate the role of astrocytes and the mechanisms involved.
1.5 Multiple sclerosis

MS is a chronic inflammatory demyelinating disease of the CNS, most likely complicated by a secondary neuro-destructive component. Even after detailed study, the precise pathogenesis remains enigmatic. However, a growing body of evidence has held an auto-immune pattern culpable (Brassat, 2012). Pathologically, it is characterised by inflammatory cells producing focal lesions, typically involving demyelination and axonal damage with gliosis of the astrocytes, particularly within WM of the brain and spinal cord (Milo and Kahana, 2010). Clinically, however, the signs and symptoms are quite diverse, depending on the area of the CNS being affected by the lesions (Tsang and Macdonell, 2011). Patients may have physical, mental or psychological symptoms. MS is very rare before four years of age, with the majority of the cases reported to happen in early adulthood between 20-40 years; incidence rates in females is double that of males (Milo and Kahana, 2010). Despite extensive research the exact causes of MS or the initial triggering factor have not been identified. Both genetic and environmental factors seem to play a fundamental role in susceptibility to the disease, such as viral infection particularly during childhood, occupational exposure to toxins, smoking and stress (reviewed in Marrie, 2004, Hauser and Oksenberg (2006)).

Traditionally, myelin destruction in MS is thought to be the result of CD4 +ve T cell mediated induced immunity attacking either myelin sheath directly or their parent cells, i.e. oligodendrocytes (Platten and Steinman, 2005). Nonetheless, the inflammatory cells promoting myelin lesions as a sole mechanism of myelin ruination has been challenged. Recent neuropathological evidence suggests that primary oligodendropathy in the absence of lymphocyte and macrophage infiltration may be responsible for myelin damage in MS (Henderson et al., 2009, Barnett and Prineas, 2004). Consequently, the auto-immune like reactions that accompany lesions in MS might be merely an epiphenomenon i.e. a secondary scavenging process working to clear the debris from oligodendrocyte apoptosis. If oligodendrocytes are primarily affected in MS, the inevitable question will be what causes oligodendrocyte injury in the first place? To date, this fundamental question has remained unanswered. In the
light of current knowledge (Barnett and Prineas, 2004) have proposed that the following sequences of events may happen in MS lesions. Within hours, apoptotic oligodendrocytes appear in the lesions with decreased immune-reactivity to myelin associated proteins (no or very few inflammatory cells in this stage). After one to two days, oligodendrocyte disappearance and myelin degeneration becomes visible. This stage will be followed by a prominent infiltration of the affected tissues by T cells, macrophage, reactive astrocyte and microglia, accompanied by appearance of oligodendrocyte precursor cells (OPCs). After two to three weeks, numerous differentiated OPCs reappear in the lesion with attempts of re-myelination. Remyelination in MS represents a significant incident, because the myelin sheath around axons not only increases the velocity of action potential (AP) conduction, but also provides axons with necessary metabolic support to prevent axonal degeneration (Funfschilling et al., 2012). In addition to losing their metabolic support, axons in MS lesion may also face a higher metabolic demand. In normal myelinated axons, APs have a salutatory pattern between the nodes of Ranvier (NoR) where Na$^+$ ion channels are concentrated. However, this electro-genic machinery is lost in unmyelinated axons (Craner et al., 2003), and Na$^+$ channels are distributed throughout the whole nerve. Subsequently the APs become more demanding of energy but less efficient. As discussed above, attempts at remyelination by OPCs and presence of differentiated oligodendrocytes in MS lesion have been noticed repeatedly. However, remyelination is usually incomplete (Patrikios et al., 2006). Although a single casual factor for remyelination failure as yet has not been identified, many causes for remyelination failure have been speculated. One such factor is reactive astrocytes (section 1.10 Reactive astrocytosis, page 18), which is thought to be an early response to demyelination in MS lesions (Ayers et al., 2004). Glial scar, which is a dense matrix of glial processes, has been viewed for a long time as an inhibitor of myelin regeneration by depriving the demyelinated axons from repair mediated cells (reviewed in Stichel and Muller (1998b)). This view is not universal however, and has been challenged heavily. In fact, reactive astrocytes have been demonstrated to secrete mediators for remyelination in demyelinated axons (Murtie et al., 2005). Therefore, the role of astrocytes in MS lesions seems more complex than initially envisaged. The exact roles that reactive astrocytes play in demyelinated lesions and during repair are not clear
and further research is required to clarify the ambiguity surrounding the astrocyte role. Furthermore, it is clear that WM injury is critical in the pathology of MS, and a clearer understanding of the processes involved may indicate novel therapeutic strategies for treatment.

1.6 Glial cells

More than one and a half centuries have passed since the concept of neuroglia was first brought to the attention of scientific community by Rudolf Virchow, a young German scientist at the time. Given the novelty of the subject and the lack of modern day sophisticated analytical methods, it is unsurprising that Virchow was unable to recognise different kinds of glia. In fact, it took nearly half a century before Michael von Lonhossek recognised and coined the term astrocyte in 1893 in reference to astrocyte’s star like morphology (reviewed in Kettenmann and Verkhratsky (2008)). When Virchow first noticed glial cells, he considered them to be a substance that only holds proper neural tissues together and hence named it as glia, or glue. This unfortunate nomenclature, although still in use to date, produced the misconception that glial cells are inactive cells and simply serve as a cement to hold neurons together. As we shall see later, glial cells are active participants in normal brain functioning and they are far from being only glue. Conversely, the numbers of functions attributed to glia are always expanding. Many thrilling and unanticipated findings have already ascribed to glial cells. Nonetheless, these fascinating cells are persistently surprising scientists and these findings seems to represent only the tip of the iceberg.

Broadly speaking, glial cells are divided into two major categories; macroglia and microglia. Macrogial cells, which principally comprise of astrocytes and oligodendrocytes, are derived embryonically from neuro-epithelium (i.e. an ectodermal origin), while microglia, which are modified macrophages acting as active defence immune cells, have a mesodermal origin (Rowitch and Kriegstein, 2010).
Traditionally, it was believed that the glial cells outnumber the neurons in mammalian brains by as much as 10:1. Thus, it is assumed that neurons make up 10% of the cellular elements of the brain and glial cells make up the remaining 90% (Nishiyama et al., 2005). However, this view has been challenged by recent studies which suggest that the actual numbers of neuronal and non-neuronal (glial) cells are similar in human brain (Azevedo et al., 2009). It is important to notice that the equality of glial/neuronal ratio is correct if the whole brain is put into consideration. Glial/neuronal ratio in different regions of human brain can be significantly different from each other; for instance the glial/neuronal ratio in cerebellum was estimated to be 0.23, while the same ratio in the grey matter of cerebral cortex was 1.48. These values are in agreement with similar results from other labs (Pelvig et al., 2008). While the former study has estimated the total number of neuronal versus non-neuronal cells in the whole human brain, no attempts were made to differentiate the relative numbers of different types of glial cells. In the latter study, different glial cells (astrocytes, oligodendrocytes and microglia) were estimated, albeit only in the neocortex. Based on that study, the total numbers of glial cells in neocortex of females are 27.9 billion cells; while the total numbers of glial cells in male are 38.9 billion cells. In females; astrocyte, oligodendrocytes and microglia numbers were estimated as 4.8, 21 and 1.8 billion respectively. In males the numbers were 7.8, 28.8 and 2 billion respectively. Accordingly, the majority of the glial cells in neocortex (approximately 75%) are oligodendrocytes with a smaller portion (approx. 17%) being astrocytes. Glial cells appear to be ubiquitous in brains of all vertebrate and invertebrates as well, although their morphology may differ between different species. In addition, glial cell numbers seem to correspond to animal’s size; for example, a tiny nematode worm has only a few glial cells while glia may form as much as 97% of an elephant’s brain (Allen and Barres, 2009).
1.7 Astrocytes

Camillo Golgi in 1886 made a significant observation. He noticed that glial cells (although still not recognised as astrocytes nor was the term astrocyte coined) make contact with blood vessels through long fine processes. Furthermore, a common view at the time was that dendrites were performing a nutritive function. As glial cells have many dendrite-like fine projections, he speculated that these cells transform nutrients from blood vessels through fine processes to the neurons. Later on, the well-known Spanish scientist Ramon y Cajal provided a much more detailed insights to the structure and function of the glia (Bouzier-Sore and Pellerin, 2013). The term astrocyte was coined in 1893 by a Hungarian neuroanatomist Michael von Lenhossek (Kettenmann and Verkhratsky, 2008). Shortly afterwards (Andriezen, 1893) described two types of glial cells; neuroglia fibre cells and the protoplasmic neuroglia cells. These two types were later verified as two different kind of astrocytes; fibrous and protoplasmic astrocytes respectively. In 1983, Raff et al. distinguished two types of morphologically different astrocytes: type I with a fibroblast appearance and found in both grey and WM, while type II was only present in WM and had a neuron like appearance. This classification, however, was only recognised in tissue culture. It was also shown that type II astrocytes develop from a common progenitor cell with oligodendrocytes called the O-2A cell which was migratory and was thought to travel in the central nervous system to wherever myelination was required (Raff et al., 1987).

Astrocytes are connected through a network of gap junctions, which in turn, are formed by assembly of two hemi-channels. Hemi-channels are formed by structurally related trans-membrane proteins called connexins, of which, connexin 43 and connexin 30 are the two most common subtypes, particularly abundant in astrocytic end-feet around the blood vessels (Rouach et al., 2008). Gap junctions are poorly selective channels which allow trans-cytoplasmic passage of many small molecules such as ions (Na+, K+, Ca2+), energy metabolites (lactate, glucose, ketone body, fatty acid) and neurotransmitters (glutamate, gamma-aminobutyric acid (GABA)) (Giaume et al., 2010). Furthermore, even hemi-channel openings are now considered to be functional in physiological and pathological conditions (Orellana et al., 2012).
Astrocytes, through their receptors, transporters and gap junctions can respond to changes in neuronal metabolic demands and keep the microarchitecture of the brain at optimal conditions. In addition, astrocytes can regulate the blood flow (Attwell et al., 2010), take up glucose and convert it to glycogen (Hertz et al., 2014), supply energy metabolites to the neurons (Bouzier-Sore and Pellerin, 2013), regulate pH and sustain ion homeostasis (Bevensee et al., 1997), induce myelination (Nash et al., 2011), shape synaptic transmission (Dallerac et al., 2013, Oliet et al., 2001), guide the axons during development (Powell and Geller, 1999) and regulate K+ concentration (Bay and Butt, 2012) (Also see section: 1.8 Potassium regulation, Page 14). However, it is not known how changes in these astrocyte functions impact on neuronal injury. Given this critical role of astrocytes in metabolic support, the studies presented in this thesis aimed to ascertain to what extent neuronal damage occurring during ischemia is dependent on astrocyte function, and whether the age-dependent susceptibility to damage (see page 8) relates to stages of astrocyte development.

1.8 Potassium regulations by astrocytes

For normal neuronal excitability and AP conduction, stringently regulated extracellular K+ concentration is essential. An unmanageable surge in extracellular concentration of K+ ions as a result of efflux during axonal AP propagation or inter-neuronal communication will suppress neuronal activity. This strict regulation is one of the vital functions attributed to astrocytes. The astroglial membrane potential is close to the Nernstian equilibrium for K+ ions (E_K) (Bay and Butt, 2012). Thus, in both vertebrates and invertebrates, astrocytes have a high resting membrane conductance for K+ ions (Kuffler et al., 1966). This remarkable high conductance furnishes the astrocytes with the necessary machinery to sense the changes in concentration of extracellular K+ ions associated with normal neuronal activities (Amzica, 2002). The nature of K+ conductance, however, remains a subject of debate even to date. Strong evidence supports the involvement of inward rectifying channels, particularly the subtype 4.1 (Kir 4.1) in high K+ conductance. For example, results from studying Kir
knockout mice and pharmacological blockade of Kir 4.1 by BaCl₂ showed that deletion of the gene or pharmacological blockade of Kir resulted in marked reduction of inward currents and hampered the regulation of the extracellular K⁺, thereby diminishing the axonal AP conduction (Bay and Butt, 2012). Using a specifically ablated Kir 4.1 gene in a mouse model, Haj-Yasein et al. (2011) showed that the Kir 4.1 gene deletion greatly compromised K⁺ clearance after synaptic activation in stratum radiatum of hippocampus slices by perturbing the K⁺ spatial buffering. Given the aforementioned significance of Kir4.1 subtype on astroglial membrane potential and their major impact on determining the passive conductivity of K⁺ ions, it is therefore anticipated that the deactivation of these channels will have a major influence on K⁺ buffering.

Another set of K⁺ channels that are seemingly engaged in setting the resting membrane potential of astrocytes are the novel two pore K⁺ channel or K2P channel (reviewed in Lesage and Lazdunski (2000)), which can be found in both excitable and non-excitable cells. These channels are unusual in that they are non-inactivating channels and operate at all membrane potentials. Therefore these channels are envisaged to play a substantial role in determining the resting membrane conductance of K⁺ ions. In addition to the significant roles of K⁺ rectifying channels, it seems that most of the K⁺ ions that are released to the extracellular fluid during neuronal excitation are removed by Na⁺-K⁺ ATPase located in glial and neuronal plasma membrane (D'Ambrosio et al., 2002). This conclusion is drawn from observations which showed that di-hydro-ouabain 5μm, a selective inhibitors of Na⁺- K⁺ pump, not only increased the baseline K⁺ level, but also diminished K⁺ recovery after high frequency stimulation of hippocampal slices.

However, it is not known how changes in these astrocyte functions impact on neuronal injury. Given this critical role of astrocytes in metabolic support, the studies presented in this thesis aimed to ascertain to what extent neuronal damage occurring during ischaemia is dependent on astrocyte function, and whether the age-dependent susceptibility to damage relates to stages of astrocyte development.
1.9 Glial fibrillary acid proteins (GFAP)

Immunohistochemical techniques that allow the detection of specific biomarkers at the cellular level have been used widely to determine the distribution and localization of different cell types in healthy and pathological tissues. One such marker that has been used as a prototypical marker for immunohistochemical identification of astrocytes is GFAP. GFAP is a member of the cytoskeletal protein family which also include vimentin, nestin, desmin, synemin, neurofilament, peripherin and other proteins that collectively serve a cyto-architectural function and provide the cell with integrity and resilience. Initially, GFAP was found in 1969 in brains of patients with MS in which it was purified from tissue samples fixed in absolute ethanol (Eng et al., 2000). Later, in the same year, the amino acid composition of purified GFAP was determined at the Stanford Medical Centre and presented at the second international meeting of the International Society for Neurochemistry in Milan (Eng et al., 2000). Since then, GFAP has been studied extensively. It is now well established that GFAP is the principal 8-12 nm intermediate filament (IF) in mature astrocytes of CNS. Although different kinds of IFs share basic characteristics, IFs have been further subdivided into six types based on their amino acid sequence and protein structure. GFAP together with vimentin, desmin and peripherin are called type III IF proteins. However, GFAP has fewer acidic and basic amino acids and the head domain is much shorter compared to other type III IF proteins (Geisler and Weber, 1983). Also, different isoforms and splice variants of GFAP have been detected that are expressed in specific subsets of astrocytes in healthy and pathological specimens such as GFAPα, β, Y, δ and κ, with GFAPα being the most abundant isoform and the first one to be identified (Andreiuolo et al., 2009, Blechingberg et al., 2007, Choi et al., 2009). However, the exact roles and distribution of GFAP isomers remains elusive for now. Although GFAP was initially thought to be an astrocyte specific IF (Eng et al., 2000), more recently, GFAP has been shown to be expressed by many neuronal and non-neuronal cell types including ependymal cells, Schwann cells, glomeruli, Leydig cell of the testis and many more (Roessmann et al., 1980, Davidoff et al., 2002, Buniatian et al., 1998, Bianchini et al., 1992).
The molecular identity of GFAP in CNS astrocytes may differ from GFAP in PNS or non-neuronal cells. For example, Jessen et al. (1984) used a monoclonal antibody to GFAP (anti GFAP-3) in immunohistochemistry and immunoblotting studies to determine the structural difference between central and peripheral nervous system GFAP. While astrocytic GFAP was easily detected after reacting with the antibody, most peripheral glia failed to be detected as judged from their reaction with this antibody. Post-translational modification was suggested as a possible reason for the apparent difference between GFAP that is abundantly expressed in astrocytes and GFAP that is found in most peripheral glia. Despite the fact that GFAP has been established as predominant IFs of astrocytes, it is vitally important to recognise its distribution within astrocytes and determine its limitation as a tool to visualize astrocytes in healthy and pathological tissues. In normal healthy CNS, GFAP is detectable in many, but not all, astrocytes. However, many cells that morphologically would be identified as healthy astrocytes do not show detectable level of GFAP readily (Sun et al., 2010, Walz and Lang, 1998). In addition, GFAP expression shows a great regional and local variability. Normally GFAP is only expressed abundantly in large processes, less in the soma and completely devoid in fine branches. Thus, GFAP delineates around 15% of the total volume of astrocytes (Sun et al., 2010). Consequently, using GFAP to visualize astrocyte immunohistochemically can underestimate the true size and complex branching of astrocytes.

GFAP is a highly regulated protein whose expression is regulated by many factors such as brain injury (Eng et al., 2000) or ageing (Baltan et al., 2008). The exact age at which GFAP appears in human tissue is not clear. Different studies have reported the first appearance of GFAP protein in radial glial cells around 13 weeks after gestation in normal developing human cortex (Middeldorp et al., 2010). These GFAP +ve cells, however, disappear before birth. Subventricular zone cells which are derived from radial progenitors (radial glial cells) (Volpe, 2009a) and give rise to both glia and neurons (Zecevic et al., 2005) are also GFAP +ve and stay till adulthood (Middeldorp et al., 2010). Furthermore, GFAP in astrocytes increases gradually throughout the life span of both rats and humans (Eng et al., 2000, Nichols et al., 1993). Moreover, it is known that astrocytes respond to all kinds of injury by a process
known as reactive astrocytosis (RA), where up-regulation of IFs in astrocytes, particularly GFAP is a hallmark. RA occurs along a gradual continuum i.e. it is not an all or none process, and hence the severity of RA and GFAP up-regulation responds to the severity of the insult (Sofroniew, 2009). As RA is associated with an increase in the number and cell volume of astrocytes, in addition to GFAP up-regulation, it has been argued that acute injury to astrocytes after any insult may be obscured by up-regulated GFAP in immunohistochemistry studies (Shannon et al., 2007).

**1.10 Reactive astrocytosis**

Astrocytes become activated after any kind of CNS insult such as stroke, neurodegenerative and neuro-inflammatory diseases in a process called RA, which is a spectrum of changes that happens in astrocytes. Up-regulation or re-appearance of different IFs, particularly GFAP, has become the hallmark of RA (Eng et al., 2000). IFs become particularly abundant in the main processes and the soma of astrocytes. Furthermore, astrocytes undergo hypertrophy of the processes and cell proliferation (Barreto et al., 2011), which together with increased production of GFAP will result in high level of GFAP which is commonly observed with RA. While severe insults such as ischemia can result in irreversible cell injury and potential cell death, RA is considered a major response of astrocytes to a milder form of injury or when astrocytes survive a period of non-lethal injury (Salter and Fern, 2008). As mentioned before, RA is not an all or none phenomenon; rather, it happens a long a gradated continuum from mild astrogliosis, which is also called isomorphic astrogliosis, to severe astrogliosis (anisomorphic astrogliosis) (Liberto et al., 2004). In case of mild astrogliosis, astrocytes show a relatively low level increase in GFAP expression, slight increase in hypertrophy of the processes and cell proliferation. In contrast, severe astrogliosis results in much higher (than mild type) GFAP expression with greater process hypertrophy and cell proliferation; which in extreme cases culminate in glial scar formation (Stichel and Muller, 1998a).
Historically, RA has been considered detrimental to neuronal recovery and axonal regeneration (Stichel and Muller, 1998a). That view was rooted in the fact that reactive astrocytes secrete molecules that can affect the composition of extracellular milieu surrounding injured axons and neurons (Jones et al., 2003); some of these molecules such as proteoglycan are important modulators of neuronal outgrowth and their presence inhibits neuronal recovery after injury (Jones et al., 2003). Over the last few decades, owing to gradual increasing interest in the study of the biology and pathology of astrocytes, a wealth of data concerning the detailed molecular and functional characteristics of healthy and reactive astrocytes have accumulated. The available data clearly indicate that RA is a very complex and multi-directional process that may worsen (Hayakawa et al., 2010) or enhance (Sofroniew, 2005) CNS recovery after injury. For example, most of the insults to CNS are associated with a clear increase in the level of pro-inflammatory cytokines such as TNFα which may even precede the activation of astrocytes and formations of astrogliosis (Liberto et al., 2004). In addition, reactive astrocytes can also release the arachidonic acid metabolite, nitric oxide and reactive oxygen species (ROS) which can adversely affect cell survival and inhibit neurite outgrowth (Liberto et al., 2004). Nonetheless, other reports have shown that reactive astrocytes can also release other cytokines such as interleukin-1β (IL-1β) which can promote CNS recovery (John et al., 2003, Mason et al., 2001). When activated, astrocytes have the potential to gain abnormal or lose normal physiological functions; and thus, overall, it has been suggested that RA can have two stages, an initial beneficial stage acutely after injury which promotes CNS recovery, followed by a detrimental stage later on when the lesion becomes chronic and reactive astrocytes form glial scar, in which CNS recovery may be inhibited (Pekny and Nilsson, 2005).

Astrocytes can become activated as a result of stimulation by a wide variety of molecules that can be secreted by many cell types such as neurons, oligodendrocytes, microglia or even other healthy astrocytes, or from injured or dead cells in CNS lesions (Sofroniew, 2009). These molecules can be growth factors and cytokines such as IL-6, TNFα, ciliary neurotrophic factor; molecules that are released as a result of cell injury such as adenosine tri-phosphate (ATP), glutamate, nitric oxide or ROS; mediators of
innate immunity such as lipopolysaccharides and other Toll-like receptors; others such as ammonia (NH$_4^+$), amyloid β, and hypoxia (Sofroniew, 2009, John et al., 2003, Neary et al., 2003). CNS insults are usually accompanied by a surge of ATP in the extracellular fluid due to ATP release from injured and/or dead cells (Domercq et al., 2010), and as shown by Neary et al. (2003), ATP induced activation of P2X1 and P2X2 receptors on cultured astrocytes can result in astrocyte activation (astrogliosis).
1.11 Problems of astrocyte identity

Since the late nineteenth century, two distinct classes of astrocytes have been identified based on their cellular morphological appearance and anatomical location (Andriezen, 1893). Since then, tremendous advances in our technical ability have allowed scientists to further explore the physiological and morphological properties of these cells. As a result, over the years, a wealth of data has accumulated about the appearance and electrophysiological properties of the astrocytes. Nonetheless, not all the astrocytes share similar properties. These differences are surprisingly diverse and have raised doubts about what an astrocyte really means.

GFAP, which is the most abundant IF in mature astrocytes, is expressed by developing, mature and reactive astrocytes and has been used as a standard prototype antigen marker in astrocyte identification (Eng et al., 2000). Unfortunately, not all astrocytes express GFAP and even when expressed, GFAP displays a great regional and local variation. For example, in stratum radiatum of the hippocampus in Wistar rats, approximately 40% of the astrocytes as recognised by the expression of astrocyte specific enzyme glutamine synthetase (GS) did not show a detectable level of GFAP (Walz and Lang, 1998). This phenomenon of unevenly distribution of GFAP-immunoreactive astrocytes is well documented in many situations (Ling and Leblond, 1973, Kalman and Hajos, 1989), with GFAP negative astrocytes particularly abundant in grey matter compared to WM (Walz and Lang, 1998). To address this issue, many papers have suggested that GFAP messenger ribonucleic acid (mRNA) may be a better alternative to determine the identity of cells as astrocytes (Nolte et al., 2001, Nichols et al., 1993). Nevertheless, detection of GFAP mRNA also proved not to be absolutely astrocyte specific. For instance, 74% of acutely isolated glial cells from rat hippocampus showed GFAP mRNA (Zhou et al., 2000), but these cells also showed characteristic complex electrophysiological properties which are different from those of astrocytes. Although these GFAP mRNA positive cells were initially thought to be immature astrocytes, as they also stain positive for predominantly astrocytic marker protein S100β, we now know that they are actually not astrocytes and have been classified as a new class of glial cells called NG2 glia (See section: 1.12 NG2 cells, page
22). Furthermore, as NG2 cells show morphological similarities to astrocytes, such as a thin organelle-poor rim of cytoplasm that surrounds a nucleus within the soma and thick main processes that arise from the soma and taper gradually to thin branches, they were labelled as a kind of smooth protoplasmic astrocytes (Levine and Card, 1987). Nonetheless, ultra-structurally GFAP (Nishiyama et al., 2005) and gap junctions (Wallraff et al., 2004, Bergles et al., 2000) have not been detected in NG2 glia which are two prominent components of astrocytes. However, electron microscopy has revealed functional synaptic junctions between NG2 cell processes and axon terminals which, when activated, induced an α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor induced calcium current inside NG2 cells (Bergles et al., 2000). These dissimilarities clearly demonstrate that the sole presence of GFAP mRNA does not necessarily positively identify cells as astrocytes. In fact, using GFAP alone in the past to identify astrocytes led to other cells erroneously being labelled as astrocytes. One example of such cells is radial glia, which are bipolar shaped cells in developing CNS and serve as the key element in neurogenesis (Malatesta et al., 2008). In addition to the expression of GFAP in radial glia (except rodent radial glia in which GFAP has not been detected), they also share other characteristics of astrocytes including the presence of astrocytic glutamate transporter (GLAST), the astrocytic enzyme GS, protein S100β, vimentin and even glycogen granules inside the cytoplasm (reviewed in (Malatesta et al., 2008)).

Although GFAP is the most abundant IF in astrocytes and has been used as a prototypic marker, other less abundant IFs have also been utilized to visualize astrocyte, such as vimentin and nestin (Chiu et al., 1981, Schnitzer et al., 1981); albeit much less frequently than GFAP. Again, these markers are not totally astrocyte-specific and their expression has been detected in many other cell types. S100β, which is a member of S100 protein family, is considered to be a glial specific marker, particularly for astrocytes (Steiner et al., 2007), but like other cell markers, it is not a pure astrocytic marker. It has been found in human ependymal cells, choroid plexus epithelium, vascular endothelial cells, lymphocytes, oligodendrocytes, neurons (Steiner et al., 2007) and NG2 cells (Zhou et al., 2000). In fact, it has been speculated that its specificity to identify astrocytes is even less than that of GFAP (Steiner et al., 2007).
Furthermore, astrocytes show position dependant distinct subtypes in different regions of CNS, such as ventral spinal cord (Hochstim et al., 2008) and olfactory bulb (Bailey and Shipley, 1993). In spinal cord, at least three subtypes of astrocytes were identified; they were derived from progenitor domain expressing different homeodomain transcription factors, namely Pax6 and Nkx6.1, which in turn, controlled the expression of Reelin and Slit1 respectively (Hochstim et al., 2008) (Reelin and Slit1 are secreted proteins involved in CNS development). In conclusion, it is increasingly likely that our ideas about astrocytes being a homogenous population of cells are wrong. After all, there may not be a single method which allows the identification of all astrocytes. Different subpopulations of astrocytes may express different markers. A morphologically astrocyte-like cell may stain immuno-positive for one marker such as S100β but not for others such as GFAP (Steiner et al., 2007). In view of these problems with using GFAP as an astrocyte marker, in the current study astrocyte counting was based on the number of cells that were positively stained by propidium iodide (PI) (see section: 2.3.4 Analysing data using ImageJ, page 64), and since PI is non-specific, subsequently some of the counted cells might not be actually astrocytes. However, this problem was dealt with more efficiently by identifying astrocytes ultra-structurally using electron microscopy. Therefore it is clear that there is some ambiguity in interpretation of immunohistochemical data in terms of the structural and, more importantly, functional integrity of astrocytes. By using several complimentary methods (immunohistochemistry, electrophysiology and electron microscopy), we aimed to assess to what extent these methods could be used to identify injured cells, and critically, to determine which cellular markers gave a valid indication of cell functionality.
1.12 NG2 cells

Classically, glial cells are divided into only two major categories; macroglia and microglia. However, recent advances in immunocytochemical staining and electrophysiological recording have revealed a distinct type of glial cells with an unusual set of morphological and functional properties that distinguish them from other well-known glia. This new kind of cell is now known as polydendrocytes or NG2 cells in reference to the expression of NG2 chondroitin glycan sulphate proteoglycan on their surface. NG2 cells exist uniformly in white and grey matter of CNS; they represent the major proliferative cell population of mammalian adult brains (Dawson et al., 2003). While the full extent of the roles that NG2 cells play in the CNS is not established yet, the general consensus is that NG2 cells are OPCs; thus, NG2 cells can produce mature oligodendrocytes for re-myelination in the CNS after demyelination by demyelinating diseases such as MS (Zawadzka et al., 2010) or spinal cord injury (McTigue et al., 2001). The mechanisms by which NG2 cells may be activated to produce mature oligodendrocytes remain poorly understood. However, NG2 cells express receptors for many of the neurotransmitters that are functioning in the CNS, thus NG2 cells are equipped with the necessary machinery to listen to the neuronal activities. Lin and Bergles (2004) showed that quantal release of GABA from interneurons induced GABA<sub>A</sub> currents in hippocampal NG2 cells. The authors suggest that GABA induced currents may represent a kind of stimulation that regulates NG2 cells development. Likewise, Bergles et al. (2000) demonstrated that the quantal release of vesicular glutamate from hippocampal axons located adjacent to NG2 cells produced an inward current in NG2 cells that was caused by AMPA receptor activation.

If NG2 cells are able to produce new mature oligodendrocytes in adults, one might speculate that sufficient re-myelination may occur to overcome demyelinated lesion resulting from demyelinating disease. However, such complete myelin regeneration is usually not observed clinically. Chang et al. (2002) observed the presence of high number of OPCs in chronic lesions of MS that was not significantly different from control brains. These cells extended multiple processes that have close
relationship to demyelinating axons, although they failed to re-myelinate them. However, it is very important to notice that the axons in lesion areas are dystrophic and dystrophic axons may fail to give enough signals for regeneration.

NG2 cells, like astrocytes, have a stellar morphology and send their processes to close proximity either with NoRs (Butt et al., 1999) or synapses (Bergles et al., 2000) where they are well positioned to interact with neuronal activity. Nonetheless, antigenic and ultra-structural studies have proposed that they are two separate glial populations (reviewed in Nishiyama et al. (2005)). GFAP, which is the most common intermediate filament in astrocytes, is not detected in NG2 cells, neither by antigenic staining nor by ultra-structural examination. Furthermore, GS and S100β, the two other markers abundant in astrocytes, including GFAP –ve astrocytes, have generally not being detected in NG2 cells (Nishiyama et al., 2005), although one study (Zhou et al., 2000) did detect S100β. Interestingly, studies have showed that NG2 cells are not connected through gap junctions (Wallraff et al., 2004, Bergles et al., 2000) the extensive networks of intercellular connection whose presence is well documented in astrocytes. In fact, they revealed the complete absence of connexion 43, the major protein unit that forms gap junctions. This finding represent an important functional difference between astrocytes and NG2 cells since gap junctions are heavily involved in spatial buffering of ions and signalling molecules in astrocytes. Deciphering the specific interaction between astrocytes and NG2 cells on the one hand, NG2 cells and neurons on the other hand can therefore be envisaged as a crucial area of research interest in the forthcoming years.

1.13 Microglia

Microglia are the resident macrophages of the CNS, which were first recognised nearly a century ago by a student of highly celebrated scientist Ramón y Cajal, named del Rio Ortega in 1919 (Rezaie and Male, 2002), who also coined the name. Microglial cells have a myelomonocytic origin (Kreutzberg, 1996), unlike other glial cells of the brain which have an ectodermal origin (Vaughn, 1969); i.e. they originate from
hematopoietic stem cells of the bone marrow after they have differentiated to monocytes which then invade brain before the closure of the brain blood barrier (BBB). Microglia are highly plastic cells which can attain various morphological shapes depending on their location and the level of activity. A resting state microglia cell has a characteristic small soma with little peri-nuclear cytoplasm and numerous thin, highly branched processes (Kreutzberg, 1996).

Microglia play an important role in defending the brain parenchyma, and is the first and the main line of defence. One of the major characteristics of microglia is their sensitivity to alteration in microenvironment status of their domain; their activation often precedes that of other cell types of the brain (Kreutzberg, 1996). This rapid response is critical in safeguarding the CNS, since few antibodies naturally are small enough to cross BBB, and hence microglial ability to react quickly to recognise foreign bodies and present their antigens to the T cells is vital (Dissing-Olesen et al., 2007). Although normally microglia exist in rather inactive and down-regulated phenotype, still they are constantly foraging the brain for damaged cells or infectious agents. Many agents have already been identified that can activate microglia and inducing substantial morphological and functional transformation: for example complement fragment C5a and C3a, ATP, chemokines and cytokines, glutamate, cell wall components of gram positive and gram negative bacteria and many more (Moller et al., 1997, Trang et al., 2012, Taylor et al., 2005). Microglia activating agents may be released as a result of a wide variety of neurological diseases including AD, stroke, MS, amyotrophic lateral sclerosis, tumour, human immunodeficiency virus (HIV) and infection, among others (Ransohoff and Perry, 2009).

Historically, the appearance of active immune cells within brain and spinal cord has been recognised as a malicious sign of pathology and linked to impending tissue damage. However, microglia, like reactive astrocytes (Sofroniew, 2005, Hayakawa et al., 2010), may contribute to both neuro-degeneration and neuro-protection. Since microglia express different kinds of metabotropic glutamate receptors and respond to fluctuation in extracellular glutamate level, the final outcome of their activation may be neuro-protective or neuro-toxic after acute and chronic injury partly as a result of glutamate receptor activation (Ransohoff and Perry, 2009).
Microglial cells have been shown to contribute to neurotoxicity through multiple pathways such as secretion of injurious cytokines, production of free radicals and enhancing excitotoxicity (Volpe, 2009b, Volpe, 2009a). Furthermore, microglial activation has been detected as a result of treatment by tissue plasminogen activator which is the only routine treatment currently available for ischemic stroke (Doyle et al., 2008). Using a microglial inhibitory factor, tuftsin fragment 1-3, which inhibits microglia activation, Wang et al. (2003) showed an improvement in the neuro-behaviour outcome of mice and a significant reduction of the stroke lesion volume. In addition, handling specific aspects of microglial reaction to neuro-inflammation has been suggested to provide neuro-protection in neuro-inflammatory diseases such as AD (Taylor et al., 2003). Furthermore, retinal neovascularization has been blamed as culprit for vision loss in many ischemic conditions such retinopathy of prematurity and diabetic retinopathy. Although microgli were thought to promote vascular destruction, Ritter et al. (2006)) showed that in fact microglial cells enhanced vascular normalization.

Microglia express both ionotropic P2X and metabotropic P2Y receptors (Ransohoff and Perry, 2009). Like in other types of brain cells (Domercq et al., 2010), elevation of extracellular concentration of ATP as a result of massive release of ATP during ischemia from injured cells will lead to an increase in adenosine level that may be sufficient to activate microglial cells. Therefore, the available data suggest that for microglial targeted therapy to be feasible, it is important to first recognise the distinct components of neuro-immune response and how these components change over time as a result of pathology.

1.14 Fluorocitric acid

Fluoroacetate (FA) is the toxic element of South African plant *Dichapetalum chymosum* and other *Dichapetalum* plants. It was the first known chemical substance to undergo “lethal synthesis”; a term coined by Peters (van der Kamp et al., 2011) in the middle of last century to describe enzymatic formation of highly toxic substance
from non-toxic precursors in living tissues. Over subsequent years, the effects of fluorotoxins (the term fluorotoxins has been used to mean both fluoroacetate and its toxic metabolite FC throughout this study) have been studied in several different tissues in a number of animal species.

Functionally, FC has to be considered a reversible inhibitor of aconitase enzyme. Different lines of evidence support its reversibility. Paulsen et al. (1987) showed that the function of FC is reversible after 24 hours based on a detailed biochemical, enzymatic and electron microscopic examination. There were significant changes in the level of various amino acids including glutamine, glutamate and GABA accompanied by enzymatic changes in glial cells collected from brains after 4 hours of a single injection of 1nmol FC. Electron microscopic examination also revealed ultrastructural changes in brain slices suggestive of injury in glial cells. All the changes were almost reversed toward normal 24 hours after the FC treatment. However, these changes persisted after 2nmol FC injections. Notably, after a single injection of 2nmol, similar changes occurred, but they were still present 24 hours after the injection, which suggest that FC is causing reversible inhibition of glial metabolism in specific range of dose and time. Villafranca and Platus (1973) also noticed that aconitase which is inactivated and radiolabelled by $^{14}$C-FC lost its radioactivity when passed through G-50 Sephadox or precipitated by ammonium sulfate. Failure of aconitase to retain its radioactivity was interpreted as a sign of regaining function by aconitase enzyme and hence implying the reversibility of FC function. (Hosoi et al., 2004) observed that 4 hours after fluorocitrate (1nmol/L) infusion, acetate uptake in rat brains decreased by around 80%, but the decrease almost completely disappeared 24 hours later. Therefore, FC has to be considered a reversible inhibitor. Lastly, as a fluoride ion is released in the reaction of FC with aconitase enzyme, FC has been termed as a suicidal substrate for the enzyme (Clarke, 1991).
1.14.1 Mechanism of action and consequences of FC treatment

Citrate formation by the enzyme citrate synthase from oxaloacetate and acetyl-coenzyme A (CoA) is the first chemical reaction in tricarboxylic acid (TCA) cycle. Acetyl-CoA itself is formed from acetate and CoA by the enzyme acetyl-CoA synthetase inside mitochondrial matrix (Schwer et al., 2006). When the fluorinated form of acetate in the form of FA is used as a substrate for the reaction, the previous reaction will produce the fluorinated form of citrate; FC, which is toxic in different living tissues. FC acts by binding to the aconitase enzyme; the second enzyme in TCA cycle (van der Kamp et al., 2011). FC, which is the toxic derivative of FA, is responsible for the lethal toxicity of FA in humans and other mammals; these toxic effects include cardiac arrhythmias and neurological manifestations such as tremor, tetany and convulsion after oral administration of FA (Bosakowski and Levin, 1986). Blocking the conversion of citrate to isocitrate in the TCA cycle using fluorotoxins (see Figure 1-1) will lead to a decrease of carbon flux through the cycle with subsequent increase in citrate level in both brain and other tissues (Goldberg et al., 1966).

![Figure 1-1: A brief diagram showing the action of FC](image)

Figure 1-1: A brief diagram showing the action of FC
Oral administration of 3 mg/kg of FA in rats caused a 15 fold increase in citrate concentrations in the heart (Bosakowski and Levin, 1986), while Patel and Koenig (1971) showed a significant increase in citric acid content in rat brain and cat spinal cord after FA treatment. Likewise, Hassel et al. (1997) observed 200% increase in citrate level in FA treated mice. In addition, FC can have an inhibitory effect on mitochondrial citrate carrier which can affect how cells handle increased citrate level in the presence of FC (Brand et al., 1973). The origin of the increased citrate is thought to be astrocytic because inside brain tissues, it has been shown that astrocytes are the only cell type to export citrate to the extracellular space in detectable amounts (Sonnewald et al., 1991). Intracerebral injection of FC in adult rats almost invariably produced epileptiform EEG (14 out of 15 experiments) (Willoughby et al., 2003), and some of the rats even exhibited convulsive seizures. Given that citrate is a known chelator of calcium ions (Hornfeldt and Larson, 1990) and low level of calcium is a known cause of increased neuronal excitability and seizure, then increased citrate with subsequent hypocalcaemia may be the reason behind such observations.

Most of the cellular ATP is produced by oxidative metabolism of glucose or other metabolites that can enter the TCA cycle in mitochondria. Since FC halts the Krebs cycle, then cellular ATP would be expected to diminish. Such an expectation was supported by early studies that showed the ATP level was decreased by 46% in heart tissue after FA administration (Bosakowski and Levin, 1986). However, there is a discrepancy in the results of ATP measurement in brain tissue. FC treatment for 60 minutes in Muller cells of chick retina (a type of retinal glial cells, similar to astrocytes) did not affect ATP level significantly (Zeevalk and Nicklas, 1997); also (Patel and Koenig, 1971) detected no decrease in ATP in rat brain or cat spinal cord. Likewise, Benjamin and Verjee (1980) found no decrease in rat brain ATP after 3mM of FA, although glutamine synthesis was diminished significantly. Voloboueva et al. (2007) showed a gradual, but moderate depletion in cellular ATP level after FC treatment in cultured astrocytes which only reached significant level after 4 hours. (Keyser and Pellmar, 1994) showed that guinea pig’s hippocampal ATP level decreased by 35% after incubation with FA. The decrease was even more in C6 cells which are a pure glial preparation from rat astroglial tumor cell lines. Voloboueva et al. (2007) showed that
in pure astrocytic culture medium, 0.25mmol FC decreased ATP level by 53 and 84 % after 2 and 3 hours respectively, but after 4 hours the ATP had decreased to almost undetectable level. It should be noted, however, that they performed their experiments in zero-glucose medium. ATP level in zero-glucose control culture without FC exposure remained largely stable. Possible explanations for this discrepancy are that astrocytes can highly upregulate glycolysis to compensate for oxidative metabolism blockade (Swanson and Graham, 1994) and it is been shown that lactate production is increased after FC treatment which can indicate a shift to anaerobic glycolysis within glial compartment (Zeevalk and Nicklas, 1997). Alternatively, the type of preparation and time of measurement is hugely significant in determining the level of ATP because ATP level may take a long time to decrease significantly and measuring it soon after fluorotoxin treatment may give confusing results.

Glutamine is synthesized exclusively inside glial cells (Martinez-Hernandez et al., 1977) either from glutamate by the action of GS, an enzyme to be found exclusively in glial cells (Martinez-Hernandez et al., 1977) or de novo from TCA intermediates. As fluorotoxin treatment is assumed mainly to affect the TCA cycle function in glial cells, it also causes glutamine depletion. This effect was first speculated to be due to the inhibitive effect of FC on GS (Lahiri and Quastel, 1963); however, this possibility was later proved to be extremely unlikely. Swanson and Graham (1994) showed that fluorotoxins caused a large reduction in glutamine production in astrocyte culture when exposed to FC or FA. However, the reduction was reversed when glutamate was added to the medium. If fluorotoxins block the action of GS, then addition of glutamate should not affect glutamine formation. The normal glutamine production that was observed after addition of glutamate excludes this possibility. In fact, FC treatment has been observed to even increase GS activity (Paulsen et al., 1987). The authors proposed that increased activity could be secondary to loss of standard cellular proteins that normally inhibits GS activity. In agreement with the previous study, Hassel et al. (1992) noticed that glutamine formation after FC injection into neostriatum of adult rats decreased by 95-98%. The percentage was derived from counting radiolabel incorporation of different glial specific substrates “acetate, citrate and 2-oxoglutarate” into glutamine. Zielke et al. (2007) also showed that 2 hour
infusion of 0.1mmol FC microdialysis in rats’ hippocampus decreased glutamine level by 86%. The profound decrease in glutamine level after fluorotoxin treatment can be the result of arrested Krebs’s cycle in astrocytes. In addition, the astrocytic enzyme GS, uses cellular ATP as a cofactor in converting glutamate to glutamine (Hassel et al., 1992). As cellular ATP is ultimately expected to diminish after FC treatment due to its effect on TCA cycle function, then the decreased activity of GS may be another reason for glutamine depletion. Lastly, inhibition of astrocytic TCA cycle will lead to glutamate being fed up into the cycle as a substrate beyond the blockade instead of normal conversion to glutamine with subsequent glutamine depletion (Hassel et al., 1992).

Glutamine serves as the main precursor for neurotransmitters in nervous tissue such as glutamate and GABA. Glutamate is formed from glutamine by the action of glutaminase enzyme and glutamate decarboxylation by the enzyme glutamate decarboxylase, in turn, produces GABA.

As the major effect of FC is to decrease glutamine formation, thus a decrease in glutamate level seems inevitable as well. Paulsen et al. (1987) showed a decrease of 41 % in glutamate level after 4 hours of 1nmol FC injection into striatum of rats. Likewise, Zielke et al. (2007) showed a 67% decrease in glutamate level after FC microdialysis.

However, during the initial phase of FC treatment, some increase in glutamate level has been noticed in microdialysis of striatum (Paulsen et al., 1988). Elevated glutamate level has also been observed in hippocampus (Largo et al., 1996) and in Muller cells of chick retina (Zeevalk and Nicklas, 1997). These increases may be due to diminished uptake by astrocytes as showed by Voloboueva et al. (2007); in astrocytic cultures exposed to 0.25mmol of FC, the rate of glutamate uptake was decreased by 58% and 74% after 2 and 3 hour respectively. Alternatively, it may be a result of cell swelling (Kimelberg et al., 1990), poor K⁺ controlling in extracellular space (Largo et al., 1996), and/or cell death that may occur after FC treatment.

In a similar way, GABA also follows a biphasic change in its concentration after FC exposure; an initial increase in concentration was followed by a gradual depletion. After injection of 1 nmol FC into striatum of adult rats, GABA increased initially after 2
hours and then decreased thereafter. In agreement, Hassel et al. (1992) found that radiolabel incorporation into GABA from acetate decreased by 75% in the presence of FC. The initial increase in GABA that was observed in the former study could be due to decrease of either its metabolism or uptake by glial cells. In fact, the latter study found that in the presence of FC 25% less radiolabelled GABA was incorporated into glutamine which supports the view that GABA metabolism by glial cells decreases in the presence of FC. Nonetheless, GABA production itself is dependent on glutamine production in glial cells. Therefore, when glutamine level falls significantly, eventually GABA will decrease and this has been noticed in both studies. This biphasic changes in GABA level was also previously noticed in rat brain and cat spinal cord (Patel and Koenig, 1971).

In brief, FC exposure can be expected to yield two key biochemical changes, namely elevation of citrate level and depletion of glutamine level within neural tissues. Citrate and glutamine changes seem to be more sensitive that decreasing ATP level within glial compartment. Since glutamine is an astrocyte specific product, its production in astrocytes is severely affected from all precursors. An initial increase in glutamate level is followed by a gradual depletion. Although GABA level is less sensitive to FC inhibition and only decreased when glutamine level is very low; it decrease eventually nonetheless.

1.14.2 Use of fluorotoxins in the study of brain metabolism

The use of FA in the study of glial function was first considered when Lahiri and Quastel (1963) showed that FA depressed the incorporation of radioactivity from radiolabelled glucose and glutamate into glutamine in adult rat brains. Clarke et al. (1970) later showed that fluorotoxins inhibit the incorporation of radioactivity from various radiolabeled precursors into glutamine much more effectively than into glutamate. They also showed that the effect was not due to the inhibition of the enzyme GS, a conclusion that was suggested by the former study. Importantly, Clarke et al. (1970) suggested that the effect was on the small compartment of glutamate,
which later proved to be glial in origin. Further support for the use of fluorotoxins as a gliotoxic substance appeared when Cheng et al. (1972) showed that formation of glutamine, a substance that only forms inside glia, was inhibited by FC. In contrast, acetylcholine, a substance that only forms inside neurons, was unaffected. Fluorocompounds, therefore, attracted attention as a possible substance to be used as a selective gliotoxins to inactivate glial components in the study of brain metabolism and physiology. Thus, it seemed that using fluorotoxins can produce a model in which metabolically active glial cells can be rendered inactive, thereby providing a way to study metabolic interaction and substrate trafficking between neurons and glia. Nowadays, a considerable body of data supports the view that fluorotoxins have been successfully applied to study glial contribution in health and disease. Both FC and FA can be administered in a variety of ways to achieve selective inhibition of glial metabolism. They can be directly injected into brain (Hassel et al., 1992), used in microdialysis of brain in vivo (Largo et al., 1996), in in vitro tissue slices (Berg-Johnsen et al., 1993), and cell culture (Voloboueva et al., 2007). Fluorooacetate, but not FC, can also be used by systemic administration (Bosakowski and Levin, 1986).

Acetate is preferentially taken up and metabolized by glial cells in CNS (Waniewski and Martin, 1998). However, the biochemical basis for this selectivity is not clear yet. Waniewski and Martin (1998) observed at least 18 times faster $^{14}$CO$_2$ production from $^{14}$C-acetate in cortical astrocyte culture compared to cortical synaptosomes. This difference was attributed to preferential uptake of acetate by cultured astrocytes rather than its metabolism. In fact, the activity of acetyl CoA synthetase, the first enzyme required in the first step of acetate metabolism, was higher in synaptosomes than in astrocyte culture which suggests that lack of enzymatic activity was not the reason for glial specificity. Thus, there is no glial specific localization of the enzymes required for acetate metabolism. It seems therefore, that glial specificity is due to uptake mechanism to glial compartment. The authors suggested that acetate uptake was facilitated predominantly by monocarboxylate-like transporters. In addition, an element of passive diffusion to both astrocytes and synaptosomes seemed likely, albeit imported acetate at much lower rate. Citrate, an intermediate substrate in TCA cycle, is likely also to be taken up
selectively by astrocytes. Similarly, neurons do not appear to be deficient in enzymes required for citrate metabolism (Hassel et al., 1992). Taking into account the chemical similarities between (acetate, citrate) and (FA, FC), it is very probable therefore that FA is taken up selectively by astrocytes by similar mechanisms.

1.14.3 Support for glial specificity of fluorotoxins

Results from a wide range of studies in different laboratories suggest that acetate is a substance that is selectively taken up and metabolized by glial cells in brain tissue (Hassel et al., 1992, Hosoi et al., 2004, Hassel et al., 1997, Waniewski and Martin, 1998). In contrast, glucose can enter both glial and neuronal tissue. Both substances can be used as a precursor for different amino acid formation. The notion that acetate is selectively taken up and metabolized by astrocytes made it a very good choice to be used for studying selective astrocyte metabolism and physiology. Radiolabelled acetate, therefore, can enter TCA cycle and yield different amino acids. In accordance with that view, Hassel et al. (1992) showed that radiolabelled acetate and glucose that were injected stereotactically into the neostriatum of adult rats were incorporated into glutamine, glutamate, aspartate and GABA. FC decreased the incorporation of radiolabels from acetate into all the aforementioned amino acids. In contrast, amino acid formation from radiolabel glucose remained unaffected, with the exception of glutamine. These results were ascribed to selective inhibition of glial metabolism by FC. In agreement with the previous study, Willoughby et al. (2003) observed reduced radiolabel incorporation from acetate to glutamine, while labeling from glucose to glutamate remained unaffected. Likewise, Paulsen et al. (1987) showed that 1 nmol FC selectively and reversibly affected the glial compartment. The principal type of cells to be affected was astrocytes as evidenced structurally and biochemically. Astrocytes showed pale cytoplasm, loss of glycogen content with swollen and fragmented endoplasmic reticulum. Mitochondria were also swollen with a decreased number of crests. These changes were reversed 24 hours later. However, when they doubled the dose of FC, the injury was extended to involve neurons as well. Similar results were obtained by Largo et al. (1996), in which they showed that 0.1
mM of FC affected only astrocytes histologically in hippocampal region of rats, sparing the neurons. Thus, it is important to note that fluorotoxins are glial specific only at a specific range of concentration and time.

The amount of cyclic adenosine monophosphate (cAMP) released in response to local application of beta-receptor activation was measured in rat brain cortex, after microdialysis infusion of 1 mM FC. It was found that metabolic inhibition of glial cells by FC blocked cAMP response almost completely (90%). Since beta-adrenoreceptors in rat brain cortex are predominantly localized to glia, these data were interpreted as being the result of specific glial inactivation by FC. This conclusion was further supported by infusion of brain with kainic acid, a specific neurotoxin, which showed no effect on the previous response (Stone and John, 1991). In fact, similar conclusions were reached a year before by the same group of researchers (Clarke, 1991) when they showed that incubation of brain slices with FC decreased cAMP response by 75-79%, but intracellular recording of neurons remained unaffected. Again, kainic acid only affected the neurons. Keyser and Pellmar (1994) also showed that in guinea pig hippocampal slices; FA caused a decline in excitatory post synaptic potential. This decline was reversed in the presence of 1-3 mM of glial specific substrate; isocitrate. As isocitrate is only taken up by glial cells, the authors believe that the original effect should have been on glial cells.

Results from tissue culture also support the glial specificity of FC. Voloboueva et al. (2007) exposed pure neuronal culture to 0.25 mmol of FC followed by glutamate exposure. Neurons showed the same level of cell injury as in control culture when exposed to glutamate. Thus, FC did not show any effects on neuronal cells. In contrast, astrocyte culture exposed to FC showed a significant cell death after 4 hours. Astrocytes were almost completely dead by 5 hours. Specific effects of FC on astrocytes were also confirmed following the observation that both plasma membrane and mitochondrial membrane were depolarized after FC treatment. In addition, astrocytic ATP level was significantly depleted.

Furthermore, Zeevalk and Nicklas (1997) used Muller cells of chick retina to show glial specificity of FC (Muller cells are similar to astrocytes of brain and behave
similarly as well). They showed that radioactive acetate provided a higher relative specific activity of glutamine compare to radioactive glucose. FC significantly decreased incorporation of radioactivity from acetate to glutamine but not from glucose to glutamate, which suggests inhibition of glial compartment only. They also noticed histologically that FC treatment did not affect neuronal population of the retina. Together these experiments, using a variety of different preparations across several species, strongly suggest a specific effect of FC on astrocyte metabolism only.

1.15 Glutamate

Glutamate is the major excitatory neurotransmitter in the brain (Derouiche and Frotscher, 1991, Fonnum, 1984, Lacomblez et al., 1996, Erecinska and Silver, 1990). During normal physiological processes of the brain, glutamate is released from the presynaptic nerve terminals into the synaptic cleft; however, glutamate concentration has to be regulated tightly for the brain to function properly. If glutamate concentration is not regulated adequately, it causes excitotoxicity, a process which has been involved in a wide variety of neurological disorders such as stroke, MS, seizure and amyotrophic lateral sclerosis among many others (Lacomblez et al., 1996, Goff and Wine, 1997, Bordi et al., 1997, Hauser and Oksenberg, 2006, Zhao et al., 2015).

Normally, the brains’ extracellular glutamate concentration is about 2 µM (Erecinska and Silver, 1990), which is thousands of times lower than the intracellular glutamate concentration in most of the cellular subtypes of the brain; intracellular glutamate concentration ranges between 1-10 mM in different cell types of the brain (Erecinska and Silver, 1990). Yet, against this concentration gradient, glutamate is efficiently taken up by the brain cells, predominantly the astrocytes, by means of an efficient transport system which will be discussed in detail later. In addition to its role as the major excitatory neurotransmitter, glutamate is also an important metabolic fuel which can be metabolised oxidatively inside the mitochondria (McKenna et al., 1996). Since part of the glutamate is degraded oxidatively, a continuous supply of glutamate is necessary to sustain a balanced glutamate pool. Synthesis of glutamine, a major glutamate precursor, is an astrocyte specific process; in addition, astrocytes uptake,
metabolize and release glutamate, and therefore, putting the astrocytes in the central stage in controlling glutamate activities. Since many aspects of glutamate biochemistry are handled by astrocytes, their role in glutamate regulation is discussed in more detail.

1.16 Roles of astrocytes in glutamate handling

1.16.1 Glutamate synthesis and metabolism

It is commonly accepted that glutamate formation from glutamine is the main synthetic route for replenishing synaptically released glutamate in neurons. Neurons, however, cannot synthesize glutamine by themselves; instead, they depend on a continuous supply of glutamine from their neighbouring astrocytes and therefore, a continuous movement of carbon skeleton between the neurons and astrocytes is fundamental to maintaining the glutamate concentration at the required level. This sequence of events by which an adequate supply of glutamate is sustained within CNS is called glutamate-glutamine cycle (van den Berg and Garfinkel, 1971, McKenna, 2007, Hertz, 2013). This cycle is operated by an uneven distribution of the enzymes responsible for synthesis and break down and the transport system for glutamine and glutamate between different cellular components of the CNS, particularly neurons and astrocytes. For example, GS, the enzyme responsible for glutamine synthesis, is an astrocyte exclusive enzyme (Martinez-Hernandez et al., 1977, Derouiche and Frotscher, 1991), while phosphate activated glutaminase, the enzyme responsible for conversion of glutamine to glutamate, is particularly abundant in the neurons (Salganicoff and Derobertis, 1965, Hogstad et al., 1988). Furthermore, as shown by Derouiche and Frotscher (1991), astrocytic fine processes that are in close contact with glutamatergic synapses are rich in GS, which supports the idea that synaptically released glutamate is taken up easily by strategically positioned processes and convert to glutamine.

Glutamine is then released by the astrocytes to the extracellular space by a transporter system referred to as system N, which include SNAT3 and 5 (Broer and
Brookes, 2001). Subsequently, glutamine is taken up by the neurons by system A of transporters which include SNAT 1, 2 and 4 (Broer and Brookes, 2001). Once inside the neurons, glutamine is converted to glutamate by the action of phosphate activated glutaminase, with the release of ammonia as a by-product of that conversion (Marquez et al., 2009). It has been known for a long time that accumulation of ammonia in neurones, is neuro-toxic (Hindfelt et al., 1977, Bosoi and Rose, 2009) causing a major disturbance of cerebral carbohydrate, amino acid and energy metabolism. In addition, ammonia is also needed by the astrocytes in the processes of amination of α-ketoglutarate to form new glutamate molecules (α-ketoglutarate + NH₄⁺ → glutamate). Therefore, a shuttle mechanism must exist to support the flux of nitrogen from the neurons to the astrocytes to balance the rate of de novo synthesis of glutamate. Ammonia exists in gaseous (NH₃) and ionic (NH₄⁺) forms. NH₃ is a lipid soluble compound and therefore can diffuse through the lipid bilayer of cell membrane easily. However, under normal physiological conditions, more than 98% of ammonia exists in its ionic form (NH₄⁺) in the brain which is less likely to penetrate the cell membrane thorough diffusion (Bosoi and Rose, 2009). Nonetheless, NH₄⁺ has very similar ionic properties to K⁺ which enables NH₄⁺ to compete with K⁺ for crossing the cell membrane through K⁺ channels or N⁺-K⁺ ATPase transporter, in addition to the possible existence of specific ammonia transporters in mammals (Bosoi and Rose, 2009). Furthermore, nitrogen may flux from neurons to astrocytes through an amino acid shuttle mechanism, for example alanine or branched chain amino acids (Lieth et al., 2001), or through aquaporin channels (aquaporin 8) (Saparov et al., 2007). Overall, the exact mechanism by which nitrogen compounds are transferred from neurons to astrocytes is yet to be fully elucidated (Bosoi and Rose, 2009).

In addition to the formation of glutamate from glutamine in neurons as mentioned above, there are other routes by which neurons can regain part of their released glutamate. These routes include re-uptake of a portion of the released glutamate through glutamate transporters (Holmseth et al., 2012) or glutamate synthesis from TCA cycle intermediates (Hassel, 2001, Chambers et al., 2014) through the enzymatic reactions of glutamate dehydrogenase and aminotransferases; these intermediates can be either synthesized neuronally or imported from the astrocytes.
These routes, however, remain less important than glutamate formation from glutamine in replenishing neuronal glutamate.

1.16.2 Glutamate release and reuptake

Excitatory neurotransmission in the mammalian brain is largely controlled by glutamate which is released from synaptic vesicles into the synaptic cleft during AP conduction. However, glutamate in the synaptic cleft has to be removed quickly to prevent over excitation and killing the neurons by excitotoxicity. To achieve a controlled concentration of glutamate at the synaptic cleft, released glutamate has to be taken up again by either the neuronal or non-neuronal components, or diffuse out inter-synaptically. Reuptake into the presynaptic or postsynaptic terminals has been shown in different regions of the brain and its contributions to the overall control of the brain glutamate concentration seems to be region specific (Kim et al., 2008, Otis et al., 1997, Rossii et al., 2000, Shigeri et al., 2004). However reuptake into the non-neuronal components, namely astrocytes, is the major contributor to glutamate clearance (Oliet et al., 2001). The remarkable ability of the astrocytes to accumulate glutamate is due to the presence of glutamate transporter proteins which are also called excitatory amino acid transporters (EAATs). To date, 5 subtypes of EAATs have been identified and termed EAAT-1-5 in human (Shigeri et al., 2004); however, in rodents, EAAT-1-3 are labelled as GLAST, GLT1 and EAAC1 respectively (Shigeri et al., 2004), while EAAT4 and 5 retain their names.

EAAT1/GLAST and EAAT2/GLT1 are generally found in membranes of glial cells, while EAAT3/EAAC1 and EAAT4 are considered to be neuronal specific, and lastly, EAAT5 is only found in photoreceptors and bipolar neurons of the retina (Shigeri et al., 2004). However, there are certain variations between the different subtypes in their regional distribution or developmental expression patterns. For example, GLT1 is highly expressed in brain, specifically in astrocytes, but low level of expression has also been detected in neurons (Sheldon and Robinson, 2007). Likewise, GLAST has also been reported in cerebellar neurons Golgi bodies (Rothstein et al., 1994).
Furthermore, GLT1 is undetectable in brain at birth; it appears after two weeks in forebrains and at three weeks in cerebellum and finally it reaches the adult pattern of expression at five weeks of age. In contrast to GLT1, GLAST is present in significant amount at birth in both forebrain and cerebellum (Ullensvang et al., 1997). Of note, however, these investigators failed to determine either GLAST or GLT1 in neuronal elements of the brain.

GLAST and GLT1 appear to have similar binding affinities for glutamate and together it is estimated that they re-uptake about 80% of the released glutamate (Lehre et al., 1995). Since these transporters are using Na⁺ ion trans-membrane gradients, they are termed sodium dependant glutamate uptake systems and are responsible for the majority of the glutamate uptake (O'Kane et al., 1999). The roles of these transporters in regulating normal brain functioning are fundamental, especially in protecting the neurons from the excitotoxic effects of glutamate; for instance, compared to astrocyte rich cultures, neurons in astrocyte poor cultures are a hundred times more vulnerable to glutamate excitotoxicity (Rosenberg and Aizenman, 1989). Furthermore, knock down of GLAST1 or GLT1 is reported to cause elevated glutamate level and neuro-degeneration (Rothstein et al., 1995), lethal spontaneous seizure (Tanaka et al., 1997) or increased susceptibility to cerebellar injury (Watase et al., 1998).

Under pathological conditions, these transporters can reverse the transport of glutamate, i.e. efflux of glutamate from the astrocytes with debilitating effects on the neurons. This is particularly prominent during condition of energy deprivation such as ischemia. Ischemia can cause marked depletion of cellular ATP and elevated level of extracellular K⁺ and glutamate. Experiments have shown that the majority of glutamate in the core of ischemic lesion comes from the astrocytes (Szatkowski et al., 1990, Rutledge and Kimelberg, 1996). In addition, astrocytes can release glutamate non-vesicularly by cystine-glutamate anti-porter. These transporters typically mediate the release of L-cystine from the astrocytes to the extracellular space in exchange for glutamate (Bridges et al., 2012) which consequently represents an important process in the formation of glutathione and ultimately protection of the neurons from damage by oxidative stress. Therefore changes in glutamate levels are clearly implicated in
ischaemic damage, including that seen in WM. However, it is not clear whether prevention of glutamate receptor mediated effects would be neuroprotective during ischaemic insult. Using antagonists at glutamate receptors, we aimed to investigate whether blocking glutamatergic neurotransmission could protect the neurons from damage.

1.17 Glycogen

Branched polymers of glucose are generally used as an energy store in different living cells. While plants store their energy in the form of multi-branched polysaccharides of glucose called starch, animals store their energy in the form of glycogen, primarily in liver and muscles where it serves as the second most important long term energy storage behind adipose tissues. While the use of glycogen as reserve energy has long being known in animals, microorganisms including yeast and bacteria are also capable of utilizing glycogen to fight the starvation conditions (Wilson et al., 2010). In animals, glycogen is usually located intra-cytoplasmically as fine granules which measure 100-400 ångströms (10-40 nm) in diameter (Melendez et al., 1999). In addition, owing to its simple branching pattern, glycogen has emerged as the first real candidate to be a biological fractural structure (fractal object is a complex structure build with a simple procedure involving little information) (Melendez et al., 1999). This branching pattern is fundamental in providing fast energy release during periods of energy requirement for three reasons. Firstly, branched glycogen has a higher number of ends per unit volume. Secondly, synthesis and degradation of glycogen can only occur from the non-reducing ends of α 1-4 of the glucose chain (Brown and Ransom, 2007). Finally, branching increases water solubility of glycogen (Roach et al., 2012).
1.18 Glycogen in brain

In mammalian brain parenchyma, glycogen is localized almost exclusively in the astrocytes (Brown et al., 2003, Cataldo and Broadwell, 1986). Although glycogen has been known for a long time, especially in the liver (Young, 1957), its function in the brain is not fully understood. Furthermore, glycogenolysis until recently was regarded as a simple procedure to produce energy during periods of increased energy demands when the availability of readily accessible substrates such as glucose became scarce. A growing body of evidence has emerged, especially in the last two decades, which argues that glycogen is more fundamental to the normal operations of the brain processes than simply providing an emergency fuel source.

Glucose is the main carbon source of energy which can cross BBB and enters the brain (Zielke et al., 2007); even other substrates that are able to support brain functions such as lactate and pyruvate are essentially derived from glucose. Furthermore, many researchers have hypothesized that glycogen breaks down to a mono-carboxylate, most probably lactate, which then can be released by astrocytes and taken up by the neural elements where it is metabolised oxidatively to produce energy (Brown et al., 2003, Dringen et al., 1993). Thus, it is likely that glycogen breaks down to lactate rather than glucose. This conclusion is based on experiments in which primary astrocyte cultures from brains of new born rats release only lactate, but not glucose, to the medium when astrocytic glycogen was depleted as a result of exposing the medium to glucose deprivation condition (Dringen et al., 1993). The roles that lactate play in the brain seems to be different from its role in the periphery where it is usually viewed as a waste product which forms when glucose cannot be fully metabolised oxidatively. In brain, however, lactate seems to be preferentially taken up and metabolised by neurons to produce energy (Wyss et al., 2011), especially during periods of high demands. The importance of lactate as a fuel source to brain is particularly evident during early stages of development when its concentration is higher in body liquids compared to adults and seems to be preferred for utilization by the neurons over glucose (Zilberter et al., 2010).
When glycogenolysis is required, the enzyme phosphorylase kinase activates the inactive glycogen phosphorylase-α to its active form which is glycogen phosphorylase-β (Hertz et al., 2014). As a result, glycogen breaks down from the non-reducing end of the chain to glucose 6-phosphate. However, a special de-branching enzyme is required to remove α1-6 branches and change the glycogen to a linear form (Brown and Ransom, 2007). Inside the brain, many agents have been recognised that can induce glycogenolysis including elevated K⁺ level, glutamate formation and uptake, β-adrenergic agonist such as isoproterenol, noradrenaline, adenosine and ATP, among others (Hertz et al., 2014). In addition, glycogenolysis provides the necessary fuel for many essential processes such as the uptake of extracellular K⁺ following neuronal excitation (Hertz et al., 2014, Xu et al., 2013) and glutamate formation (Xu et al., 2013).

1.19 Role of lactate in brain metabolism

More than a century ago, pioneering neuroanatomists speculated that astrocytes are strategically located within the brain parenchyma enabling them to transfer nutrients from the circulation to the neurons (Andriezen, 1893). This speculation was further supported in the following years by observations that astrocytes indeed send processes which form specialised structures at their ends called end feet which ensheath blood vessels of the brain on one hand, and other astrocytic processes which ensheath the synapses on the other hand, and hence, providing an interface where astrocytes can communicate and sense neuronal activity (Pellerin and Magistretti, 1994). This anatomical regulation of astrocytes in relation to the blood vessels and neurons seemed to put the astrocytes in a privileged site to take up nutrients from the blood vessels and transfer it to the neurons. Initially, based on the findings that (1) multiple glucose transporter proteins exist on the surfaces of astrocyte processes that facilitates the transport of glucose (Maher et al., 1994, Morgello et al., 1995) and (2) under normal physiological conditions glucose is the main, if not exclusive, substrate that can be used by the brain (Pellerin et al., 1998), it was proposed that the substrate transferred from blood vessels through the
astrocytes to the neurons must be glucose. However, in a set of experiments conducted by Tsacopoulos et al. (1988) who used honey bee retina, a highly organised neural tissue composed of two distinct metabolic compartment in which the photoreceptors (sensory neurons) form a rosette–like structure surrounded by glial cells, it was found that upon stimulation of the photo-sensory cells by light, an astrocyte specific, but not neuronal, conversion of 2-deoxy [3H] glucose to 2-deoxy [3H] 6-phospahte happened. Nonetheless, an increased consumption of O2 was detected inside the neurons. The authors concluded that these results represent a clear case of inter-cellular communication between glial and neuronal compartments and the observed increase in the rate of oxidative metabolism in the neurons must be down to an increased supply of carbohydrate by glial cells. Over the years, many lines of evidence accumulated suggesting that the speculated carbohydrate substrate that was transferring from astrocytes to the neurons is lactate. Evidence includes the ability of slices of cerebral cortex to remain excitable in the presence of lactate (Pellerin et al., 1998), lactate-induced prevention of neuro-degeneration (Izumi et al., 1997), and the increased concentration of lactate in the culture medium after glutamate stimulation (Pellerin and Magistretti, 1994). It was in the last work that the authors suggested that glutamate, which is the main excitatory neurotransmitter in brain, in addition to its receptor mediated neuronal excitation, causes an elevated level of glycolysis and lactate production within the astrocytes. Furthermore, they provided evidence that the increased intracellular sodium concentration, which occurs concomitantly with glutamate uptake into the astrocytes, was responsible for the observed increase in lactate concentration was not receptor mediated. With later refinements, this work formed the basis of what has become known as astrocyte neuron lactate shuttle (ANLS), which states that, especially during periods of increased activity, astrocytes release lactate which is then taken up by neurons as an energy substrate (See Below).
1.20 Astrocyte-neuron lactate shuttle

As discussed earlier, and was pioneered by Pellerin and Magistretti (1994), a wealth of data has accumulated over the years supporting the view that lactate, which is released in glutamate stimulated manner from the astrocytes, will be taken up by the neighbouring neurons and may contribute efficiently to maintain synaptic transmission. Since the brain is privileged by the presence of BBB, blood born lactate cannot easily cross to the neurons and hence, the astrocyte produced lactate represents an essential source. Furthermore, for lactate to exchange inter-cellularly, it has to be transported across the cell membrane since it cannot readily diffuse through the lipid bilayer of the cell membrane. Lactate transport in brain is facilitated by members of a family of proton linked monocarboxylate transporters (MCTs), of which to date, 14 members have already been identified in mammals (Pierre and Pellerin, 2005). In addition to lactate, these transporters can also transport other monocarboxylate compounds such as pyruvate, ketone bodies, β-hydroxybutyrate and acetoacetate (Pellerin et al., 1998). The different properties of each member, based on extensive in vivo and in vitro experiments, have recently emerged (reviewed in Pierre and Pellerin (2005)), demonstrating that MCTs play a fundamental roles in lactate transportation from astrocytes to the neurons.

Inside the brain, three main types of MCTs have been identified and studied extensively; they are MCT1, MCT2 and MCT4. Of interest, however, is the finding of another neuron specific sodium coupled lactate transporter called SMCT1 by (Martin et al., 2006). This transporter was exclusively found in neurons, based on in situ hybridization and immunofluorescence analysis, which showed co-localization of SMCT1 protein with neuron specific MCT2, but not with MCT1. The exact contribution of this novel transporter to lactate uptake, its regulation or its kinetics, is yet to be fully elucidated. The distribution of MCTs among different cellular components of the brain is in parallel with ANLS hypothesis based on the kinetic properties of these transporters. MCT1 and MCT4 are low affinity transporters and, in general, have 10-fold lower affinities for substrates than MCT2 (Halestrap and Price, 1999). MCT2 are usually found in cells that readily uptake lactate even in low concentration such as
proximal kidney tubules and sperm tails in addition to the neurons (Halestrap and Price, 1999). MCT4 are expressed exclusively on the astrocytes, especially in the processes that are in contact with the synapses, MCT1 are expressed on astrocytes in addition to oligodendrocytes and endothelial cells forming the blood vessels (Pellerin and Magistretti, 2012, Halestrap and Price, 1999). Unsurprisingly, this cell specific distribution of MCTs which favours lactate release from the astrocytes and lactate uptake by the neurons is matched by similar cell specific distribution of lactate dehydrogenase (LDH) isoforms (LDH enzymes catalyses the inter-conversion of lactate and pyruvate). LDH5 is abundant in glycolytic cells, namely the astrocytes, and favours the formation of lactate from pyruvate, while LDH1 preferentially drives the reaction toward pyruvate production from lactate and is abundant in the neurons (Bittar et al., 1996). Therefore, lines of evidence based on the preferential metabolic profiles of each cellular components (astrocytes and neurons), in addition to the likely lactate production from glycogenolysis in the astrocytes (see above), and the ability of lactate to support different functions of the neurons, as discussed earlier, provide a solid base to support ANLS hypothesis which implies that lactate is shuttled from astrocytes to the neurons, especially during periods of increased activity. Two clear and testable hypotheses derive from this discussion. First, that lactate is a suitable alternative (to glucose) energy source in WM; and second that increased neuronal activity, and so increased energy demand, enhances the utilisation of lactate as an energy source. On this basis, experiments described in this thesis aimed to test this theory, in the rat optic nerve model of WM injury.

From the discussion so far, it is clear that WM injury is an important component of ischaemic brain damage, and understanding the mechanisms of WM damage an area of critical interest in several degenerative brain diseases. However, many WM tracts in the brain are difficult to access making fundamental research of this kind difficult or impossible. The rat optic nerve (RON) is an excellent model system for studying these processes, as it is an easily accessible WM pathway in the CNS, which contains all the major elements of other WM tracts. On this basis, the experiments described in this thesis use the RON model system (see section: 1.21 Rat Optic Nerve, page 48).
1.21 Rat Optic Nerve

RON is composed of retinal ganglion cell axons with supportive glial cells without neuronal cell bodies. Like other CNS structure, RONs are covered by all the three layers of meninges (dura, arachnoid and pia matter). Similar to other CNS and WM tissue tracts, the myelin sheath that surrounds individual axons in RON is formed by oligodendrocytes and not Schwann cells as is the case in peripheral nervous system (PNS). As RON has a high degree of structural and functional homogeneity, it has been used for a very long time with a considerable success as an excellent tissue tract for morphological, physiological and biochemical studies of WM. Another advantage of using RON as a model for WM study lays in the fact that it is relatively easy to dissect and remove the entire nerve trunk from all postnatal ages in rats. Thus, RON is an excellent and technically relatively straightforward model for WM research. RON develops in an orderly manner i.e. it develops from an entirely unmyelinated tract at birth to a virtually completely myelinated tract at adulthood (Hildebrand and Waxman, 1984). This is particularly advantageous for developmental studies as any possible change in conduction properties of the axons, composition of extracellular milieu or channel kinetics can be followed up. At P0, all the axons are unmyelinated. By P10, almost one third of the axons have already gone through radial expansion and have increased in diameter as a preliminary step to initiate myelination, while the remainder of the axons are still small and pre-myelinated (Alix and Fern, 2009). By adulthood, 97% of the axons are myelinated (Hildebrand and Waxman, 1984).

The first contact between oligodendrocytes and unmyelinated axons usually happens beyond six days postnatally, but the most dramatic increase in the number of myelinated axons which corresponds temporally to a dramatic fall in the number of unmyelinated axons usually happens during the third postnatal week (Hildebrand and Waxman, 1984). During this time in development of RON, the most abundant increase in oligodendrocyte generation also happens (Vaughn, 1969).
1.22 Summary, Aims and Hypotheses

In summary, astrocytes appear to be a fundamental participant in determining how the brain functions in normal physiology and pathology. The interactions between astrocytes and other cellular components of WM appear to be multiple and happen through different routes. However, when astrocytes are selectively damaged, it is not clear how this may impact on functioning of neighbouring cellular components. Therefore, the present study aimed to characterise how ischaemic insult affects astrocyte structure and function, and how this affects neighbouring cells. The developmental age of the astrocytes also seems to be important in determining their response to insult (Salter and Fern, 2008), and different mechanisms of injury may come into play as the astrocytes develop. Given this age-dependent change in mechanisms of response to insults it is likely that there will also be an age-dependent change in the effects on neighbouring cells. The second aim of the study was therefore to determine whether there was a change how astrocytes respond to insult in different developmental ages of tissue, to assess whether the changes relied on different mechanisms, and to discover whether this impacted differently of the viability of the surrounding tissue, in particular axon integrity.

Glutamate is the major excitatory neurotransmitter in brain and is heavily implicated in mechanisms underlying neuronal degeneration after ischaemic injury. Since astrocyte injury is known to affect the ability of the neurons to tolerate increases in glutamate levels, a third aim of the study was to ascertain whether glutamate receptor blockade was able to protect the axons in case of astrocyte injury.

Lactate is normally produced as a result of glucose metabolism in all the tissues including muscles and brain (Dienel, 2014), and in peripheral tissue, lactate is usually seen as a waste product. However, in the brain lactate appears to play a more fundamental functional role as a source of energy (see section: 1.19, Role of lactate in brain metabolism, page 44), but it is still not clear whether lactate alone can support axonal functions. Based on the available data, it appears that lactate is particularly effective in supporting axonal functions during periods of high energy demands. Therefore, the exact roles of lactate in brain and the time when brain is able to use
lactate efficiently to produce energy remains an interesting area of research. A further aim of the study was to confirm the hypothesis that lactate is taken up by neuronal components, and used as a source of energy, and to assess whether this is a general phenomenon, or whether lactate usage is dependent on the level of energy demand.

Lastly, certain experimental methods, such as immunohistochemistry, have been used widely in the past to assess cell functions and viability. The dependability of immunohistochemistry to assess cell viability has been questioned recently (Shannon et al., 2007). If immunohistochemistry fails to accurately predict cell viability, then it is important to establish the flaws in the method or to modify it in a way that truly reflects the status of examined tissue. In the studies reported here, different techniques were used to assess cell integrity and functionality (immunohistochemistry, electron microscopy, electrophysiology), with a view to better understanding the limitations of each technique, and allowing more definitive interpretation of the changes observed in terms of tissue damage.

Therefore, in the light of the literature reviews, we hypothesized that if astrocytes were restricted selectively by inhibiting their metabolism, then the neighbouring elements i.e. axons and oligodendrocytes would suffer as a result. Further, the extent of injury to each element would be age dependant and would correspond to the extent of injury in their astrocyte counterparts. In addition, we would predict that glutamate receptor antagonists should protect neurons from damage during astrocyte injury. Finally, we hypothesized that if lactate is normally released and taken up by the axons (see section: 1.20 Astrocyte-neuron lactate shuttle, page 46), then exogenous lactate will be able sustain axonal functions even in the absence of glucose.
Chapter 2  Establishing the role of FC

2.1 Introduction

Immunohistochemistry, which is the process of detecting a specific antigen within a tissue section by directing a specific antibody, has been used in research for a long time with considerable success. In the current study, immunohistochemistry was used to determine the basic changes that occur as a result of inactivating astrocytes by an astrocyte specific toxin called FC. The consequences on neighbouring elements were also evaluated immunohistochemically. To do so, astrocytes were visualized by anti-GFAP, axons by anti-NF and oligodendrocytes by anti-O4 antibodies.

Although conceptually simple, immunohistochemical technique has its own hurdles. To overcome some of these difficulties, the immunohistochemical technique was modified in different ways to obtain the best possible outcomes. In addition, the interpretations of the results were performed in different ways to reflect the most likely outcomes of the tissues in control and test conditions. One advantage of immunohistochemistry is its ability to detect more than one antigen within a single section of a tissue. In the current study, this approach was utilized to determine the concomitant changes in cellular and nuclear morphology by using specific antibodies against each component. The results are shown in the current chapter.

Based on an extensive literature review (see section: 1.14.3 Support for glial specificity of fluorotoxins, page 35), FC has been identified as an astrocyte specific toxin in a specific range of concentration and time. To establish the basic effects of FC on astrocytes, immunohistochemical staining was used to determine the changes in astrocyte viability following FC treatment. GFAP is the most abundant IF in astrocytes (see section 1.9 Glial fibrillary acid proteins (GFAP), page 1.9) and the most widely used marker that has been used in immunohistochemical study. Therefore, GFAP was chosen to visualize the effects of FC on astrocytes. In addition, two other cellular components of WM, axons and oligodendrocytes, were also visualized by using specific immunohistological markers for each. Anti-neurofilament antibodies were used to visualize axons and anti-O4 antibodies to visualize oligodendrocytes. We
expected to see a decrease in GFAP staining as a result of injury to the astrocytes by FC. Since astrocytes respond to injury in an age-dependant manner, RONs of 3 different and critical ages representing early development (P0), mid-term development (P10) and adult were chosen to be studied. Therefore it was expected that the FC produces age-dependant damage to the astrocytes. Finally, since it has been suggested that lactate can be as effective as glucose in supporting the axons, the effect of substituting glucose with lactate on astrocytes and axons after FC treatment were examined. We anticipated that lactate can support the axonal functions when the astrocytes are damaged.
2.2 Materials and Methods

2.2.1 Tissue preparation

Optic nerves from Wistar rats of either sex were used as a model for investigating how selective injury of astrocytes inside WM of the CNS affects the health of neighbouring axons. Other than being almost completely myelinated, rat optic nerve is structurally and functionally similar to other CNS fibre tracts (Hildebrand and Waxman, 1984) and can be regarded as a practical model for studying brain white matter. In essence, the RON is composed of central nervous system axons myelinated by oligodendrocytes and accompanied by astrocytes (Barreto et al., 2011). Wistar rats of different ages P0-2, referred to as P0; P8-12 referred to as P10; and adults (50 days of age or more) were killed by cervical dislocation, in accordance with UK Home Office regulations, before being decapitated. The head was then dissected, starting by cutting the connective tissue around the eyeballs until the eyeballs were totally free inside the eye cavities. Then fine scissors were put behind the eyes to cut the eye-end of the optic nerves. The skull was then opened up to entirely expose the brain. The brain was then put into a small petri dish (35mm*10mm, Greiner Bio-one), and flooded immediately in artificial cerebrospinal fluid (aCSF), to avoid drying and preserving normal texture and physiology of the nerves. Optic nerves were then dissected free by cutting distally of the optic chiasm. Both optic nerves were then separated by cutting through the chiasm; one nerve was used as control and the other as test in all the experiments that are included in this study.
2.2.2 Protocols of immunostaining:

When the project was started, immunostaining did not produce the results that were expected. A number of obstacles were faced; different problems were dealt with by modifying the protocol in different ways. In general, oligodendrocyte staining was the most challenging, although both astrocyte and axon staining had their problems as well. In brief, two different protocols were followed and they are outlined in detail below.

2.2.2.1 First protocol

After dissection, each optic nerve was placed in a small chamber in 0.5 ml of aCSF for control nerves or 0.5 ml of aCSF mixed with 0.125mM of FC barium salt (Sigma-Aldrich) for test nerves. The chamber was transferred to a pre-heated (to 37°C) and pre-oxygenated (bubbled with hydrated 95% O₂ - 5% CO₂) chamber. Nerves were kept under the stated conditions for a specified time period (2, 3, 4 or 5 hours) before they were removed and washed in physiological buffer solution (PBS 0.1M, without CaCl₂ and MgCl₂; Invitrogen), at least once. Nerves were then transferred into paraformaldehyde 2% (Agar Scientific) in the fume cupboard and kept at 4°C for 30 minutes in P0 and P10 or for one hour in adult rats. Nerves were then washed in PBS x3 before being put in 20% sucrose as a cryo-preservative for 5 minutes at 4°C in P0 and P10 rats, and 10 minutes in adult rats. Nerves were washed again with PBS x3 before transferring to a small disposable base mould (7mm*7mm*5mm, Polyscience Inc), and embedding in Tissue Tek (Sakura), prior to freezing quickly in hexane, anhydrous 95% (Sigma-Aldrich) and dry ice.

Nerve blocks were then sectioned using a Bright cryostat (Model OTF) to 20μm sections at -15°C and placed on superfrost plus slides (25mm X 75mm X 1mm; Thermoscientific; Menzel-Glæzer). After being left to dry, a small circle was drawn by a hydrophobic pen (ImmEdge pen; Vector laboratories) around each section on the slide to minimize the amount of antibody required. Approximately 50 μL of 2%
paraformaldehyde was put on each section and the slides were placed in a container with the base covered by a soaked tissue to keep it moist. Slides were kept for 20 minutes at 4°C in paraformaldehyde before being washed again with PBS 0.1M, 2x15 minutes. Then a blocking serum solution was prepared by mixing goat serum 10% (Sigma-Aldrich), PBS 0.1M and Triton X-100, 0.1% (Sigma-Aldrich). Each section was then flooded by 50 μL of the solution and either left for at least two hours or overnight in room temperature. Sections were then washed 3x10 minutes with PBS before adding primary antibody.

Primary antibody was prepared by mixing either anti-glial acidic fibrillary protein (anti-GFAP) alexa flour 488 (mouse monoclonal), conjugate (Millipore), or anti-neurofilament (anti-NF) 70 kDa clone DA2 (mouse monoclonal), unconjugated (Millipore) with the previously prepared blocking serum solution at a 1:100 ratio; 50μL of the primary antibody solution then added to each section and left overnight at 4°C.

Next day, slides with unconjugated anti-NF were washed 3x3 with PBS. Secondary antibody Alexa flour 488 F(ab)2 fragment of goat anti-mouse IgG (Molecular probes) diluted 1:100 in blocking serum solution, was added and left for at least one hour at 4°C. Finally all the slides were washed 3x10minutes in 0.1M PBS. After being dried, a drop of perma flour mounting medium (Thermoscientific) was added to each section and the slides were covered by a 22*55mm coverslips (Scientific Laboratory Supply). A brief flow-chart of the steps is shown in Figure 2-1.
2.2.2.2 Second protocol

In the second protocol, the same materials were used as before, but with some modifications. After keeping the nerves for a specified time under the control or test conditions, the nerves were transferred to the small disposable moulds and then frozen immediately in Tissue Tek, i.e. the fixation step in paraformaldehyde and sucrose cryo-preservation were omitted. Both control and test nerves were fixed in 2% paraformaldehyde for 25 minutes in all age groups after sectioning, followed by blocking of the sections by blocking serum solution. Albumin from bovine serum 96-99% (Sigma-Aldrich) was added to the blocking solution (the full recipe is shown in: 2.2.3.2 Blocking serum solution, page 57) and left on the nerve sections for one hour. Primary and secondary antibody was used as before, with the exception of diluting anti-NF to 1:200 ratios. All the washing steps performed as 3x10 minutes. A brief flow-chart of the steps is shown in Figure 2-1.

Figure 2-1: a brief over-view of the steps of immunohistochemistry protocols

The steps which are highlighted were included in the first protocol, but they were eliminated in the second protocol.
2.2.2.3 Oligodendrocyte Staining

Oligodendrocyte staining was performed by using conjugated O4 antibody, Alexa fluor 488 (Millipore). The steps of staining were identical to GFAP and NF staining. However, as shown in (Figure 2-6), the quality of staining was very poor. Therefore, oligodendrocyte staining presented a major challenge as the staining protocol had to be modified in a variety of way to obtain a decent cell staining. Alterations in protocols centred on paraformaldehyde and Triton by inclusion or exclusion of one or both of them simultaneously. The clearest result was obtained by excluding both the fixation step with paraformaldehyde and the permeation step with Triton (Figure 2-6).

2.2.3 Solutions

2.2.3.1 Artificial cerebrospinal fluid

aCSF solution was composed of the following (in mM):
126 NaCl, 3.0 KCl, 2.0 MgCl₂, 2.0 CaCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, 10 glucose and pH 7.45. The osmolarity of the solution was measured and adjusted to 310-320 mOsmol by addition of NaCl. The solution was bubbled with 5% CO₂ and 95% O₂ during experiments. In zero glucose conditions, glucose was omitted and replaced with 10mM sodium L-lactate.

2.2.3.2 Blocking serum solution

Blocking serum solution was used to block non-specific binding sites and reduce not-specific staining. The solution was prepared by mixing 0.1M PBS + 0.1% Triton with goat serum 10% and 10% Albumin from bovine serum 96-99% (Sigma-Aldrich).

The recipe per one ml of the solution is:

800 μL of 0.1M PBS + 0.1% Triton
100 μL of 10% bovine serum albumin
100 μL of goat serum
2.2.3.3 Fluorocitric acid, L-lactate and propidium iodide

Fluorocitric acid barium salt (Sigma-Aldrich) solution was prepared by mixing it with H\textsubscript{2}O and hydrochloric acid (HCl) 1M to form 0.125 mM solution stocks, and then mixed with zero-glucose aCSF. L-lactate was added to the zero-glucose aCSF solution at a concentration of 10mM. Propidium iodide (Sigma-Aldrich) was purchased as a 1.0 mg/ml aqueous solution, and diluted 3000 times to make 500nM solutions.

2.2.4 Visual analysis scoring

A protocol was developed to allow the visual scoring of cellular injury. The images were analysed blind for features that indicate the health or injury of astrocytes. The nerves were sectioned and the sections were given alphabetical codes (for example A, B, C and etc) by Miss. Natalie. The images then collected from the coded sections and scored by a person blinded to the ages and experimental conditions of the sections (whether it is control or test). The axons’ appearance was very similar in both control and test nerves, and therefore, have not been quantified. The scoring system for each of the GFAP and PI staining is described below, with a numerical score given according to the following categories (representative images shown below):

2.2.4.1 GFAP

1-normal cellular pattern

Occasional cell body staining with multiple long, intact processes that arose from the soma and ran in different directions

2-Reactive pattern

Appearance of dense, tightly packed meshwork of disorganized processes and somas. Both the soma and processes appeared to be hypertrophied with increased staining intensity. GFAP labelling was more prominent and cells were obscuring each other.
3-significantly damaged pattern

Obvious disruption of astrocytic branches was indicated by disturbed GFAP staining. Remnants of astrocytic processes may still present with rare cell body staining.

4-Dead cell pattern

Dissociated cellular branches and cell bodies, only background staining with no clear branches.

Figure 2-2: samples of GFAP scoring system.

The numbers represent how each image is numbered in the scoring system. In image number 1, some cell bodies are stained with GFAP and appear as dots, with multiple processes arising from cell bodies and radiating in different directions. Image number 2 shows many prominent GFAP staining of cell bodies and thicker processes. Image number 3 has a clear reduction in positively stained cell bodies and processes, while image number 4 has lost almost all recognizable cell components, although some background staining is still visible. Scale bar is 40µm.
2.2.4.2 Propidium iodide

Viable cells

Cells that had a clear centre with a ring of stain at the periphery, or stained lightly, but uniformly throughout the entire nucleus but with no clear border.

Dead cells

Clear nuclear chromatin condensation indicated by round or nearly rounded shape nucleus. Nuclear border is clearly seen with bright staining.

Figure 2-3: samples of PI scoring system

A: some examples of what have been considered to be still viable cells (arrows). Note that a cell that has stained even inside the nucleus has been considered viable, in cells like that, both the intensity and the shape of the cells have been taken into account. B: some cells that have counted as dead cells (arrows). Note that those cells have intense staining and generally rounder with clearly defined borders. Scale bars = 10µm.
2.2.5 Confocal Microscopy

Images were collected by using an Olympus (Fluoview FV 1000) confocal microscopy as single-plane fluorescent sections, with a 60X oil-immersion objective. The same parameters were used for collecting both control and test images so that direct comparison can be made. Image analysis was performed by measuring either mean pixel intensity in ImageJ software (US National Institutes of Health, Bethesda, Maryland, USA) or visually in a blinded fashion as described before.

2.2.6 Statistical analysis

Data are expressed as means ± standard errors of mean (SEM). P values are indicated by asterisks, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001. P values were determined by unpaired Student’s t test for comparison between two groups using GraphPad Prism version 6.04 for Windows, (GraphPad Software, La Jolla California USA). Both control and test data are collected from the same animal for comparison i.e. each animal provided one control and one test optic nerve. Numbers (N) are expressed as numbers of images analysed/number of nerves. For statistical analysis, the number of images analysed was used. For propidium iodide cell death counting, 4 images from each control and test condition per animal were counted. Dead cells counted in the entire field (approximately 230*220 µm).

2.3 Results

2.3.1 First protocol

With the first protocol, which was longer and more complex than the second, the results were inconclusive. The confocal microscopy images did not show cell parts clearly; cells were ill-defined and not readily identifiable. Repeated experiments
produced similar outcomes and therefore, a modification of the staining protocol was necessary. Consequently, we changed the staining protocol as described in (2.2.2.2 Second protocol, page 56), with satisfactory results. Examples of both astrocyte and axon staining with anti-GFAP and anti-NF are shown in Figure 2-4.

![Images of GFAP and NF staining](image)

**Figure 2-4: images of the first protocol**

Images of both GFAP (Left) and NF (Right) staining collected from first protocol usage in P0 RON after 3 hours in control aCSF. In Both cases, cellular details of astrocytes and axon are ill-defined and not clear.

### 2.3.2 Second protocol

Slides that were prepared by using second protocol produced images with a significant improvement in quality of both GFAP and NF staining. Samples are shown in Figure 2-5.
2.3.3 Oligodendrocyte staining

Different protocols for oligodendrocyte staining with anti-O4 antibody were used by inclusion or exclusion of fixation and/or permeation steps. Furthermore, the nerves were exposed to O4 antibody for various amounts of time, ranging from one hour to overnight exposure. A sample of each condition after overnight exposure is shown in (Figure 2-6). The best result was obtained after removing both fixation and permeation steps as the cells were more clearly defined compared to other conditions (Figure 2-6D). Initially, we were intending to examine the effects of astrocyte damage on oligodendrocytes by immunohistochemistry and that is why we stained oligodendrocytes; however, following the unsatisfactory outcome of these pilots staining experiments, this part of the project was not pursued further.
Figure 2-6: oligodendrocyte staining

Oligodendrocyte staining was performed under various conditions where either fixation by paraformaldehyde and/or permeation by Triton is either excluded or included in the staining steps. A: both steps are included. B: only fixation is included. C: only permeation is included. D: both steps are excluded. Scale bars = 20µm.

2.3.4 Analysing data using ImageJ

At the start of the project, images were processed by using ImageJ software to evaluate the degree of damage caused by the toxic effects of FC based on the mean pixel intensity (MPI) of NF and GFAP staining in regions of interest. Three areas of interest per section, representative of the whole section, were selected and analysed for MPI. A minimum of three sections were analysed in this way per nerve. Mean pixel
intensity of images was then averaged for both GFAP and NF staining; results were then compared for control versus test nerves.

Visual inspection of the images showed no signs of injury in either GFAP or NF staining in test condition; however, mean pixel intensity measurement showed a slight decrease at three hours in both GFAP and NF staining, but an increase afterwards (Figure 2-7). As we did not detect any observable injury at 0.125 mM FC, we increased the concentration of the toxin 4-fold to 0.5 mM; but again, astrocyte injury was still not seen either visually or as judged from measuring MPI (Figure 2-8). In addition, NF staining was also largely intact. It is worth remembering that we were using aCSF that contained 10 mM glucose at this stage of experiments, and FC inhibits the oxidative metabolism of astrocytes inside the mitochondria only, but not glycolysis. Furthermore, astrocytes are known to be able to produce enough energy to survive from glycolysis alone (Fonnum et al., 1997). It is possible therefore, that glycolysis was providing enough energy for astrocytes to survive the effects of the toxin.

At this point, normal glucose aCSF was changed to zero-glucose aCSF by substituting glucose with 10 mM L-lactate. Lactate is known to be taken up by axons and utilized as a fuel to support their function (Brown and Ransom, 2007, Wender et al., 2000). This protocol produced robust injury in astrocytes which was seen as loss of GFAP from astrocyte cell body and processes. However, uncertainty arose about the dependability of MPI measurement of GFAP staining by using ImageJ when data were analysed in the presence of L-lactate in zero-glucose aCSF. For example, in two sets of experiments where the test nerves appeared clearly damaged upon visual inspection, region of interest analysis yielded variable results. In one nerve, MPI decreased by only 3%; while in the other MPI decreased by nearly 45% (Figure 2-9). As can be clearly seen in the figures, the background staining of GFAP staining is prominent in both nerves, but it is much higher in the 1st nerve and possibly obscuring any effects that might have arisen from injury to astrocytes. Therefore, it was concluded that employing ImageJ to measure MPI does not give a real indication of astrocyte injury. Subsequently, a protocol was developed (as described before in 2.2.4.1 GFAP, page 58) which allowed the injury to be quantified by a visual scoring system performed by a blind observer to the data. A nuclear specific dye was also utilized called propidium

65
iodide (PI) to visualize nuclear changes. As PI is normally membrane impermeable and generally excluded from viable cells, it can be used as a good indicator of cell death. Nuclear injury was also quantified via blinded visual scoring (as described in 2.2.4.2 Propidium iodide, on page 60).

Figure 2-7: intensity measurement using ImageJ software
Mean pixel intensity measurement of GFAP staining (A) and NF staining (B) in both control and test nerves after the times specified in P0 RONs. Test nerves were exposed to 0.125 mM FC in aCSF containing 10 mM glucose. N= (63/7, 18/2, 18/2), (56/7, 18/2, 18/2) at 3, 4 and 5 hours in GFAP and NF staining respectively.
Figure 2-8: concentration of FC increased to 0.5 mM

GFAP staining in control (A) and test (B) nerves in P0 RONs at 3 hours. Test nerves were exposed to an increased concentration of FC (0.5 mM). Note that even at that concentration, no difference in GFAP staining can be seen. Scale bar is 40 µm, and applies for both images. Comparison between their MPI measurements is shown in C. C: normalized MPI measurement of control and test nerves for both GFAP and NF staining in P0 RONs at 3 hours in 0.5mM FC (4-fold higher than the concentration normally used). Note that in test nerves, MPI was only slightly lower than control nerves. N=18/2 in both control and test nerves in both GFAP and NF staining.
Figure 2-9: substitution of glucose for L-lactate.

Two pups (P10) were obtained from the same litter, and experiments performed simultaneously for 3 hours. Compared to controls (1st pup (A), 2nd pup (C)), visual inspection showed massive destruction of astrocytes in both rats in test condition (B and D). However, MPI measurement by using ImageJ yielded variable results as shown in E. Scale bar = 40μm and applies for all the figures. E: normalized MPI measurement of two nerves which shows various rates of intensity changes. N= 9/1 in all cases.
Figure 2-10: ImageJ analysis of GFAP (A, B and C) and NF (D, E and F) staining in P0, P10 and adults respectively. As can be seen, there was not any specific pattern of injury in any ages since the significance was random at different time points. * p < 0.05, ** p < 0.01, *** p < 0.001; significant difference between control and test nerves; Student’s t test. N= (12/2 in 2, 3 and 4 hours; 18/3 in 5 hours in both GFAP and NF staining in P0 nerves); (24/4, 18/3, 24/4 and 24/4 in 2, 3, 4 and 5 hours respectively in both GFAP and NF staining in P10 nerves) and (18/3 in all the time points in both GFAP and NF staining in adult nerves).
2.3.5 Astrocyte staining in P0 RONs

Images of astrocytes co-stained with anti-GFAP and PI collected from P0 RON at different time points are shown in (Figure 2-11). In control nerves at 5 hours, many processes could be seen radiating from cell bodies in different directions. PI stain stained many nuclei of astrocytes, but majority of nuclei were viable. Nonetheless, the numbers of dead cell were more frequent compared to the shortest time point in the current study (2 hours). In test nerves, even at 5 hours, many astrocytes appeared to be intact. The amounts of visible processes were usually comparable to control nerves; however, the processes generally appeared thicker and more cell bodies were also stained. Likewise, PI stain had stained many nuclei, but in general, more dead cells were apparent in test nerves compared to control nerves.

Injury to astrocytes was then quantified according to the visual scoring system which was developed for both GFAP and PI staining. All the scoring was performed by a scorer blind to the experimental condition. At 2 hours, no change in GFAP score was detected in test nerves compared to controls, and in all the images, GFAP staining appeared normal. However, at 3 hours, the score of test nerves was significantly higher than controls (1.5 ± 0.15 vs. 2.5 ± 0.15, N=12/3 in both cases, P= 0.0001) (Figure 2-12). It is worth noting, that 3 hours was the only time point in which GFAP score was different between control and test nerves, no significant change was detected afterwards at either 4 (2.4 ± 0.18 vs. 2.38 ± 0.14, N = 31/5, P= 0.9491) or 5 hours (2.05 ± 0.16 vs. 1.9 ± 0.12, N= 40/6, P= 0.4463) (Figure 2-12).

Quantification of injury using PI also showed no significant increase in the number of dead cells at 2 hours (1.16 ± 0.4 vs. 0.16 ± 0.11, N= 12/3 in both cases). However, at 3 hours, there was a sharp increase in the number of dead cells in test nerves (0.58 ± 0.43 vs. 17.83 ± 3.08, N= 12/3 in both cases, P< 0.0001). The number of dead cells remained almost constant afterwards; it was (2.75 ± 0.6 vs. 12.83 ± 1.82) at 4 hours and (8.75 ± 2.77 vs. 17.92 ± 4.88) at 5 hours. In control nerves, the number of dead cells showed a significant increase at 4 hours compared to 5 hours (2.75 ± 0.6 vs. 8.75 ± 2.77, N=12/3 in both, P= 0.0462) (Figure 2-13).
Figure 2-11: GFAP and PI co-staining in P0 RONs

Examples of co-staining of astrocytes by anti-GFAP and PI at the time points specified at the right sides of images. In A (control) and B (test), images are organized in a way that the first column is GFAP staining for astrocytes, the second column is PI staining for nuclei, and the third column is merging of the two previous columns. White rectangles show the magnified areas of both control and test images at 5 hours and are shown in C and D respectively. Scale bars = 40 µm. C: a magnified area of the control nerve at 5 hours, showing many astrocyte processes which are stained with GFAP. Many nuclei are also lightly stained with PI; while majority of the stained cells are viable, a few of them are dead (arrow). Scale bar = 10 µm. D: a magnified area of the test nerve at 5 hours, showing many astrocyte processes which are stained with GFAP; note that the processes appear thicker than control and more cell bodies are also stained. Many nuclei are also stained with PI; while many of the stained cells are viable, some of them are dead (arrows). Scale bar = 10 µm.
Figure 2-12: visual analysis of GFAP changes in P0 RONs

GFAP changes in astrocytes using the numerical visual analysis in P0 RON after the times specified. Control nerves were exposed to normal glucose aCSF, while test nerves were exposed to zero-glucose aCSF in the presence of 0.125mM FC plus 10 mM L-lactate. Note that the result is only significant at 3 hours. N= 12/3, 12/3, 31/5, 40/6 at 2, 3, 4 and 5 hours respectively for both control and test nerves. *** p < 0.001; significant difference between control and test nerves; Student’s t test.

Figure 2-13: cell death count in P0 RONs detected by PI staining

Cell death count in P0 RONs detected by staining of nuclei with PI at the time points specified. Control nerves were exposed to normal glucose aCSF, while test nerves exposed to zero-glucose aCSF in the presence of 0.125mM FC plus 10 mM L-lactate. Note a sharp increase in the number of dead cells in test nerves at 3 hours; however, the number of dead cells remained near the same level afterwards. N=12/3 at any time point for both control and test nerves. *** p < 0.001; significant difference between control and test nerves; Student’s t test.
2.3.6 Axons staining in P0 RONs

While GFAP staining to quantify astrocyte injury has been questioned in the past (Shannon et al., 2007), NF-staining appears to be a reliable way to quantify axons injury (Alix and Fern, 2009). The path of axons in the optic nerve is tortuous; therefore, following individual axon along its entire length is usually not possible, especially in 2D single plane images as in the current study. Occasionally, however, an axon could be followed for a long distance along its length (Figure 2-14 D). In addition, axons in test nerves were largely similar to control nerves; no physical abnormalities could be detected upon visual inspection.

In addition to NF staining of the axons, we also used PI to co-stain the nuclei of the astrocytes with axons, as we did with GFAP staining. Similar to GFAP-PI co-staining, NF-PI co-staining did not show significant changes in the number of dead cells at 2 hours (0.08 ± 0.08 vs. 0.0 ± 0.0, P= 0.3282, N=12/3 in both cases). However, at other time points (3, 4 and 5 hours), the number of dead cells increased significantly in test nerves. At 3 hours, the number of dead cells in control vs. test nerves were (0.25 ± 0.25 vs. 3.58 ± 1.52, P= 0.0416, N=12/3 in both cases), while at 4 hours it was (1.4 ± 0.74 vs. 5.1 ± 1.57, P= 0.0479, N=12/3 in both cases), and finally at 5 hours (4.5 ± 0.9 vs. 17.08 ± 5.4, P= 0.0316, N= 12/3 in both cases). Given that PI is staining dead nuclei, it was expected that co-staining of PI with either GFAP or NF would produce similar results. Although this was the case at 5 hours when the number of dead cells was comparable (17.92 ± 4.88 vs.17.08 ± 5.4 in PI-GFAP vs. PI-NF co-staining, respectively), at 3 hours the number of dead cells was considerably higher in GFAP-PI co-staining (17.83 ± 3.08) compared to NF-PI co-staining (3.58 ± 1.52) (compare Figure 2-13 vs. Figure 2-15).
Figure 2-14: NF and PI co-staining in PO RONs

Examples of co-staining of axons by anti-NF and PI at the time points specified at the right sides of images. In A (control) and B (test), images are organized in a way that the first column is NF staining for axons, the second column is PI staining for nuclei, and the third column is merging of the two previous columns. White rectangles show the magnified areas of both control and test images at 5 hours and are shown in C and D respectively. Scale bars = 40 µm. C: a magnified area of the control nerve at 5 hours, showing many axons which are stained with NF; axons appear as fine threads which usually have a tortuous course. Many nuclei are also lightly stained with PI. Scale bar = 10 µm. D: a magnified area of the test nerve at 5 hours, showing many axons which are stained with NF; note that in some cases an individual axon can be followed along part of its length (arrow heads), also note that axon appearance is comparable to control axons in C. Many nuclei are also stained with PI; while many of the stained cells are viable, some of them are dead (arrows). Scale bar = 10 µm.
Figure 2-15: cell death count in P0 RONs detected by PI staining in conjunction with NF staining

Cell death count in P0 RONs detected by staining of nuclei with PI at the time points specified. Control nerves were exposed to normal glucose aCSF, while test nerves exposed to zero-glucose aCSF in the presence of 0.125mM FC plus 10 mM L-lactate. Note that the number of dead cells increases gradually from 3 hours until it reaches highest level at 5 hours. The number of dead cells in control is also increasing gradually. N=12/3 at any time point for both control and test nerves. * p < 0.05, significant difference between control and test nerves; Student’s t test.

2.3.7 Astrocyte staining in P10 RONs

Images of astrocytes co-stained with anti-GFAP and PI collected from P10 RON at different time points are shown in (Figure 2-16). In control nerves at 5 hours, some cell bodies were occasionally stained (arrow head in Figure 2-16 C) with many processes radiating from cell bodies in different directions (arrows). PI stained many nuclei of astrocytes, but majority of nuclei were viable. In test nerves, at 5 hours, many astrocytes were clearly damaged. The amounts of visible processes were considerably less compared to control nerves; only occasionally cell processes could be seen (arrow head in Figure 2-16 D). In addition to loss of GFAP stained processes, PI had stained many nuclei, and in general, many of the stained cells were dead cells in test nerves compared to control nerves (arrows).
Injury to astrocytes was then quantified according to the visual scoring system for both GFAP and PI staining. At 2 hours, no change in GFAP score was detected in test nerves compared to controls (1.75 ± 0.21 vs. 2.16 ± 0.2 in control vs. test nerves, N=12/3 in both cases, P= 0.1794); however, at 3 hours, the score of test nerves was significantly higher than controls (1.5 ± 0.16 vs. 3.66 ± 0.11, N=18/3 in both cases, P< 0.0001) (Figure 2-17). Significant changes were also detected at 4 (1.25 ± 0.09 vs. 3.25 ± 0.17, N = 24/4, P< 0.0001) and 5 hours (2.06 ± 0.24 vs. 3.96 ± 0.03, N= 30/5, P< 0.0001) (Figure 2-17).

Quantification of injury using PI also showed no significant increase in the number of dead cells at 2 hours (3.25 ± 1.53 vs. 1.25 ± 0.71, N= 12/3 in both cases, P=0.2514). However, at 3 hours, there was an increase in the number of dead cells in test nerves (6.9 ± 1.9 vs. 39.5 ± 5.69, N= 12/3 in both cases, P< 0.0001). The number of dead cells was largely similar at 4 hours (9.33 ± 1.31 vs. 42.67 ± 3.35, N= 12/3 in both cases, P< 0.0001), but increased even more at 5 hours (4.91 ± 1.0 vs. 71.8 ± 7.2, N= 12/3 in both cases, P< 0.0001).
**Figure 2-16 : GFAP and PI staining in P10 RONs**

Examples of co-staining of astrocytes by anti-GFAP and PI at the time points specified at the right sides of images. In A (control) and B (test), images are organized in a way that the first column is GFAP staining for astrocytes, the second column is PI staining for nuclei, and the third column is merging of the two previous columns. White rectangles show the magnified areas of both control and test images at 5 hours and are shown in C and D respectively. Scale bars = 40 µm. C: a magnified area of the control nerve at 5 hours, showing many astrocyte processes which are stained with GFAP (arrows) arising from cell bodies which are occasionally stained as well (arrow head). Many nuclei are also lightly stained with PI; but only very few cells were dead. Scale bar = 10 µm. D: a magnified area of the test nerve at 5 hours, showing a prominent loss of many astrocyte processes; note that very few processes (arrow head) appear in the field, other processes appear to have fragmented and appear as short pieces. Many nuclei are also stained with PI; while some of the stained cells are viable, many of them are dead (arrows). Scale bar = 10 µm.
Figure 2-17: visual analysis of GFAP changes in P10 RONs

GFAP changes in astrocytes using the numerical visual analysis in P10 RON after the times specified. Control nerves were exposed to normal glucose aCSF, while test nerves were exposed to zero-glucose aCSF in the presence of 0.125mM FC plus 10 mM L-lactate. Note that the score is not significantly higher only at 2 hours. N= 12/3, 18/3, 24/4, 30/5 at 2, 3, 4 and 5 hours respectively for both control and test nerves. *** p < 0.001; significant difference between control and test nerves; Student’s t test.

Figure 2-18: cell death count in P10 RONs detected by PI staining

Cell death count in P10 RONs detected by staining of nuclei with PI at the time points specified. Control nerves were exposed to normal glucose aCSF, while test nerves exposed to zero-glucose aCSF in the presence of 0.125mM FC plus 10 mM L-lactate. Note a sharp increase in the number of dead cells in test nerves at 3 hours; however, the number of dead cells remained near the same level at 4 hours, but increased even further at 5 hours. N=12/3 at any time point for both control and test nerves. *** p < 0.001; significant difference between control and test nerves; Student’s t test.
2.3.8 Axons staining in P10 RONs

Like in P0 nerves, axons were visualized by anti-NF staining and the images are shown in (Figure 2-19). Axons in control and test nerves were very similar; no physical abnormalities could be detected upon visual inspection.

In addition to NF staining of the axons, PI was also used to co-stain the nuclei of the astrocytes with axons, similar to P0 nerves. Similar to GFAP-PI co-staining, NF-PI co-staining did not show significant changes in the number of dead cells at 2 hours (0.25 ± 0.17 vs. 1.5 ± 0.66, P=0.0847, N=12/3 in both cases). However, at other time points (3, 4 and 5 hours), the number of dead cells increased significantly in test nerves. At 3 hours, the number of dead cells in control vs. test nerves were (4.41 ± 0.57 vs. 27.25 ± 2.88, P < 0.0001, N=12/3 in both cases), while at 4 hours it was (2.25 ± 0.64 vs. 31.00 ± 4.86, P < 0.0001, N=12/3 in both cases), and finally at 5 hours (1.75 ± 0.44 vs. 64.25 ± 11.15, P < 0.0001, N= 12/3 in both cases). As would be expected, the number of dead cells in GFAP-PI and NF-PI co-staining was very similar (Compare Figure 2-18 vs. Figure 2-20).
Figure 2-19: NF and PI staining in P1O RONs

Examples of co-staining of axons by anti-NF and PI at the time points specified at the right sides of images. In A (control) and B (test), images are organized in a way that the first column is NF staining for axons, the second column is PI staining for nuclei, and the third column is merging of the two previous columns. White rectangles show the magnified areas of both control and test images at 5 hours and are shown in C and D respectively. Scale bars = 40 µm. C: a magnified area of the control nerve at 5 hours, showing many axons which are stained with NF; axons appear as fine threads which usually have a tortuous course. Many nuclei are also lightly stained with PI. Scale bar = 10 µm. D: a magnified area of the test nerve at 5 hours, showing many axons which are stained with NF; note that in some cases an individual axon can be followed along part of its length (arrow heads), also note that axon appearance is comparable to control axons in C. Many nuclei are also stained with PI; while some of the stained cells are viable, many of them are dead (arrows). Scale bar = 10 µm.
Figure 2-20: cell death count in P10 RONs detected by PI staining in conjunction with NF staining

Cell death count in P10 RONs detected by staining of nuclei with PI at the time points specified. Control nerves were exposed to normal glucose aCSF, while test nerves exposed to zero-glucose aCSF in the presence of 0.125mM FC plus 10 mM L-lactate. Note that the number of dead cells increases gradually from 3 hours until it reaches highest level at 5 hours. N=12/3 at any time point for both control and test nerves. *** p < 0.001; significant difference between control and test nerves; Student’s t test.

2.3.9 Astrocyte staining in adult RONs

Images of astrocytes co-stained with anti-GFAP and PI collected from adult RON at different time points are shown in (Figure 2-21). In control nerves at 5 hours, some cell bodies were occasionally stained (arrow head in Figure 2-21C) with many processes radiating from cell bodies in different directions (arrows). PI stained many nuclei of astrocytes, but majority of nuclei were viable. In test nerves, at 5 hours, many astrocytes were clearly damaged. The amounts of visible processes were considerably less compared to control nerves; only occasionally cell processes could be seen. In addition to loss of GFAP stained processes, PI had stained many nuclei, and in general, many of the stained cells were dead cells in test nerves (arrows in Figure 2-21 D).
Quantification of injury using GFAP scoring showed a significant injury in adult nerves at all the time points that are tested. At 2 hours, the values were (1.0 ± 0.0 vs. 1.55 ± 0.12, N=18/3 in both cases, P< 0.0001). It is worth noting that adults were the only group in which GFAP scoring was significantly different at 2 hours. Likewise, at 3 hours, the score of test nerves was significantly higher than control nerves (1.11 ± 0.07 vs. 1.5 ± 0.14, N=18/3 in both cases, P = 0.0239); at 4 hours (2.05 ± 0.22 vs. 3.27 ± 0.10, N = 18/3, P< 0.0001) and at 5 hours (1.66 ± 0.24 vs. 3.22 ± 0.15, N= 18/3, P< 0.0001) (Figure 2-22).

Surprisingly, quantification of injury using PI did not show any significant effect at 2 and 3 hours. A 2 hours, the number of dead cells were (2.64 ± 0.83 vs. 3.47 ± 0.74, N= 18/3 in both cases, P= 0.4668); at 3 hours (3.38 ± 0.91 vs. 4.88 ± 0.88, N= 18/3 in both cases, P= 0.2469). However, at 4 hours, the number of dead cells saw a steep increase and reached statistically significant level in test nerves (6.5 ± 1.0 vs. 16.5 ± 1.83, N= 18/3 in both cases, P= 0.0001), and the number of dead cells increased slightly at 5 hours (3.83 ± 1.17 vs. 21.08 ± 1.98, N= 18/3 in both cases, P< 0.0001).
Figure 2-21: GFAP and PI staining in adult RONs

Examples of co-staining of astrocytes by anti-GFAP and PI at the time points specified at the right sides of images. In A (control) and B (test), images are organized in a way that the first column is GFAP staining for astrocytes, the second column is PI staining for nuclei, and the third column is merging of the two previous columns. White rectangles show the magnified areas of both control and test images at 5 hours and are shown in C and D respectively. Scale bars = 40 µm. C: a magnified area of the control nerve at 5 hours, showing many astrocyte processes which are stained with GFAP (arrows) arising from cell bodies which are occasionally stained as well (arrow head). Many nuclei are also lightly stained with PI; but only very few cells were dead. Scale bar = 10 µm. D: a magnified area of the test nerve at 5 hours, showing a prominent loss of many astrocyte processes; note that all the processes appear to have fragmented to pieces. Many nuclei are also stained with PI; while some of the stained cells are viable, many of them are dead (arrows). Scale bar = 10 µm.
**Figure 2-22: visual analysis of GFAP changes in adult RONs**

GFAP changes in astrocytes using the numerical visual analysis in adult RON after the times specified. Control nerves were exposed to normal glucose aCSF, while test nerves were exposed to zero-glucose aCSF in the presence of 0.125mM FC plus 10 mM L-lactate. Note that the results are significant at all-time points. N= 18/3 in all the times for both control and test nerves. * p < 0.05, *** p < 0.001; significant difference between control and test nerves; Student’s t test.

**Figure 2-23: cell death count in adult RONs detected by PI staining**

Cell death count in adult RONs detected by staining of nuclei with PI at the time points specified. Control nerves were exposed to normal glucose aCSF, while test nerves exposed to zero-glucose aCSF in the presence of 0.125mM FC plus 10 mM L-lactate. Note a sharp increase in the number of dead cells in test nerves at 4 hours. N=18/3 in all the time points for both control and test nerves. *** p < 0.001; significant difference between control and test nerves; Student’s t test.
2.3.10 Axon staining in adult RONs

Similar to other ages, axons were visualized by anti-NF staining and the images are shown in (Figure 2-24). Axons in control and test nerves were stained similarly; no physical abnormalities could be detected upon visual inspection.

In addition to NF staining of the axons, PI was used to co-stain the nuclei of the astrocytes with axons, similar to other ages. Similar to GFAP-PI co-staining, NF-PI co-staining did not show significant changes in the number of dead cells at 2 hours (3.55 ± 0.61 vs. 3.61 ± 0.56, P = 0.9475, N=18/3 in both cases) and 3 hours (3.5 ± 0.41 vs. 3.94 ± 0.97, P=0.6784, N=18/3 in both cases). However, at other time points (4 and 5 hours), the number of dead cells increased significantly in test nerves. At 4 hours, the numbers of dead cells in control vs. test nerves were (7.33 ± 1.76 vs. 17.83 ± 2.17, P = 0.0011, N=18/3 in both cases), while at 5 hours it was (4.25 ± 0.68 vs. 19.0 ± 2.47, P < 0.0001, N=18/3 in both cases) (Figure 2-25). As would be expected, the number of dead cells in GFAP-PI and NF-PI co-staining was similar (Compare Figure 2-23 vs. Figure 2-25).
Figure 2-24: NF and PI staining in adult RONs

Examples of co-staining of axons by anti-NF and PI at the time points specified at the right sides of images. In A (control) and B (test), images are organized in a way that the first column is NF staining for axons, the second column is PI staining for nuclei, and the third column is merging of the two previous columns. White rectangles show the magnified areas of both control and test images at 5 hours and are shown in C and D respectively. Scale bars = 40 µm. C: a magnified area of the control nerve at 5 hours, showing many axons which are stained with NF; axons appear as fine threads which usually has a tortuous course. Many nuclei are also lightly stained with PI, but none of them appear to be dead. Scale bar = 10 µm. D: a magnified area of the test nerve at 5 hours, showing many axons which are stained with NF; note that in some cases an individual axon can be followed along part of its length (arrow head), also note that axon appearance is comparable to control axons in C. Many nuclei are also stained with PI; some of them are dead (arrows). Scale bar = 10 µm.
**Figure 2-25: cell death count in adult RONs detected by PI staining in conjunction with NF staining**

Cell death count in adult RONs detected by staining of nuclei with PI at the time points specified. Control nerves were exposed to normal glucose aCSF, while test nerves exposed to zero-glucose aCSF in the presence of 0.125mM FC plus 10 mM L-lactate. Note a sharp increase in the number of dead cells in test nerves at 4 hours. N=18/3 in all the time points for both control and test nerves. ** p < 0.01, *** p < 0.001; significant difference between control and test nerves; Student’s t test.

### 2.4 Discussion

When the research was started, the difference in the measurement of GFAP intensity was intended to be used as a way of determining the effects of FC on astrocytes. Although FC treatment produced widespread depletion of GFAP staining by visually inspection, measuring GFAP intensity did not show a measurable and conclusive change in astrocytes viability. This necessitated a change in the method and propidium iodide, which stains the nuclei of dead cells, was included in the study. In addition, the images were quantified using a visual scoring system instead of intensity measurement.

Astrocytes of all the ages showed injury features when quantified by visual scoring system after FC treatment, which implies the effectiveness of FC as an
astrocyte specific toxin. Although it was hypothesized that the axons would be damaged after astrocyte injury, axon staining using anti-NF antibodies did not show signs of injury in any age groups that have been tested. Lactate has been suggested as an effective metabolic fuel to support axonal survival, and the results showed that lactate could indeed support axonal function, even when astrocytes were compromised.

2.4.1 Interpretation of GFAP staining intensity is questionable

GFAP is the main intermediate filament protein of mature astrocytes and an important component of the cytoskeleton. It has been widely used as an astrocyte marker to visualize astrocytes in the past (see section: 2.2.4.1 GFAP, page 58). However, the dependability of GFAP staining in the study of astrocytic viability has been questioned recently. Members of our lab (Shannon et al., 2007) have shown that 30 minutes of OGD in isolated RONs did not cause significant change in GFAP intensity measurement in control versus test nerves in spite of the significant increase in the number of dead cells that were determined by nuclei staining. The current results are in agreement with that observation. In two sets of data that were collected from rats of the same litter and experiments performed simultaneously, GFAP staining analysis yielded variable results (Figure 2-9). Also, we have shown that although the number of dead astrocytes was significantly different in control vs. test nerves as determined by PI staining of the nuclei, MPI measurement of GFAP staining did not provide the same level of significance in different ages of RONs (Figure 2-12, Figure 2-17 and Figure 2-22, compare them to Figure 2-13, Figure 2-18 and Figure 2-23 respectively).

Normally GFAP delineates only around 15% of the total volume of an astrocyte (Bushong et al., 2002), leaving the majority of an astrocyte invisible by GFAP staining. In addition, there are an array of different shapes, sizes and orientation of astrocytes within WM (Butt and Ransom, 1993), which adds to the difficulty of GFAP staining interpretation. Furthermore, astrocytes respond to almost any kind of injury by a
process known as RA (see section: 1.10 Reactive astrocytosis, page 18). There are reports to show that RA happens as early as 30 minutes after ischemia with features of cell division become apparent after 3 hours (Petito, 1986, Petito and Halaby, 1993), and generally, it is believed that RA is associated with increased expression of GFAP protein which may obscure any early signs of injury when GFAP is used as an indicator of cell injury. Furthermore, even when an astrocyte is damaged, GFAP does not disappear instantaneously; rather, it may leave behind a fluorescent “island” surrounding the damaged cell for sometimes (Back et al., 2007a) or GFAP may leak to the surrounding tissue from the damaged cell (Garcia et al., 1993). Similarly, in the current study, different levels of GFAP immunoreactivity have been encountered in the background (Figure 2-9) which surely can interfere with the sensitivity of GFAP staining to determine the true level astrocyte damage. Lastly, it has been suggested that exposure of astrocytes to acidic medium solution increases GFAP immunoreactivity, an effect that was encountered by exposing astrocyte to acidic Ringer solution and mitochondrial inhibition (Hulse et al., 2001). Similarly, in the current study, as FC treatment and subsequent cell death is associated with lowering pH (Largo et al., 1996), an alteration in the level of GFAP expression as evidenced by immunohistochemical staining can be envisaged.

Since GFAP does not reveal the complete morphology of an astrocyte, more recently, transgenic mouse model has been generated that express enhanced green fluorescent protein (eGFP) under human or mouse GFAP promoter gene in a subset of astrocytes which allows astrocytes to reveal their complete morphology in normal and pathological states (Salter and Fern, 2008, Sun et al., 2010, Shannon et al., 2007). This approach has the advantage of highlighting individual cells which can be followed serially at different time points, as in (Shannon et al., 2007). One disadvantage, however, is neither transgenic mice nor GFAP immunostaining allow the cellular ultrastructural details to be examined. Electron microscopy, therefore, has been suggested as a more reliable technique to investigate the ultrastructural changes of damaged astrocytes (Fern, 2001), and that is something that has been done and the results are shown later (See: Chapter 4, page 135).
2.4.2 Astrocytic cell damage did not affect axon staining in RONs

Astrocytes are considered to be fundamentally important for normal functioning and maintenance of CNS (Salter and Fern, 2008, Benarroch, 2005, Re et al., 2006). In addition, death of astrocytes is also associated with release of substances that can affect the viability of the neurons such as excitotoxic glutamate, cytokines, reactive oxygen species, endothelial growth factor, chondroitin sulphate proteoglycan and TNF (Fern, 1998, Sun et al., 2010). Taking together, these factors have led to the hypothesis that astrocyte injury can influence axon structure and function (Thomas et al., 2004, Fern, 1998, Sun et al., 2010). Here, the consequences of selective astrocytic damage induced by FC treatment in zero-glucose aCSF solution on neighbouring axons were investigated, and the results indicate that selective astrocytic damage did not have any effects on axon structure assessed by anti-NF staining in RONs (Figure 2-14, Figure 2-19 and Figure 2-24). However, these results cannot explain if axon functions and ultrastructure have been preserved. Furthermore, it is difficult to assume that these results reflect the true well-being of the axons since it is not currently known how long will it take for axons to disintegrate after a lethal injury. For example, treatment of spinal cord dorsal column with glutamate did not induce any detectable structural injury to the axon cylinders as detected by spectrin breakdown even after 3 hours (Li and Stys, 2000). In addition, OGD caused delayed axonal degeneration in murine brain slices which was occurring 4 to 10 hours after the insult depending on the age of the animal (McCarran and Goldberg, 2007). Of note, however, the latter study, like the current one, has used immunohistochemistry to determine injury to the axons, and it is likely that the axonal integrity may take longer to be disturbed enough to be detectable by immunohistochemistry methods after an insult. However, the functional integrity may be lost much earlier (Li and Stys, 2000). To tackle this issue, evoked compound action potential (CAP) measurement and ultrastructural analysis using electron microscopy were performed and the results are shown in (Chapter3, page 94 and Chapter4, page 135).
2.4.3 Lactate supported axon integrity

Astrocytes play actively in removing excessive extracellular K+ which is elevated during neuronal activity (see section: 1.8 Potassium regulations by astrocytes, page 14). In addition, astrocytes are the principal cell type in CNS that handle glutamate metabolism (see section: 1.16 Roles of astrocytes in glutamate handling, page 38). Although WM is an area devoid of synapses, there are ample sources of glutamate release within WM which include axons, oligodendrocytes and astrocytes (Back et al., 1998). Maintaining ionic gradients and glutamate metabolism is highly energy dependant and performed, primarily, by astrocytes. Nearly 50% of the brain energy is used to maintain ionic gradient, mainly through Na+ - K+ ATPase activity (Brown et al., 2001). FC, a selective metabolic poison for astrocytes, was used initially to selectively kill astrocytes through the inhibition of oxidative metabolism and subsequently diminishing ATP production. However, astrocytes survived even 5 hours exposure to 0.125mM FC in normal glucose aCSF (Figure 2-7); even 0.5 mM FC did not appear to damage astrocytes when applied concomitantly in normal glucose aCSF (Figure 2-8). It has been shown that during low activity of the glial cells, glycolysis can produce sufficient ATP for cell survival (Fonnum et al., 1997). Furthermore, Dringen et al. (1993) showed that glycolysis and glycogenolysis will provide most of the energy required for astrocytes during periods of rapid increase in energy demands. In addition, in primary astrocyte culture from rats, very low level of oxidative metabolism has been reported (Hertz et al., 2007). The aforementioned observations together with the current results that was showing normal astrocyte staining after FC treatment in the presence of glucose suggests the possibility that glycolysis was responsible for astrocyte survival during FC treatment. Moreover, most of the energy-requiring active processes will occur in the thin processes of astrocytes, and as these thin processes are lacking mitochondria, they depend on glycolysis and glycogenolysis for energy production (Hertz et al., 2007). Subsequently, glucose was removed from the aCSF and was substituted with 10 mM L-lactate. Application of glucose free aCSF with 0.125 mM FC clearly damaged astrocytes, but axons appeared to be normal (Figure 2-14, Figure 2-19 and Figure 2-24). Likewise, others (Wender et al., 2000, Brown and Ransom,
have shown that axons and neurons can survive for prolonged periods if glucose is substitutes for lactate. In brief, these results show that axons are staining normally in the presence of lactate even when astrocytes are dead.

2.4.4 Astrocyte sensitivity to FC is age dependant

In all the ages that were tested, FC treatment for 2 hours did not produce any significant effects on the number of dead cells as assessed by PI staining of the dead nuclei (Figure 2-13, Figure 2-18 and Figure 2-23). However, a rapid and significant increase in the number of dead cells occurred after 3 hours. The numbers of dead cells were identical when PI was used in conjunction with either GFAP or NF in all the ages (compare Figure 2-13, Figure 2-18 and Figure 2-23 to Figure 2-15, Figure 2-20 and Figure 2-25 respectively). As was discussed in the (2.2.4.2 Propidium iodide, page 60), the stained nuclei were either classified as viable or dead cells; however, there was a large group of cells that appeared to be larger than normal, but were not shrunken enough to be counted as dead cells as well, these cells were excluded from the counting. These cells might have been swollen by cytotoxic ion influx mediated by Na⁺-K⁺-Cl⁻ co-transport and Na⁺ and K⁺ dependant HCO₃⁻ transport (Salter and Fern, 2008). In addition, an increase in the extracellular level of Ca²⁺ and ATP is associated with a transient increase in the astrocytic cell volume. Since both Ca²⁺ and ATP can be released as a result of FC treatment and cell death (see section: 1.14.1 Mechanism of action and consequences, page 29), these cells most likely represent swollen cells. However, astrocytes that suffer non-fatal injury may show a large increase in the concentration of intracellular Na⁺ ions but still survive the insult (Thomas et al., 2004). Consequently, the fate of these cells cannot be determined confidently and hence are excluded from the counting.

The mechanism and sensitivity of WM astrocytes to ischemic insults have been shown to be age dependant. Astrocyte membrane expresses a large array of different ion channels, transporters and transmitter receptors which are developmentally regulated. For instance, astrocytes of actively myelinating WM in a modelled transgenic mice were shown to have a heightened sensitivity to ischemic injury, even
more than grey matter astrocytes (Shannon et al., 2007). Moreover, the mechanisms of injury changes from the cytotoxic Ca\textsuperscript{2+} influx through both L and T type voltage gated channels in neonatal astrocytes (Fern, 1998) to Na\textsuperscript{+}-K\textsuperscript{+}-CL\textsuperscript{-} co-transport mediated cell death in older ages (Thomas et al., 2004). This characterization of ion channel dependant cell death during different developmental stages is especially helpful in understanding the astrocytes response to various pathological situations. In line with this, P0 astrocytes showed lower degree of injury after FC treatment compared to other ages as assesses by the number of dead cells stained with PI. Furthermore, the amount of GFAP retained in P0 nerves at the end of 5 hours of FC treatment was clearly more than that of other ages (compare Figure 2-11 to Figure 2-16 and Figure 2-21). In P10 nerves, the numbers of dead cells were clearly much higher than that of P0 nerves and this can be due to a number of reasons. As shown by Shannon et al. (2007), WM astrocytes at P10, which corresponds developmentally to the period of active myelination, are highly susceptible to insults. In addition, pre-oligodendrocytes are abundant in this stage of life, and since pre-oligodendrocytes are intrinsically the most sensitive cells to injury (Back et al., 1998), it is likely that pre-oligodendrocytes will be injured as well. Other cells that might be also present are NG2 cells and microglia (see section: 1.12 NG2 cells, page 24 and 1.13 Microglia, page 25). Since PI is a non-specific dye and stains nuclei of dead cells generally, it is not possible to differentiate the nuclei of different cells in the current study and some of the counted nuclei may be cells other than astrocytes.

Lastly, adult nerves showed almost the same number of dead cells as in P0; however, the depletion of GFAP immuno-reactivity was much more in adults than in P0 (compare Figure 2-16 to Figure 2-21). In addition, the population of RON astrocytes have already established at birth. As development progresses, the RON expands considerably, but astrocyte number remains the same which results in a much lower density of astrocytes within adult RONs. Since the counting of dead cells were performed within the same area (220*230 \(\mu m\)), the same number of dead cells in adults clearly indicate a much higher rate of cell injury.
Chapter 3  Investigating the functionality of RON after FC treatment

3.1 Introduction

The principal aim of the project was to examine how different essential elements within white matter affect each other when they receive ischemic challenge separately. Wistar rat optic nerve was selected as a model for this study as it is a purely white matter tract without neuronal cell bodies. First, a protocol was established to allow selective damage to astrocytes by using a form of chemical ischemia and investigating the consequences on neighbouring axons with changes detected by immunohistochemistry. As injury mechanisms operating in white matter seem to be very much age dependent, three different critical age groups were chosen for the research.

While immunohistochemistry allowed visualization of the structural abnormalities of white matter, it was not possible to conclude whether axons which exhibited normal immunohistochemical staining were still functional. To pursue this issue, the same protocol was used to produce chemical ischemia and selectively kill astrocytes and functionality of the whole model was assessed by measuring compound action potentials in the nerves in vitro.

Compound action potentials were measured from three different age groups at different time points based on the preliminary results. After establishing the changes in functionality, further experiments investigated the mechanisms underlying the injury process in the model. The results are shown in the current chapter.

In the previous chapter, the role of FC on astrocytes and axons were tested and it was shown that astrocytes were injured after FC treatment as determined by loss of GFAP immunoreactivity and a higher number of dead cells as determined by PI staining. However, NF staining, which stains the axons, did not show any changes. Therefore, it was vital to examine the axons further to determine if the axons are damaged after FC treatment, because it was hypothesised in the beginning that
astrocyte injury would produce axonal injuries as well. Thus the aim of the experiments described in this chapter was to assess the functionality of the nerves by stimulating them electrically and recording their CAPs. It was important to first establish whether the RONs can produce a stable CAP over several hours. Furthermore, since lactate was used in immunohistochemical experiments, it was essential to ascertain that lactate could preserve CAPs in a similar manner to glucose.

Once the basic measurements of CAPs were done, then the CAPs were recorded in the presence of the toxin. CAPs measurement in this case could answer two fundamental questions; (1) whether astrocyte damage by FC had a deleterious effects on axonal conductivity and (2) whether axons which stained normally by NF actually remained functional?

Finally, the hypothesis that glutamate is released abundantly after astrocyte injury and causes axonal damage was tested. To that end, both classes of ionotropic glutamate receptors, i.e. NMDA and non-NMDA receptors were blocked separately or together by specific glutamate receptor antagonists to examine the effects of glutamate.
3.2 Material and Methods

CAP recording:

All the CAP recordings were obtained in vitro from freshly isolated optic nerves from decapitated rats as described before (see section: 2.2.1 Tissue preparation, page 53). Individual nerves were placed on a tissue slice chamber system (Harvard Apparatus; MA, USA) which allowed the nerve to be submerged in oxygenated aCSF (for the compositions of aCSF please see section: 2.2.3.1 Artificial cerebrospinal fluid, page 57). Artificial CSF was gravity fed and flow was regulated to a rate of 2-4 ml/min depending on the age of the rat by a flow meter (Cole-Parmer; London, UK). The surface of the chamber was covered by a piece of mesh to allow a smooth and steady flow of aCSF over the nerves. Artificial CSF stock was placed in a water bath which kept the temperature of the fluid constant at 37 °C around 1.5 meters above the recording chamber to allow gravity to draw aCSF towards the chamber. In addition the temperature of the slice chamber was measured and regulated automatically at 37 ± 0.5 °C by an independent temperature controller (Warner Instruments; CT, USA). The chamber also had an aperture through which a continuous supply of oxygen kept the nerve fully oxygenated for the duration of the study.

After dissection, individual nerves were left in place untouched for 30-60 minutes to allow the nerves to recover from the shock of dissection. CAPs were elicited by electrical stimulation at a rate of 2 stimulation / minute (stim/min) and obtained through a glass suction electrode. Suction electrodes were prepared individually from low melting points glass capillary tubes. A capillary tube was heated approximately 0.5 cm from one end over a Bunsen burner and was gently bent to an angle of approximately 45 degrees. The other end of the capillary was left undisturbed. Similar electrodes were utilized for both stimulating and recording. For the stimulating electrode, silver wire (0.25 mm in diameter; WPI, Stevenage, UK) was chlorided by soaking in a household bleach tab dissolved in water and was inserted into the lumen of stimulating electrode. A second wire was wrapped around the tip of the stimulating electrode near its opening which provided a way for the current to return and complete the electric circuit. Care was taken to insure the proper contact
between the wires and the fluid in the recording chamber at all times to avoid interruption in the continuity of the circuit. The recording electrode was accompanied by a parallel differentiated electrode to record the artefact from the stimulus. For the recording electrodes, silver wire (as above) was inserted into the lumen of each capillary (both recording electrode and the parallel differentiated electrode).

The RONs were stimulated from the distal (eye) end of the nerve, thereby insuring normal orthodromic conduction of AP. After the incubation period of at 30 – 60 minutes, the ends of the nerve were gently inserted into the lumen of the stimulating electrode at one end and the recording electrode at the other end by positioning the electrodes near the ending of the electrodes and scooping the nerves to the mouth of the electrodes, each electrode was attached to a fine manipulator which allowed gentle movement in all three planes. Normally, the recording potential was subtracted from the parallel differentiated electrode to get a pure AP signal. CAPs were evoked via constant short square wave current pulses (1µm in length; 5V in amplitude) (Isostim A320; WPI, Stevenage, UK). The signals were amplified (Cyber Amp; Axon Instruments, Union City, CA), then filtered (low pass 800-6000 Hz) depending on the age of the rat being tested, digitalized (1401 Mini; Cambridge Electronic Device(CED), Cambridge, UK ) and recorded on a personal computer running Signal Software (CED, Cambridge, UK). To produce supra-maximal stimulation, approximately 20% of the current which produced maximum stimulation was added. P0 nerves usually needed much longer stimulation to produce maximum effect (approx. 0.2-0.5 ms) compared to adult nerves (approx. 0.05 ms). All the data were stored on a hard drive for further analysis.

In all the experiments, APs were recorded in normal glucose containing aCSF for at least the first 10 minutes to set a baseline value. Then the stock solution was changed to zero glucose aCSF to produce test data. In all the conditions when drugs were used, they were added to the stock solution. AP recording from both P0 and P10 showed a typical biphasic profile, comprising one peak and one trough, while AP from adult nerves typically showed a three peak profile. In P0 and P10, AP amplitude was determined as the difference between the highest point in the peak and the lowest point in the trough. For adult AP quantification, the area under the curve was
measured and analyzed. For determining the change in individual peak amplitude in adult, the changes in the highest points of the peaks were compared to a single point in the baseline. Some of the features of CAP recording are shown in Figure 3-1.

Figure 3-1: some features of electrophysiological recording of CAPs
3.3 Statistical analysis

In all the graphs, data are expressed as Means ± SEM. P values are indicated by asterisks, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. P values were determined by unpaired Student t test for comparison between two groups and by one way analysis of variance (ANOVA) test for comparison between more than two groups followed by Tukey’s post-hoc test using GraphPad Prism version 6.04 for Windows, (GraphPad Software, La Jolla California, USA). Numbers (n) are the numbers of nerves that CAPs are recorded from and those numbers are used for statistical analysis. To get a single value reading at any point of time (such as 100 minutes), a total of 10 reading (from 98 minutes to 103 minutes) were averaged and the averaged value was determined as CAP at that point of time. The values obtained were used to determine significance. In all the experiments, the first 10 minutes were recorded in glucose containing aCSF to establish a baseline value; this time was later deducted when values were obtained at specific time point i.e. when values, for example, at 100 minutes were aimed to be determined, 110 minutes after the start of experiments were taken. This was true for all the experiments except recording in control nerves in glucose containing aCSF in which exact time points were taken. In P0 and P10 nerves, the amplitude between the highest point in the peak and the lowest point in the trough was measured to determine CAP, while in adult nerves, the area under the curve (all the three peaks) was measured to determine CAP. Therefore, the term CAP refers to amplitude in both P0 and P10 nerves and to CAP area in adult nerves. To determine changes in CAP, the first minute of recording is normalized to 100% and used as a baseline against which other time points were compared.

3.4 Results

3.4.1 CAPs recording under control condition

From the beginning of electrophysiological recording, it was important to establish whether RONs can survive over several hours in vitro. To do so, CAPs were
recorded from all the three ages that have been used in this study for a maximum of 5 hours (300 minutes). Experiments were not performed beyond 5 hours. As can be seen in Figure 3-2, CAPs were largely stable in all the ages for 5 hours. At the end of 5 hours of recording, the percentage of CAPs which remained were (88.32 ± 8.64) in P0, (96.19 ± 4.45) in P10 and (103.46 ± 6.06) in adult nerves (Figure 3-2C). As can be noted from the results, P0 nerves were the least stable among all the ages and CAPs decreased the most in them.

After establishing that the nerves were stable in glucose, it was then important to determine whether L-lactate also preserves CAPs in RONs. To do so, glucose was omitted from aCSF and replaced with 10mM lactate. There are two major benefits in replacing glucose with L-lactate. Firstly, all the experiments were in lactate containing aCSF; therefore, any effects that might be detected after FC treatment could be attributed to the toxin and not glucose replacement. Secondly, zero glucose condition is more comparable to ischemia where glucose supply is severely restricted. The results of 5 hours recording in lactate are shown in Figure 3-3. As can be noted, CAPs were stable for 5 hours in all the ages. At the end of 5 hours recording, the percentages of CAPs which remained were (91.52 ± 5.84) in P0, (93.97 ± 4.68) in P10 and (103.43 ± 4.63) in adults (Figure 3-3C).

After establishing that CAPs remained stable for at least 5 hours in both glucose and lactate, then the rate of changes in the percentage of CAPs was compared in both conditions. No statistically significant changes were found between the recordings from glucose versus lactate containing aCSF (Figure 3-4). At the end of 5 hours recording, CAPs in glucose versus lactate were (88.32 ± 8.64 vs. 91.52 ± 5.84), (96.19 ± 4.45 vs. 93.97 ± 4.68) and (103.46 ± 6.06 vs. 103.43 ± 4.63) in P0, P10 and adults respectively.
Figure 3-2: CAP recording under control condition using glucose

A: Examples of CAP recordings from the beginning (blue) and the end (green) of 300 minutes of recording from control experiments containing glucose. Scale bars are 10mV by 2 msec in P0 and P10; 20 mV by 0.5 msec in adult.

B: Continuous recording of CAPs for 5 hours (300 minutes) in glucose containing aCSF. CAPs were stable in all age groups for 300 minutes of recording. A small increase in CAP amplitude was detected after commencing of CAP recording in both P0 and P10 RONs (nearly 15 and 10 % respectively) that disappeared again after nearly two hours. Each point represents one stimulus during recording and the values are averaged from (8, 8 and 7) nerves in (P0, P10 and adults) respectively.

C: A graphical presentation of CAPs at the end of 300 minutes recording in 10mM glucose aCSF in different age groups. Note that CAP area remained highly stable in adults. Likewise, CAP amplitude remained largely stable in P10 and P0 RONs.
**Figure 3-3: CAP recording under control condition using L-lactate**

A: Examples of CAP recording from the beginning and the end of 300 minutes of recording from control experiments containing lactate. Scale Bars are 10mV by 2 msec in P0 and P10; 20 mV by 0.5 msec in adult.
B: Continuous recording of CAPs for 5 hours (300 minutes) in lactate containing aCSF. CAPs were stable in all age groups for 300 minutes of recording. Each point represents one stimulus during recording and the values are averaged from (7, 6 and 6) nerves in (P0, P10 and adults) respectively.

C: A graphical presentation of CAPs at the end of 300 minutes recording in 10mM lactate aCSF in different age groups. Note that CAP area remained highly stable in adults. Likewise, CAP amplitude remained largely stable in P10 and P0 nerves.

**Figure 3-4: CAPs were preserved by both glucose and L-lactate**

CAPs recorded from RONs of different age groups over 300 minutes in either 10 mM glucose or 10 mM L-lactate showed no statistically significant difference. P values are 0.76, 0.74 and 0.99 in P0, P10 and adult nerves respectively. P value is determined by unpaired student t test. Numbers (n) for each condition are depicted at the top of the bars.
3.4.2 CAPs recording after FC treatment

After establishing the baseline recording, the ability of the nerves to conduct APs in the presence of 0.125mM FC was then investigated. As discussed previously, in all the experiments involving FC, glucose was omitted from aCSF and replaced with lactate. FC treatment for 5 hours reduced CAPs in all ages to nearly 30-40% of their initial values. On average, the rate of CAPs decline was highest in adult nerves followed by P10 and then P0 nerves (Figure 3-5B). At the end of 5 hours recording, the percentage of CAPs that remained were (38.8 ± 5.2) in P0, (31.0 ± 4.5) in P10 and (27.3 ± 2.1) in adults.

To investigate whether the decline in CAPs were due to blockade of APs or permanent damage to the axons, the nerves were re-perfused with control aCSF containing 10mM glucose for 1 hour. Non-recoverable CAPs from RONs are attributed to permanent axon function failure (Alix et al., 2012, Alix and Fern, 2009, Fern et al., 1998). Although a small improvement was initially detected upon switching aCSF containing FC to control aCSF containing glucose, this improvement soon disappeared. At the end of reperfusion, the percentages of CAPs remained were (24.6 ± 2.07) in P0 (30.9 ± 5.6) in P10 and (22.4 ± 3.3) in adults (Figure 3-5C). In addition, statistically significant difference was found in comparison between the CAPs at the end of FC treatment and end of reperfusion in P0 (P=0.027), but the results were not significant in other ages (P10: P=0.982 and adults: P=0.241; unpaired Student t test in all the ages).
Figure 3-5: FC treatment for 300 minutes induced severe CAP decline in all age groups

A: Examples of CAP recording from the beginning (0 min), the end of 300 minutes of FC treatment (300 min) and at the end of 60 minutes of reperfusion (360 min). Scale bars are 10mV by 2 msec in P0 and P10, 20 mV by 0.5 msec in adult.

B: Treatment with FC for 300 minutes produced a continuous decline in CAPs in all age groups. At the end of experiments, CAPs reduced to 38.8±5.23, 31.07±4.51 and 27.36±2.15 in P0, P10 and adults respectively. After 300 minutes the preparation was switched to 10mM glucose containing aCSF. Although a small initial increase was noticed (indicated by arrow), CAPs decreased again soon
afterwards. At the end of 60 minutes reperfusion, CAPs were 24.61±2.07, 30.91±5.69 and 22.44±3.3 in P0, P10 and adults respectively.

C: A graphical representation of the CAPs at the end of 300 minutes of FC treatment followed by 60 minutes of reperfusion. Note that CAPs have decreased to 20-30 % of their initial values in all age groups. No significant difference was detected in comparison between the end of FC treatment and the end of reperfusion (unpaired Student t test).

Nerves were then exposed to only 100 minutes of FC treatment followed by 200 minutes of reperfusion in glucose containing aCSF to ascertain whether CAPs would recover after shorter periods of FC treatment. As can be noted in Figure 3-6, 100 minutes of FC treatment produced a continuous decline in CAPs in all ages, but CAPs were stabilized during reperfusion in both P10 and adult nerves. Although an initial small improvement was noticed in CAPs in both P10 and adult nerves with commencing of reperfusion, it did not reach statistically significant level (the comparison was made between the end of FC treatment and end of reperfusion; P10: P=0.53 and adults: P=0.75; unpaired Student t test in both ages). Unexpectedly, P0 nerves showed a unique feature in which CAPs continued to decline even during reperfusion with aCSF containing glucose and CAPs were significantly lower at the end of reperfusion when compared to the end of FC treatment (P=0.0014; unpaired Student t test). At the end of 100 minutes of FC treatment, the percentage of CAPs which remained were (88.8 ± 8.9) in P0, (79.8 ± 7.4) in P10 and (57.1 ± 8.48) in adults. At the end of reperfusion, the percentage of CAPs which remained were (35.1 ± 8.3) in P0, (72.3 ± 8.7) in P10 and (60.4 ± 5.4) in adults. As can be noted, the percentage of CAPs remained at the end of reperfusion (35.1 ± 8.3) in P0 nerves was comparable to the CAPS at the end of FC treatment for 300 minutes (38.8 ± 5.2). The percentage of CAPs at different time points after FC treatment is shown in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>300 min FC</th>
<th>300 min FC+100 min reperfusion</th>
<th>100 min FC</th>
<th>100 min FC+200 min reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>38.8 ± 5.2</td>
<td>24.6 ± 2.07</td>
<td>88.8 ± 8.9</td>
<td>35.1 ± 8.3</td>
</tr>
<tr>
<td>P10</td>
<td>31.0 ± 4.5</td>
<td>30.9 ± 5.6</td>
<td>79.8 ± 7.4</td>
<td>72.3 ± 8.7</td>
</tr>
<tr>
<td>Adult</td>
<td>27.3 ± 2.1</td>
<td>22.4 ± 3.3</td>
<td>57.1 ± 8.48</td>
<td>60.4 ± 5.4</td>
</tr>
</tbody>
</table>

**Table 1**: the percentage of CAPs at different time points after FC treatment
Figure 3-6: FC treatment for 100 minutes revealed a unique form of injury in P0 RONs

A: Examples of CAP recording from the beginning (0 min), the end of 100 minutes of FC treatment (100 min) and at the end of 200 minutes of reperfusion (300 min). Scale bars are 10mV by 2 msec in P0 and P10, 20 mV by 0.5 msec in adult.

B: Treatment with FC for 100 minutes decreased CAPs area in adults to (61.94±6.08) and CAPs amplitude in P10 to (72.36±8.76). After the preparation was switched to 10mM glucose containing aCSF, a small recovery (arrow) was followed by stabilized CAPs in both ages. However, 100 minutes...
exposure to FC uniquely resulted in a continued decline in CAP amplitude in P0 RONs even after reperfusion with 10mM glucose containing aCSF.

C: A graphical expression of the CAPs at the end of 100 minutes of FC treatment followed by 200 minutes of reperfusion. Note that no changes were detected in the percentage of CAPs remained at the end of reperfusion in P10 and adults nerves when compared to the end of FC treatment; however, CAPs in P0 nerves declined significantly, ** P ≤ 0.01 significant difference from the beginning of recording (unpaired Student t test in all the ages).

In order to compare the effects of CAPs across all four treatment groups, data were grouped by condition, and compared using one-way ANOVA. The four experimental conditions were labelled as follows:

1. Glucose: nerves were stimulated for 300 minutes in aCSF containing 10 mM glucose.
2. Lactate: nerves were stimulated for 300 minutes in aCSF containing 10 mM lactate.
3. FC, 300 minutes: nerves were stimulated for 300 minutes in aCSF containing 10 mM lactate plus 0.125 mM FC, followed by reperfusion in aCSF containing 10mM glucose for 60 minutes.
4. FC, 100 minutes: nerves were stimulated for 100 minutes in aCSF containing 10 mM lactate plus 0.125 mM FC, followed by reperfusion in aCSF containing 10mM glucose for 200 minutes.

As discussed earlier, CAPs were stable in both glucose and lactate and they produced similar trends. Expectedly, 300 minutes of FC treatment in P0 nerves produced a continuous decline in CAPs; surprisingly however, 100 minutes of FC treatment produced similar results and their trends were also similar (Figure 3-7A). At the end of 300 minutes, there was no statistical difference in CAPs between the nerves which had received 300 minutes of FC and those that had received 100 minutes of FC, but CAPs in both conditions were significantly lower than in nerves which had been perfused with either glucose or lactate, without FC (Figure 6B)
Figure 3-7: CAP recording in P0 RONs for 300 minutes under different experimental conditions

A: CAP amplitudes recorded in control condition in either 10mM glucose (blue) or 10mM lactate (red) were stable during 5 hours of recording and their trends were similar. FC treatment for 5 hours (green) produced a continuous decline in CAPs amplitude which did not recover after reperfusion. After 100 minutes of FC treatment (purple), CAPs continued to decline even after reperfusion and produced similar trends as 300 minutes of FC treatment.

B: Comparison between different experimental conditions at the end of 300 minutes of recording. There was a significant main effect of treatment; $F (3, 24) =17.57, P < 0.0001$. Note that there was no statistically significant difference between glucose vs. lactate and 300 minutes vs. 100 minutes of FC treatment. However, in FC treated nerves for either 300 minutes or 100 minutes, the CAPs were significantly lower than CAPs in glucose or lactate. Significance was determined by one-way ANOVA followed by Tukey’s post-hoc test. *** $P \leq 0.001$ significant difference from lactate, ### $P \leq 0.001$ significant difference from glucose.
Figure 3-8: CAP recording in P10 RONs for 300 minutes under different experimental conditions.

A: CAP amplitudes recorded in control condition in either 10mM glucose (blue) or 10mM lactate (red) were stable during 5 hours of recording and their trends were similar. FC treatment for 5 hours (green) produced a continuous decline in CAPs amplitude which did not recover after reperfusion. CAPs continued to decline during FC treatment for 100 minutes (purple); however, CAPs were stabilized during reperfusion.

B: Comparison between different experimental conditions at the end of 300 minutes of recording. There was a significant main effect of treatment; $F(3, 22) = 27.19, P < 0.0001$. Note that there was no statistically significant difference between glucose vs. lactate, but CAPs were significantly lower in 300 minutes compared to 100 minutes of FC treatment. In nerves treated with FC for 300 minutes, the CAPs were significantly lower than CAPs in glucose or lactate groups, but in nerves receiving 100 minutes of FC treatment, CAPs were significantly lower than only lactate. Significance was determined by one-way ANOVA followed by Tukey’s post-hoc test. *** $P \leq 0.001$ significant difference from lactate; # $P \leq 0.05$, ### $P \leq 0.001$ significant difference from glucose; $$$ P \leq 0.001$ significant difference between 300 and 100 minutes of FC treatment.
Figure 3-9: CAP recording in adult RONs for 300 minutes under different experimental conditions

A: CAP amplitudes recorded in control condition in either 10mM glucose (blue) or 10mM lactate (red) were stable during 5 hours of recording and their trends were similar. FC treatment for 5 hours (green) produced a continuous decline in CAPs amplitude which did not recover after reperfusion. CAPs continued to decline during FC treatment for 100 minutes (purple); however, CAPs were stabilized during reperfusion.

B: Comparison between different experimental conditions at the end of 300 minutes of recording. There was a significant main effect of treatment; F (3, 21) =55.08, P < 0.0001. Note that there was no statistically significant difference between glucose vs. lactate, but CAPs were significantly lower in 300 minutes compared to 100 minutes of FC treatment. In nerves treated with FC for either 300 minutes or 100 minutes, the CAPs were significantly lower than CAPs in glucose or lactate groups. Significance was determined by one-way ANOVA followed by Tukey’s post-hoc test. *** P ≤ 0.001 significant difference from lactate; ### P ≤ 0.001 significant difference from glucose; $$ P ≤ 0.001$$ significant difference between 300 and 100 minutes of FC treatment.
All the 4 conditions were then compared after just 100 minutes of recording to investigate the sensitivity of the nerves to FC treatment. In all the conditions, the values were taken after 100 minutes of recording; however, the recordings were continued as described earlier i.e. to 300 minutes in glucose, lactate and (FC, 100mins) and to 360 minutes in (FC, 300mins). It is noteworthy to mention that the nerves which are labelled (FC, 300mins) were technically similar to those labelled (FC, 100mins) at this stage, because both of them are exposed to the same concentration of FC for the same duration of time (100 minutes): it is only after 100 minutes that the two treatments differ. Therefore, both groups would be expected to have similar values and it could be argued that the two groups could be combined for this comparison. However, values from both groups have been included separately as an additional validation of consistency across the experiment. Similar to 300 minutes of recording, all the conditions were compared to each other in each age using one-way ANOVA followed by Tukey’s post-hoc test. As can be expected, there was no statistical difference in comparison between glucose vs. lactate or between 300 vs. 100 minutes of FC treatment in any of the ages. P0 nerves were the most resistant to injury, and when the 4 conditions were compared to each other, there was no significant main effect of treatment between the groups, F (3, 24) = 1.047, P = 0.389 (Figure 3-10B). P10 nerves showed a moderate degree of resistance to injury i.e. more than P0 but less than adult nerves. Comparing the groups, there was a significant main effect of treatment F (3, 22) = 8.104, P = 0.0008. In addition, FC treatment for 100 minutes decreased the CAPs significantly compared to either glucose or lactate (Figure 3-11).
**Figure 3-10: CAP recording in P0 RONs for 100 minutes under different experimental conditions**

**A:** Examples of CAP recording from the beginning (0 min) and the end of 100 minutes (100 min) of experiments. Scale bars are 10mV by 2 msec.

**B:** P0 nerves showed a high degree of resistance to FC induced injury. Although 100 minutes of FC treatment decreased CAPs amplitude, it did not reach statistically significant level; one-way ANOVA, $F(3, 24) =1.031, P=0.3966$. 

---

![Graph showing normalized CAPs for Glucose, Lactate, FC, 300 mins, and FC, 100 mins with n values for each condition.]
**Figure 3-11: CAP recording in P10 RONs for 100 minutes under different experimental conditions**

A: Examples of CAP recording from the beginning (0 min) and the end of 100 minutes (100 min) of experiments. Scale bars are 10mV by 2 msec.

B: P10 nerves showed a moderate degree of resistance to FC induced injury. FC treatment for 100 minutes decreased CAPs amplitude compared to both glucose and lactate using one-way ANOVA followed by Tukey’s post-hoc test, $F(3, 22) = 8.104$, $P=0.0008$. * $P \leq 0.05$ significant difference from lactate, # $P \leq 0.05$, ## $P \leq 0.01$, significant difference from glucose.
By comparison to other ages, adult nerves were the most sensitive to injury. Comparing the groups, there was a significant main effect of treatment, $F (3, 21) = 15.99, P < 0.0001$. In addition, FC treatment for 100 minutes decreased the CAPs significantly compared to either glucose or lactate (Figure 3-12).

**Figure 3-12: CAP recording in adult RONs for 100 minutes under different experimental conditions**

A: Examples of CAP recording from the beginning (0 min) and the end of 100 minutes (100 min) of experiments. Scale bars are 20mV by 0.5 msec.

B: Adult nerves showed the lowest degree of resistance to FC induced injury compared to other ages. FC treatment for 100 minutes decreased CAPs area compared to both glucose and lactate using one-way ANOVA followed by Tukey's post-hoc test, $F (3, 21) = 15.99, P < 0.0001$. ** $P \leq 0.01$, *** $P \leq 0.001$, significant difference from lactate; ### $P \leq 0.001$, significant difference from glucose.
3.4.3 CAPs recording with glutamate receptor blockers

To investigate the roles of glutamate after astrocyte metabolism inhibition by FC, two kinds of glutamate receptor blockers (GluRBs) were used; MK-801 (Dizocilpine) which blocks NMDA receptors and NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione) which blocks AMPA/kainate receptors. The effects of each receptor blocker were studied separately and together (which has been labelled as (All) in the figures) in all the ages. In all the conditions, GluRBs were added to the aCSF simultaneously with FC, and CAPs were recorded for 100 minutes followed by 200 minutes of reperfusion. To determine the statistically significant differences, data from the nerves which were treated with GluRBs were compared to data from nerves which were treated with FC alone for 100 minutes without GluRBs. The values at the end of 100 and 300 minutes of recording were taken and compared to each other using one-way ANOVA followed by Tukey’s post-hoc test.

In P0 nerves, FC treatment for 100 minutes showed a unique feature of injury in which CAPs continued to decline even after reperfusion. Likewise, after treatment with GluRBs, CAPs continued to decline steadily, albeit at a lower rate than FC treatment alone (Figure 3-13B). At the end of 100 minutes of recording, FC treatment alone decreased CAPs to (88.8±8.9); GluRBs improved CAPs compared to FC treatment alone, CAPs were (94.3±4.45) in all blockers, (92.4±6.5) in MK-801 and (95.0±2.8) in NBQX. However, this improvement did not reach statistically significant level; F (3, 19) =0.19, P= 0.90, (Figure 3-13C). At the end of 100 minutes of recording, FC treatment alone decreased CAPs to (88.8±8.9); although there was some indication that GluRBs improved CAPs compared to FC treatment alone (CAPs were 94.3±4.45 in all blockers, 92.4±6.5 in MK-801 and 95.0±2.8 in NBQX), the changes were not statistically significant; F (3, 19) =0.19, P= 0.90, (Figure 3-13C). At the end of reperfusion, the CAPs were (35.1±8.3) in FC treatment alone, but CAPs were (55.7±5.9) in all blockers, (74.5±9.3) in MK-801 and (64.2±5.4) in NBQX. There was a significant main effect of treatment between the groups after reperfusion; F (3, 19) = 4.957, P= 0.0104. Although some changes were evident in all three GluRB treatment groups, it reached statistically significant level only in MK-801 treatment (Figure 3-13D).
Figure 3-13: effects of glutamate receptor blockers on P0 RONs treated with FC.

A: Examples of CAP recordings from the beginning (0 min), the end of 100 minutes of FC treatment (100 min) and at the end of reperfusion (300 min). All is MK-801 and NBXQ together. Scale bars are 10mV by 2 msec. n=6 in every group except NBXQ in which n=5.

B: FC treatment for 100 minutes produced a uniquely continuous decline in CAPs amplitude that continued even after 200 minutes of reperfusion. Although GluRBs decreased the rate of CAPs decline, their final trends were similar to FC treatment in which CAPs continued to decrease even after reperfusion.
C: At the end of 100 minutes exposure to FC, GluRBs alone or in combination did not produce any significant effect; one-way ANOVA; F (3, 19) =0.19, P=0.090.

D: At the end of reperfusion, NMDA receptors blockade resulted in significantly higher CAPs amplitude compared to FC alone; one-way ANOVA followed by Tukey’s post-hoc test; F (3, 24) =4.957, P=0.0104. ** P ≤ 0.01 significant difference from FC.

In P10 nerves, FC treatment for 100 minutes decreased CAPs continuously; however, CAPs were stabilized after reperfusion and did not decrease further. In general, treatment with GluRBs produced similar trends in which CAPs continued to decline for 100 minutes and then stabilized (Figure 3-14B). At the end of 100 minutes of recording, FC treatment alone decreased CAPs to (79.8±7.4); GluRBs separately improved CAPs compared to FC treatment alone, CAPs were 84.6±3.7 in MK-801 and 83.8±6.9 in NBQX; however, the CAPs were slightly lower when both antagonists were given together (75.9±3.5) than FC alone. However, this changes did not reach statistically significant level; F (3, 19) =0.5178, P= 0.675, (Figure 3-14C). At the end of reperfusion, the CAPs were (72.3±8.7) in FC treatment alone, but CAPs were 74.8±5.8 in all blockers, 86.3±4.0 in MK-801 and 91.9±5.2 in NBQX. In addition, although CAPs were noticeably improved after GluRBs treatments, especially when MK-801 and NBQX were used alone, it did not reach statistically significant level in any case; F (3, 19) = 2.097, P= 0.1344 (Figure 3-14D).
Figure 3-14: effects of glutamate receptor blockers on P10 RONs treated with FC.

A: Examples of CAP recording from the beginning (0 min), the end of 100 minutes of FC treatment (100 min) and at the end of reperfusion (300 min). All is MK-801 and NBXQ together. Scale bars are 10mV by 2 msec. n=6 in every group except NBQX in which n=5.

B: FC treatment for 100 minutes in P10 nerves produced a continuous decline in CAPs amplitude which was stabilized after switching to normal aCSF and remained largely stable for 200 minutes of reperfusion. Treatment with GluRBs produced the same general pattern in which CAPs declined for 100 minutes and then stabilized afterwards during reperfusion.
C: At the end of 100 minutes exposure to FC, glutamate receptor antagonists alone or in combination did not produce any significant effect; one-way ANOVA; F (3, 19) =0.5178, P=0.675.

D: At the end of reperfusion, CAPs were higher after GluRBs, alone or in combination; however, there was not any statistically significant level of change in CAPs; one-way ANOVA F (3, 19) =2.097, P=0.1344.

In adult nerves, FC treatment for 100 minutes decreased CAPs continuously similar to what is observed in P10 nerves; and likewise, CAPs were stabilized after reperfusion. Treatment with all GluRBs produced similar pattern but lowered the rate at which CAPs were declining; however, when MK-801 and NBQX were used alone, a different pattern was observed. CAPs declined rather sharply during the 100 minutes of treatment particularly in NBQX, but during reperfusion, the CAPs recovered to more than FC treatment alone (Figure 3-15B). At the end of 100 minutes of recording, FC treatment alone decreased CAPs to (57.1±8.4); GluRBs improved CAPs compared to FC treatment alone except in NBQX treatment in which CAPs were less that FC alone, CAPs were (88.6±6.3) in all blockers, (76.3±6.6) in MK-801 and (41.8±9.7) in NBQX. However, the observed improvement in CAPs reached statistically significant level only after treatment with all blockers; F (3, 19) =6.859, P =0.0025 (Figure 3-15C). At the end of reperfusion, the CAPs were (60.4±5.4) in FC treatment alone, but CAPs were (72.8±8.7) in all blockers, (78.3±4.6) in MK-801 and (71.0±5.0) in NBQX. In addition, although CAPs were noticeably improved after GluRBs treatments, the changes did not reach statistically significant level in any case; F (3, 19) = 1.472, P= 0.2539 (Figure 3-15D).
Figure 3-15: effects of glutamate receptor blockers on adult RONs treated with FC.

A: Examples of CAP recording from the beginning (0 min), the end of 100 minutes of FC treatment (100 min) and at the end of reperfusion (300 min). All is MK-801 and NBXQ together. Scale bars are 20mV by 0.5 msec. n=6 in every group except NBQX in which n=5.

B: FC treatment for 100 minutes in adult nerves produced a continuous decline in CAPs area which was stabilized after switching to normal aCSF and remained largely stable for 200 minutes of reperfusion. However, treatment with GluRBs produced different results. The declines in CAPs were more dramatic in MK-801 or NBQX treatment alone when compared to all blockers.

C: At the end of 100 minutes exposure to FC, CAPs were significantly higher in all blockers compared to FC, one-way ANOVA followed
by Tukey’s post-hoc test; F (3, 19)= 6.859, P= 0.0025. In addition, CAPs in all blockers and MK-801 were significantly higher than CAPs in NBQX. * p < 0.05 significant increase from FC alone

D: At the end of reperfusion, CAPs were higher after GluRBs, alone or in combination; however, there was not any statistically significant level of change in CAPs; one-way ANOVA; F (3, 19) =1.472, P=0.2539.

Finally, the rate at which each of the three peaks of adult APs was declining was measured. To do so, a horizontal line was drawn along the base of the AP which served as the baseline; then the highest point in each of the peaks was determined and the distance between the peak and the baseline was measured as peak amplitude. The results are shown in Figure 3-16. As can be seen, the effects of FC treatment were most prominent on the second peak.
Figure 3-16: change in the peaks of adult APs

In all the FC treated cases, 2nd peak was decreased the most compared to other peaks. Also note that in glucose there was some increase in all the peaks, while in lactate some decrease, especially in the second peak and sometimes the 1st peak. The legend and Y axis label are true for all the figures. N= 6 in all cases except glucose and NBQX in which n=7 and 5 respectively.
3.5 Discussion

RONs have been used extensively in the past to study WM physiology and pathology. In this study, it was shown that RONs can be used successfully to record CAPs over several hours in control condition. In addition, CAP recordings were stable for several hours when lactate was used as a source of energy instead of glucose. This provides further supporting evidence that lactate is an essential energy nutrient inside the brain. The results of electrophysiological recording also show that even though immunohistochemically the axons appeared normal, these axons were not fully functional, which supports the hypothesis that astrocyte damage can have serious consequences on the functionality of the axons. In addition, it also raised the possibility that if an axon is stained normally immunohistochemically, it does not necessarily indicate that it is still healthy. Furthermore, it might be that the axons might take a long time to loss their NF-immunoreactivity and hence, the axons stained normally but were unable to conduct CAPs.

The severity of injury and the degree of CAPs decline were different in different ages with adult nerves being the most sensitive to injury and suffered the highest decline in their CAPs percentage. This age dependant injury pattern is similar to the injury pattern that happened in the astrocytes of different ages as determined immunohistologically (previous chapter). These results support the hypothesis that the degree of axonal injury corresponds to the degree of injury of the neighbouring astrocytes. Finally, the role of glutamate was examined by using glutamate receptor antagonist and the results showed that when astrocytes are injured glutamate is not the only element in determining axonal damage. Although glutamate is the main excitatory neurotransmitter, nevertheless astrocytes injury can activate other pathways that may lead to axonal damage.
3.5.1 RONs as a model to study WM injury

RONs have been used with considerable success for a long time in studying WM physiology and pathology (see section: 1.21 Rat Optic Nerve, page 48). In addition, ultrastructural details of different cellular elements of RONs have been investigated and established long times ago (Vaughn, 1969). Compared to tissue culture where cells are separated from their true environment, using RONs represent a model which is more comparable to true in vivo preparation. Nonetheless, RONs are different from in vivo model in two distinctive ways. Firstly, the axons of RONs are separated from their parental neuronal perikarya (retinal ganglion cells) and their target cells as well. Secondly, as the blood supply is ablated, nutrients and any chemical agents that are used during studying of RONs have to arrive to different cellular elements by diffusion from the perfusing medium. Even with those differences, apart from mild reactive changes in astrocytes, especially in the form of darkening of cytoplasm, no other structural changes were detected when freshly isolated RONs compared to nerves 60 minutes after dissection (Wilke et al., 2004). Furthermore, electrophysiological recording of APs showed a constant AP conduction in all the ages that are tested in the current study in the presence of 10mM glucose (Figure 3-2) for 5 hours; thus, further supporting the use of RONs as a stable model which can be used for several hours without alteration in structure or function in controlled conditions.
3.5.2 Exogenous lactate supports the functional integrity of the axons

It has been hypothesized that astrocytic glycogen is utilized during periods of increased activity (section: 1.18 Glycogen in brain, page 43) and lactate would be released which can be taken up by neighbouring axons and neurons and be used as a source of energy (section: 1.19 Role of lactate in brain metabolism, page 44). In this study, evidence is provided that in fact exogenous lactate can be as efficient as glucose in maintaining CAPs in all age groups (Figure 3-4). Inhibition of astrocytic glycogenolysis is reported to accelerate CAPs failure in mouse optic nerves (Brown et al., 2005), while even 2 mM glucose aCSF had no effect on mouse optic nerves CAPs when astrocytic glycogen store was intact (Chambers et al., 2014). The current results are in agreement with those results, since RONs in the current study are expected to have a normal astrocytic glycogen store and 10 mM lactate that has been used is equivalent to 5 mM glucose. Furthermore, compared to glycolysis, glycogenolysis represents a faster and more efficient source of energy from the astrocytes. Conversion of glucose to 2 pyruvate molecules yields 2 ATP, while each glycosyl unit in a formed glycogen yields 3 ATP during its conversion to pyruvate and the reaction does not consume ATP (Yan et al., 2001). The capability of glycogenolysis to produce a faster and higher ATP rate is especially significant during periods of high energy demands. Finally, further evidences for the role of lactate in supporting axonal functions are shown in (Chapter 2, section: 2.4.3 Lactate supported axon integrity, page 91).
3.5.3 Immunohistochemically stained axons were functionally impaired

As was discussed in (section: 2.4.2 Astrocytic cell damage did not affect axon staining in RONs, page 90), axons of RONs in different ages that have been tested in the current study were stained normally immunohistochemically even when the astrocytes were clearly damaged. However, immunohistochemistry alone cannot explain if the axons were still functional. To tackle this issue, CAPs were recorded from RONs under similar experimental conditions of those nerves that were used to acquire immunohistochemical data. The results of electrophysiological study sharply contradicted the results of immunohistochemistry. After 300 minutes of recording, CAPs were severely decreased in all groups, which in turn, indicate that normally stained axons were in fact severely compromised. One significant difference between the two experimental methods that have been used is the active stimulation of the axons in case of electrophysiological recording. Stimulation induces the release of signalling molecules such as K⁺ ions and neurotransmitters such as glutamate. These signalling molecules have been shown in the past to increase and accelerate the rate of Ca²⁺ accumulation in astrocytes (Duffy and MacVicar, 1996), which in turn, can adversely affect the functionality of axons rendering them unable to conduct APs even when structurally they are still intact.

Interestingly, one study found that during FC treatment, addition of glutamate to the cultured medium restored mitochondrial potential in some cells (Voloboueva et al., 2007). Glutamate can change to α-ketoglutarate and enters Krebs’s cycle beyond the blockade point that FC produces. If similar scenario is to happen in the current study, it is more likely to have a positive effect on the nerves that were used for immunohistochemistry data. In immunohistochemistry, the nerves were left in only 0.125 mM aCSF solution for the duration of the test. In addition, there was no active stimulation of the nerves; thus, glutamate release is expected to be a gradual, continuous process. This will, in turn, provide a gradual, continuous supply of energy to the astrocytes. Astrocytes are shown to be still viable even when they are severely compromised (Voloboueva et al., 2007); thus, axons may survive for as long as the astrocytes are still viable. In contrast, nerves that were used to acquire
electrophysiological data were actively activated and the volume of aCSF solution utilized was considerably higher (0.5-1L). The higher volume dilutes glutamate significantly, which in turn, decreased the concentration of the potential nutrients to the astrocytes and subsequently shorten the astrocytes survival time. It is important to notice these explanations remain merely a hypothesis for now and currently no data can be provided to support this view in this study.

Finally, it is currently not known how long it will take for the axons to dissociate after an injury insult. McCarran and Goldberg (2007) noticed no structural changes in axons of P3 mice after ischemic insults initially but delayed axonal degeneration became visible after 9 hours. This time window is well beyond 5 hours that has been used in the current study and the delayed degeneration of the axons, similar to the previous study, cannot be excluded. Based on the current results, axons which were stained normally immunohistochemically were unable to conduct CAPs electrophysiologically after FC treatment.

3.5.4 Sensitivity of RONs to FC is age dependant

Inhibition of mitochondrial function with FC is associated with release of glutamate from the astrocytes (Fonnum et al., 1997). Furthermore, WM axons are intrinsically more sensitive to glutamate induced excitotoxicity with increasing age as was determined by measuring CAPs (Baltan et al., 2008). Similarly, Back (2006) showed that WM axons vulnerability to excitotoxicity is developmentally regulated. In agreement with the former study, the latter found axonal fragmentation and fibre loss to be more notable in older (10 and 21 days) compared to younger ages (3 and 7 days). The current results are similar to the previous studies. Adult RONs showed the highest degree of sensitivity to injury when treated for 300 minutes with FC and reached a statistically significant level after only 100 minutes of treatment. P10 RONs showed a moderate degree of sensitivity to injury by FC (i.e. lower than adults but higher than P0 RONs). FC treatment produced a significant level of CAPs decrease
after 180 minutes of treatment. P0 RONs showed the lowest degree of sensitivity and reached significant level after 240 minutes of treatment with FC.

Interestingly, recent data have shown that axonal proximity to astrocytes may predict the severity of axonal injury after an ischemic insult (Fern, 2015). Since astrocyte density within RONs reversely changes with age i.e. density decreases in older nerves and vice versa, this arrangement causes the axons in older nerves to be further away from the astrocytes, which in turn, renders them more susceptible to injury, similar to have been observed in the current study. In addition, earlier studies Cremer (1981) indicated that brains at different ages have different capabilities to metabolize different substrates. For example, lactate and ketone bodies are transported and metabolized more avidly by neonatal brains compared to adults. Since in the current study lactate has been used as a source of energy, this might have given an advantage to neonatal RONS and render them more resistant to injury. In the current study, however, no attempt was made to quantify the amount of lactate that was utilized by RONs at different ages. Additionally, some neurons also express glycogen in neonates (Ivanov et al., 2011) which after glycogenolysis may supply the axons with necessary nutrients during energy crisis and delays their demise.

Decay in CAPs can occur primarily as a result of structural injury to the axons or secondarily to uncontrolled elevation of extracellular K⁺ ions. K⁺ regulation is one of the major functions of astrocytes in normal physiological conditions (section: 1.8 Potassium regulations by astrocytes, page 14) and it is done primarily by Kir4.1 subtype. In Kir4.1 knock-out mice, extracellular concentration of K⁺ significantly rises, astrocytes display marked reduction of inward currents with subsequent depolarization, culminating in CAPs decline, which indicates reduced axonal activity (Bay and Butt, 2012). Since metabolically inactivated astrocytes are also unable to regulate K⁺ and elevated level of K⁺ has been observed with FC treatment (Largo et al., 1996), it is likely that the elevated K⁺ concentration can result in decreasing CAPs. However, non-recoverable CAPs decline has been attributed to permanent axonal damage (Alix et al., 2012, Alix and Fern, 2009, Fern et al., 1998). To investigate whether the CAPs decline were due to blockade of AP conduction or permanent axonal injury; RONs were re-perfused with normal glucose aCSF solution for 60
minutes. Re-perfusion produced only a very small recovery of CAPs (Figure 3-5), and therefore, it is likely that the CAPs decline that have been observed here is the result of permanent axonal injury. The possibility of temporary AP blockade as a result of high extracellular level of $K^+$ superimposed by permanent AP blockade as a result of loss of structural integrity of the axons cannot be excluded.

Because 100 minutes treatment with FC produced significant level of damage in adults, this time point was used to see if any recovery happens if the toxin was washed out after 100 minutes. In both P10 and adult nerves, an initial non-significant recovery was followed by stabilized CAPs (Figure 3-6). This was in direct contrast to P0 nerves, in which, unexpectedly, 100 minutes of FC treatment produced a unique form of injury after washout. In P0 nerves, CAPs amplitude continued to decrease to almost the same value as 300 minutes of treatment (Figure 3-7).

Astrocytes respond to injury fundamentally differently in neonates compared to P10 and adults. For instance, acute ischemic injury in neonatal WM astrocytes is associated with a significant $Ca^{2+}$ influx through both L and T type voltage gated $Ca^{2+}$ channels (Fern, 1998). In contrast, ischemic injury to P10 astrocytes is not mediated by $Ca^{2+}$ influx, but through the activation of Na$^+$-K$^+$-Cl$^-$ co-transporter (Thomas et al., 2004), which its expression is not even detected in P0 RON astrocytes. Furthermore, $Ca^{2+}$ release from intracellular store or $Ca^{2+}$ influx through glutamate gated channels did not contribute to rise of intracellular $Ca^{2+}$ during ischemia, which is a unique mechanism of injury in WM astrocytes of neonates (Fern, 1998). In addition, neonatal WM astrocytes tend to die during early stages of ischemia (Thomas et al., 2004) which is also in contrast to P10 and adult nerves in which cell death progresses gradually with time. This raises an interesting possibility in which, in the current study, the astrocytes in P0 nerves might be already dead or committed to die after 100 minutes of FC treatment. In fact, this feature of cell death has already been observed previously and has been termed delayed cell death (Fern, 1998). In addition, neonatal astrocytes can be very sensitive to injury and they may die even before their neighbouring axons after an ischemic insult (Fern, 2001). As astrocytes are fundamental for normal axonal conduction and death of astrocytes releases injurious substances to the axons (Liberto et al., 2004), then it is expected that the death of
astrocytes results in gradual reduction in axonal conduction. Furthermore, neonatal axons lack myelin sheath, which can act as a natural barrier to protect the axons from injurious substance that will be released as a result of astrocytic cell death.

When lactate was used in control condition to support axonal function, quantitative analysis revealed a slight decrease in the amplitude of the first two peaks of CAPs in adult nerves, however, the CAPs area remained stable (Figure 3-4). The decrease in amplitude of the peaks was compensated for by broadening of the CAPs. Therefore, although adult nerves were able to still function normally in the presence of lactate, they may not be able to conduct AP at the same speed as glucose. In addition, after FC treatment, a small initial increase in CAPs was noticed (Figure 3-5). This increase can be caused by increased glutamate concentration as a result of decreased glutamate uptake by astrocyte after FC treatment or increased glutamate release from the injured cells (Paulsen et al., 1987). Likewise, the blockers of glutamate transporters increased the amplitude of excitatory postsynaptic potentials initially (Tong and Jahr, 1994). Alternatively, the initial increase in CAPs is attributed to elevated level of K⁺ ions (Brown et al., 2003). Although neurons can regulate K⁺ ions to some degree (Largo et al., 1996), this capability is limited and fine tuning of K⁺ ions needs astrocytes contribution (section: 1.8 Potassium regulations by astrocytes, page 14). In the presence of intact astrocytes, injection of 100mM KCL caused only a minor increase in extracellular K⁺ concentration, while 30mM KCL produced robust increase in extracellular K⁺ concentration when astrocyte functions were restricted with FC (Largo et al., 1996). Interestingly, application of K⁺ current blocking agent, 4-aminopyridine, affected the second peak of adult RONs more than the first peak, which indicates a more prominent role of K⁺ ions on the slower components of CAPs (Foster et al., 1982). In agreement with the previous study, the current results imply that the slower components of adult RONs were more affected by FC treatment than the faster components. Similarly, during periods of high activity in mouse optic nerves, the amplitude of the slower components were more affected than the faster ones (Brown et al., 2003).
3.5.5 Glutamate receptor blockers partially recover CAPs

Glutamate is the major excitatory neurotransmitter in the brain (section: 1.15 Glutamate, page 37). Glutamate receptors are divided into ionotropic and metabotropic receptors. Ionotropic receptors are further subdivided into NMDA and non-NMDA receptors based on the agonists that activate them. Generally speaking, NMDA receptors are highly \( \text{Ca}^{2+} \) permeable, while non-NMDA receptors are mostly \( \text{Ca}^{2+} \) impermeable, but highly permeable to other monovalent ions such as \( \text{Na}^+ \) and \( \text{K}^+ \) (Agrawal and Fehlings, 1997). Glutamate exerts its functions through glutamate receptors which are abundantly expressed on almost all the cellular components of CNS; for example, NMDA receptors have been identified on cortical astrocytes (Lalo et al., 2006), AMPA and metabotropic glutamate receptors on grey matter astrocytes (Verkhratsky and Steinhauser, 2000) and white matter astrocytes (Hamilton et al., 2008), AMPA receptors on oligodendrocytes cell bodies (Li and Stys, 2000), NMDA receptors on oligodendrocytes processes (Salter and Fern, 2005), AMPA receptors on myelin (Li and Stys, 2000), NMDA receptors on axons (Manning et al., 2008) and on compact myelin (Alix and Fern, 2009). In addition, cellular injury during insults to CNS is inevitably associated with glutamate release from the injured cells, which in turn, can activate glutamate receptors on neighbouring cells and potentiate the injury cascade, producing a vicious cycle. To investigate the role of glutamate in the current study, two different kinds of glutamate receptor blockers were used; MK-801 which blocks NMDA receptors and NBQX which blocks AMPA/kainate receptors; either separately or in combination (section: 3.4.3 CAPs recording with glutamate receptor blockers, page 116).

Generally, in the current study, glutamate receptor blockers did not produce significant protection against FC-induced CAPs decline except at the end of 300 minutes recording in P0 nerves when MK-801 was used alone (Figure 3-13) and at the end of 100 minutes recording in adult nerves when all blockers (MK-801 and NBQX) was used. In all other cases, even though glutamate receptor blockers produce partial protection, it did not reach statistically significant level (Figure 3-13, Figure 3-14 and Figure 3-15). Failure of glutamate receptor blockers to completely protect the CAPs
decline is not surprising considering that there are other mechanisms by which astrocytic injury can diminish CAPs. ATP, which is released from the injured astrocytes and the axons as a result of normal AP conduction (Hamilton et al., 2008), can act as a potent endogenous toxin. Glial cells are known to express a heterogeneous repertoire of ATP receptors, including metabotropic (P2Y) and ionotropic (P2X) receptors. Thus, ATP stimulation of P2X and P2Y receptors can mediate glial cell injury with subsequent reduction in axonal conduction. In fact, Domercq et al. (2010) have shown that ischemia induced mitochondrial depolarisations are partially reversed by P2X7 receptor antagonist and by apyrase, which is an ATP degrading enzyme. Furthermore, they have shown an improvement in structural and electrophysiological recording of the axons. These results further support the view that glutamate induced injury is not the only injury pathway which is operational in brain during insults. Furthermore, microglia, which are the resident macrophages of the brain (section: 1.13 Microglia, page 25), are endowed with P2X and P2Y receptors. Thus, ATP can also activate microglia (Ransohoff and Perry, 2009) and the activated microglia can generate free radicals, secrete injurious substances and enhance excitotoxicity (Volpe, 2009b).

Interestingly, one study found that increased intracellular Ca\textsuperscript{2+} concentration associated with electrical stimulation of optic nerve to be decreased by suramin, a general antagonist of P2X and P2Y receptors, but not by NBQX, a general antagonist of non-NMDA receptors, prompting the authors to suggest more prominent roles for ATP in axo-glial signalling. These results however, contradict Hamilton et al. (2008) results in which NBQX markedly reduced intracellular Ca\textsuperscript{2+} currents in astrocytes. Similar results are obtained by (Agrawal and Fehlings, 1997). Overall, it seems that the mechanisms of glutamate and ATP induced injury are complex and may interact or potentiate each other. Furthermore, the presence of high concentration of glutamate (approximately 1 mM) causes desensitization of non-NMDA type of glutamate receptors and reduction of glial cell injury (Fern and Moller, 2000). In agreement with the previous study, application of AMPA or glutamate alone did not affect axons at any developmental stage (P3, P7, P21 and adult rats) (McCarran and Goldberg, 2007). However, when desensitizing agent, cyclothiazide, was added to glutamate or AMPA, it induced severe axonal injury (McCarran and Goldberg, 2007). In the current study it
is not known how high the concentration of glutamate will be. But if a surge of glutamate release happens as a result of astrocytes and possibly other cellular components injury, then it might affect the outcome and makes the interpretation of the results tricky.
Chapter 4  Investigating the ultra-structural features of RONs after FC treatment

4.1 Introduction

Immunohistochemical staining was used to recognize the effects of FC treatment on different elements of WM. After that, electrophysiological recording was used to assess the functionality of the nerves, as was discussed in chapter 2 and chapter 3 respectively. In the final experimental method, electron microscopic analysis was performed to determine the ultrastructural changes associated with FC treatment. Two different experimental conditions were tested and compared to each other and to the control condition. In the first sets of experiments the nerves were immersed in FC for 3 hours and then fixed for electron microscopic analysis, while in the second sets of experiments the nerves were stimulated for the duration of FC treatment before being processed.

Based on the well-defined ultrastructural criteria, astrocytes, axons and oligodendrocytes were identified and the injury features were assessed qualitatively and quantitatively. After recognising the normal features of different elements of WM, attempts were made to recognise injury features in experimental conditions. To quantify injury, a numerical system was used which allowed the different injury features to be quantified. The results are shown in the current chapter.

After establishing the effects of FC on the astrocytes and testing the functionality of the axons in the previous two chapters, it was important to examine the ultrastructural features of injury in different elements of WM i.e. astrocytes, axons and oligodendrocytes. The aim of the electron microscopic analysis of the axons was to examine the ultrastructural details of the axons to establish the reasons behind the restricted AP conductance that was detected electrophysiologically. Specifically, if the AP blockade in electrophysiological recording was due to the disturbance in the extracellular distribution of ions such as $K^+$, then the ultrastructural changes in the axons or their myelin sheath would be expected to be absent or minimal and AP
would be recoverable. However, if wide spread damage to the axons or their myelin sheath was detected, then a permanent AP conduction failure is more likely.
4.2 Electron microscopy

After dissection (see section: 2.2.1 Tissue preparation, page 53), an individual optic nerve was placed in a small chamber containing 0.5 ml of 10mM L-lactate aCSF in control cases or 0.5 ml of 10mM L-lactate aCSF mixed with 0.125mM of fluorocitric acid barium salt (Sigma-Aldrich) in test cases. The chamber was transferred to a pre-heated (37°C) and pre-oxygenated (bubbled with 95% O₂/ 5% CO₂) chamber, and nerves were equilibrated for three hours before they were removed and primarily fixed by immersion in a mixture of 2% formaldehyde/4% glutaraldehyde/ 0.1M sodium cacodylate buffer/ 2mM calcium chloride pH 7.4 overnight at 4°C. The nerves were then washed in 0.1M sodium cacodylate buffer / 2mM calcium chloride pH 7.4 and stored for at least 24 hours at 4°C. Nerves were then washed three times (15 minutes each) in distilled de-ionised water before being post-fixed in 1% osmium tetroxide / 1.5% potassium ferricyanide in distilled de-ionised water for 2 hours. Osmium fixation was also followed by washing three times (20 minutes) in distilled de-ionised water. Lastly, the nerves were tertiary fixed in 2% aqueous uranyl acetate for 1 hour at 4°C before being washed twice (10 minutes) in distilled de-ionised water. Then the nerves were dehydrated by passing through a series of increased concentration ethanol solutions as follows: 30% ethanol for 15 minutes, 50% ethanol for 15 minutes and 70% ethanol for 15 minutes before being stored overnight at 4°C in 70% ethanol.

Next day, the nerves were treated with 90% ethanol for 30 minutes before three changes of 100% analytical grade ethanol for 30 minutes each. At the end of dehydration, the nerves were infiltrated with propylene oxide and resin as follows: propylene oxide twice, 10 minutes each, followed by 2 propylene oxide : 1 modified spurr’s low viscosity resin (hard formula) for 90 minutes. Then the ratio was changed to 1 propylene oxide : 1 modified spurr’s low viscosity resin for 60 minutes before increasing the resin content to 1 propylene oxide : 2 modified spurr’s low viscosity resin for another 60 minutes. Finally the samples were transferred to 100% modified spurr’s low viscosity resin for 30 min before being left in the same concentration of resin overnight. Next day, fresh modified spurr’s low viscosity resin was used twice, 2 hours each, before the samples were embedded and polymerised at 60°C for 16
hours. Embedded samples were then cut to approximately 90 nm thick sections, transversely or longitudinally, using a Leica ultracut S ultramicrotome and collected onto copper mesh grids. Reynold’s lead citrate solution was used to counterstain the sections before viewing on the JEOL JEM-1400 TEM electron microscope with an accelerating voltage of 80 kV. Images of the samples were captured using a Megaview III digital camera with iTEM software. A brief over-view of the steps is shown in Figure 4-1.

---

**Figure 4-1:** a brief over-view of the steps of preparing samples for electron microscopy
4.3 Axon viability scoring system

Axon viability score was assigned by giving one point to the presence of each of the following components:

- The presence of an intact axolemma
- The presence of intact microtubules
- The presence of clear, debris-free cytoplasm (debris was defined as any amorphous material which usually was either not seen or very rare in control nerves).

Axons that had all the three components were given the highest score of “3”; when all the three components were lost, axons were given the lowest score of “0”. One point was deducted for the loss of each component i.e. an axon that has intact axolemma and debris-free cytoplasm but lost microtubules scores “2”, as did an axon with intact axolemma and intact microtubules but unclear cytoplasm. Thus, axon viability score does not determine the functional integrity of the axons i.e. an axon with the score of “1” does not mean that the axon exhibits one third of the functionality of a normal axon; rather, it means it has lost “2” of the “3” components of the scoring system. This scoring is based on well-established protocol that has been used previously (Alix et al., 2012, Alix and Fern, 2009). An example of each score is shown in Figure 4-2.
Figure 4-2: Example of axon viability scoring system.

The example is taken from P10 RON after three hours of FC incubation (test condition). The numbers determine how each axon is scored. The axon with the score of “0” has lost the axolemmal integrity in upper part (the arrow head) with no recognizable microtubules, in addition to hazy, unclear cytoplasm with debris especially in the right side of the axon. The axon with the score of “1” has only an intact axolemma which can be traced all around the axon circumference but there is cytoplasmic debris, and no recognizable microtubules, and hence two points have been deducted. The axon with score “2” has an intact axolemma with some intact microtubules, but the cytoplasm is not clear and hence one point has been deducted. The axon with score “3” has an intact axolemma with numerous, easily recognizable microtubules and clear, debris-free cytoplasm. Scale bar is 0.5µm.
4.4 Glial viability scoring system

Glial viability score was assigned by giving one score to the presence of each of the following components:

- Intact cell membrane
- Intact mitochondria
- Normal endoplasmic reticulum (ER)
- Clear cytoplasm devoid of large or numerous vacuoles

A cell (astrocyte or oligodendrocyte) that exhibited all the features was given the maximum score of “4”; a cell with none of the features was given the score of “0”. One point was deducted for the loss of any component; for example, a cell that exhibited only swollen mitochondria but otherwise other features appeared normal scores “3” as did a cell which showed only swollen endoplasmic reticulum. Like axon viability scoring system, glial viability scoring system does not measure functional integrity of the cell and thus a cell that scores “2” does not mean that the cell functions at half that of the normal cell; rather, it means that the cell has lost “2” of the “4” components of the scoring system. Examples of glial (both astrocytes and oligodendrocytes) viability score system is shown in Figure 4-3.
Figure 4-3: examples of glial viability scoring system
Low (left panel) and high (right panel) magnification images showing glial cells at different levels of damage. In each case, the area inside the rectangle is enlarged and shown in detail on the right side of the image. Scale bars are 4 µm in lower magnifications, and 0.5 µm in larger magnifications. A: a normal oligodendrocyte which is taken from adult control condition. It shows normal features of a healthy cell which include numerous healthy mitochondria (m), typical narrow bore ER (arrows), well defined cell membrane (arrow head) and vesicle free cytoplasm. B: an astrocyte which is taken from adult test (FC-incubated) condition. The cell shows features of a healthy cell as in (A); however, the ER (arrows) is fragmented and thus the cell is scored as (3). GFAP fibers (GFAP) can also be recognized easily as fine spindles running along the direction of the writing. C: an oligodendrocyte which is taken from adult test (FC-incubated) condition. The cell shows largely swollen mitochondria (m), swollen and fragmented ER (arrows). The cell membrane (arrow head) and cytoplasm have remained intact. A nearby axon (Ax) is also largely damaged. D: an oligodendrocyte taken from adult test (FC-incubated) condition. The cell shows hugely swollen mitochondria (m), swollen and fragmented ER (arrows) and vacuoles within the cytoplasm. Nucleus also shows clear chromatin condensation under the unclear envelope. The cell, however, has retained an intact cell membrane (arrow heads). E: an injured oligodendrocyte taken from adult test (FC-incubated) condition. The cell shows the same features of (D) in addition to losing the cell membrane integrity in different regions (arrow heads).

4.5 Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using Prism 6 (Prism, Graphpad, San Diego, CA, USA). P values were determined by Student t test for comparison between two groups and one-way analysis of variance (ANOVA) for comparison between more than two groups, followed by Tukey’s multiple comparisons test. Statistically significant level was determined to occur when \( P \leq 0.05 \). P values are indicated by asterisks, * \( P \leq 0.05 \), ** \( P \leq 0.01 \), *** \( P \leq 0.001 \).
4.6 Results

4.6.1 Normal axonal appearance

At P0, all the axons were unmyelinated; they had a relatively circular outline bordered by axolemma with largely similar diameter; few prominent, distinct microtubules dispersed within the cytoplasm; mitochondria and smooth ER could easily be seen within the cytoplasm, but not rough ER. Occasionally, small vesicles were encountered within the cytoplasm. Lamellar body-like structure, which appeared as loosely packed concentric rings, could occasionally be seen. The extracellular space between axons was usually wide and appeared largely electron-lucent. An example of P0 axons is shown in Figure 4-4.

![Figure 4-4: normal features of axons in P0 RON.](image)

The sample is taken from a control nerve. It shows numerous fairly circular axons, smooth ER (arrow heads), distinct microtubules (arrows), lamellar body-like structure (*), a small vesicle (v) in one of the axons and large electron lucent extracellular space (ecs). Scale bar is 0.2 µm.
At P10, some of the axons have undergone radial expansion and greatly increased in size, this expansion was accompanied by a clear increase in the number of microtubules. The shape of some axons also has changed from its circular profile to oval or irregular. Like in P0 nerves, axoplasm of P10 axons showed intra-cytoplasmic organelles which included smooth ER, mitochondria and occasional small vesicles.

**Figure 4-5: normal features of axons in P10 RON.**

The sample is taken from a control nerve. It shows many axons of various diameters, some of the axons have irregular outline or an oval shape. Axons that have been contacted by an oligodendrocyte process were much bigger (in diameter) than those that have not been contacted by a process yet (compare the axons labelled as 1 and 2). Note also that the numbers of microtubules are much higher in larger axons. Different levels of myelination can be identified; it ranges from the presence of a single layer of an oligodendrocyte process (encircles axon 2) to multiple layers of loosely arranged processes (axon 3) to the formation of multiple compact layers of myelin sheath (axon 4). An astrocyte process (AstP) can also be seen that partially, but not completely, surrounds a group of axons (arrow) or an individual axon (arrow head). Scale bar is 0.5 µm.
The extracellular space remained large in this stage. Oligodendrocyte processes started to wrap around the axons and form different levels of myelination; myelination could range from the presence of a single layer of oligodendrocyte process around the axon to the formation of multiple compact myelin layers (Figure 4-5). Areas of myelin shear, which is believed to be fixation artefact due to poor fixation, were commonly seen (see below for detail).

By adulthood, almost all the axons were fully myelinated i.e. axons were surrounded by multiple layers of compact myelin. Unmyelinated axons were rarely seen. Axons could have a circular, oval or irregular shape, surrounded by extracellular space which was much narrower compared to other ages. In cross section, a few axons could be cut through the nodes of Ranvier, revealing a bare segment of axon which may give the impression that they are unmyelinated nerve fibers. However, an astrocyte process could usually be detected near such cuts, in which case the astrocyte process helped to differentiate those cut-through node segments from unmyelinated nerve fibers (Figure 4-6). The distribution of axoplasmic components resembled that of P10 axons, however, the density of microtubules increased greatly with many neurofilaments distributed uniformly throughout the entire axoplasm (Figure 4-6). While individual microtubules could easily be recognized in cross sections of adult axons, neurofilaments were much harder to be seen due to their smaller diameter.

Areas of myelin disturbance (myelin shear) (Figure 4-6) were very common in the preparation; these shears are considered to be fixation artefact due to poor fixation rather than to be pathological. To prepare the samples for electron microscope analysis, diffusion-fixation by immersing the nerves in the fixative was used, as described in (Electron microscopy, page 137). The problem of myelin fixation has been encountered frequently by others who have also used diffusion-fixation. Myelin sheaths of perfusion-fixation nerves are usually preserved much better compared to diffusion-fixation, but in the current preparation, this would have affected how the nerves would respond to the toxin and interpretation of the data would have been affected hugely. Nonetheless, even in perfusion-fixation, myelin fixation is never optimal (Sandell and Peters, 2001), not only in old but also in young
ages. The issue of how to fix myelin properly is an important point in studies which try to determine age related changes, because it creates difficulties to distinguish fixation artefacts from real changes in myelin due to aging process. As this kind of research is not the main theme of interest, fixation problem was not of fundamental importance to this study. Other features of myelin sheath which were occasionally encountered were myelin split, myelin balloon, redundant myelin and double myelin. Although these features were seen even in control condition, they were much more common after FC treatment, and hence they are discussed in detail later in (section: 4.6.3.2 Effects of FC on axons, page 171).

Figure 4-6: normal features of axons in adult RON.

The sample is taken from a control nerve. Virtually all the axons are fully myelinated. An unmyelinated axon (1) can be seen next to another axon (2) which has a bare segment without myelin sheath, and it is partially surrounded by a glial process (3), possibly an astrocyte process, which makes it a candidate to be a cut-through node axon, although a final confirmation usually needs a longitudinal view of the section. The axoplasm is filled uniformly with many microtubules which appear as small black dots (for example the axon labelled 4) dispersed with many microfilaments. Occasionally small vesicles can also be seen (arrow head), in addition to mitochondria and smooth ER. Areas of myelin disturbance (myelin shear) are very common (arrows) in the samples, which is believed to be fixation artefact. Scale bar is 1 µm.
4.6.2 Normal macro-glial appearance

In the early stages of life, virtually all the macro-glial cells are astrocytes. In P0 rats, oligodendrocytes have not yet appeared in the optic nerve; the first signs of oligodendrocyte appearance are reported to be 6 days postnatally (Vaughn, 1969). Even in P10 nerves, only around 20% of glial cells appear to be oligodendrocytes (Vaughn, 1969). Astrocytes show characteristic features which include an irregular, multi-lobed nucleus with chromatin dispersed evenly throughout the nucleus. No nucleolus was detected. Inside a rather dark cytoplasm, ER can easily be identified which is regularly studded with ribosomes and usually wider than that in oligodendrocytes. Also, mitochondrial numbers are usually less than in oligodendrocytes, and some glycogen particles may present among a plethora of free ribosomes which are dispersed evenly throughout the entire astrocytic cytoplasm and the main processes. GFAP filaments, which are the main intermediate filaments of astrocytes, were also seen as thread-like structure; these filaments are particularly common in the main processes. Golgi apparatus is usually seen as stacks of multiple semicircular membrane structures, most often in the paranuclear region, but it can be seen further away from the nucleus within the cytoplasm, but not inside the processes. Also inside the cytoplasm, small vesicles were frequently seen that normally had a clear electron-lucent core, these vesicles were usually less than 200 nm in diameter. Very rarely they measured more than 300 nm. Sometimes they were grouped together and formed a multi-vesicular body, but even then, the individual vesicles remained separate from each other and retained their size.

At lower magnification, astrocytes were seen giving rise to a few main processes which in turn ramified to finer processes and surrounded groups of axons; occasionally finger like projection arose from these fine processes and framed a single axon (Figure 4-5). In addition, astrocytes extended processes to form glia limitans, which is a thin barrier of astrocyte foot processes separating the brain and spinal cord from the covering meninges (Figure 4-9D). In general, astrocytes of other ages, namely P10 and adults, were very similar to P0 astrocytes and hence have not been described separately. One notable exception in adult astrocytes, however, was the presence of
chromatin condensation under the nuclear envelope. Although this feature was not present in all adult astrocytes, it was not uncommon. Conversely, chromatin condensation was not routinely encountered in P0 and P10 astrocytes. However, there were some cells which exhibited nuclear condensation under the nuclear envelope or within more central portion of the nucleus. This feature has been attributed to a group of cells called large glioblasts (Vaughn, 1969). These cells are in early stages of prophase in cell division cycle before the disappearance of the nuclear membrane.

Oligodendrocytes start to appear 6 days postnatally as ovoid cells which extend processes to wrap around the axons and form the myelin sheath. In P10 nerve, which is an age of active myelination, all stages of myelination were observed, ranging from a single layer of an oligodendrocyte process around an axon to the formation of multiple layers of compact myelin (Figure 4-5). Some of these processes could be traced back to an oligodendrocyte soma and helped in recognizing the parent cell as oligodendrocyte. In addition, oligodendrocytes can be identified by the presence of an oval or circular nucleus which mostly resides in one pole of the cell and leaves the majority of cytoplasm in the other pole. Sometimes, the nucleolus can be detected as a lump of discrete electron dense structure within the nucleus. Since astrocytes do not have nucleolus, the presence of a nucleolus can positively identify a cell as an oligodendrocyte. In adults, oligodendrocytes share similar features as P10 oligodendrocytes. However, like in astrocytes, chromatin condensation was only seen in adult oligodendrocytes. In P10, chromatin was usually dispersed uniformly throughout the nucleus. Apart from that difference in chromatin shape, P10 and adult oligodendrocytes were largely similar in their appearance and structure (Figure 4-8).

Most of the cytoplasmic organelles of astrocytes were identified in oligodendrocytes as well, albeit there were differences in their morphology. When compared to astrocytes, cytoplasm in oligodendrocytes appeared lighter; mitochondria usually more abundant; although studded with ribosomes like in astrocytes, ER were much narrower. Other organelles, like Golgi apparatus, small vesicles and free ribosomes were similar to what was described for astrocytes. However, GFAP and glycogen particles were not seen in oligodendrocytes. These
ultrastructural features were used for identification of oligodendrocytes and differentiating them from astrocytes (Figure 4-9).

**Figure 4-7: normal features of astrocytes**

A: An astrocyte from a P10 control nerve. Note the nuclear chromatin is dispersed uniformly throughout the entire nucleus. The black square is shown in detail in B, scale bar is 2µm. B: inside a rather dark cytoplasm, few mitochondria (m) are shown, which have normal crests. Many characteristic wide bore ER (arrow) can also be seen; in addition, Golgi apparatus (GA) and many free ribosomes are also dispersed throughout the cytoplasm. The cell has a clearly defined cell membrane (arrow head), scale bar is 0.5µm.
Figure 4-8: normal features of oligodendrocytes

A: An oligodendrocyte from a P10 control nerve. The chromatin is dispersed uniformly throughout the entire nucleus. The nucleus has occupied one pole of the cell, leaving most of the cytoplasm in the opposite pole with only a small rim of cytoplasm around the nucleus. The white square is shown in detail in B. Scale bar is 2 µm. B: an oligodendrocyte (OL) next to an astrocyte process (Ast); they are separated by their plasma membrane which can be seen clearly at the arrows. Oligodendrocyte shows characteristic narrow bore ER which is studded with ribosomes (compare both arrow heads). Mitochondria (m) can be seen in both cells. Scale bar is 0.5 µm. C: An oligodendrocyte from an adult control nerve. Chromatin condensation can be seen under the nuclear envelope (arrow) and within the deeper portion of the nucleus. Inside the cytoplasm, many mitochondria can be easily recognized which is a usual feature of oligodendrocytes. The white square is shown in detail in D. scale bar is 2 µm. D: many characteristic narrow bore ER (arrow head) can be seen with numerous mitochondria (m) in a light color cytoplasm. Golgi apparatus (GA) is also seen. In addition, a few small vesicles (arrows) which usually have an electron-lucent core can also be seen. Scale bar is 0.5µm.
Figure 4-9: oligodendrocytes vs. astrocytes

Astrocytes and oligodendrocytes can be identified and distinguished based on well-defined ultrastructural features. A: an astrocyte (Ast) versus an oligodendrocyte (OLi) in a P10 nerve. The astrocyte extends a process (arrows) all the way toward the edge of the nerve and forms glia limitans (arrow heads). In addition, the astrocyte shows a characteristic irregular nucleus, while oligodendrocyte has an oval nucleus. The rectangular area is shown in detail in B. scale bar is 10 µm B: astrocyte (Ast) has a darker cytoplasm, characteristic wide bore ER (lower arrows) and fewer mitochondria. In contrast, oligodendrocyte (OLi) has brighter cytoplasm, narrow bore ER (upper arrows) and numerous mitochondria. Scale bar is 1 µm. C: an oligodendrocyte (OL) in adult nerve identified by forming the myelin sheath of an axon (Ax). Scale bar is 5 µm. D: an example of astrocytes sending processes to form glia limitans in P0 nerve, a finding that can positively identify a cell as an astrocyte. Scale bar is 5 µm.
4.6.3 Effects of FC on RON

As discussed in the “Introduction”, effects of FC treatment in two different experimental conditions have been tested. In the first set of experiments, the nerves were simply immersed in the toxin for 3 hours with no electrical stimulation; this condition was labelled as the “before stimulation” condition throughout this study. In the second set of experiment, the nerves were stimulated throughout the incubation period of the nerve in the toxin. The nerves were stimulated at a rate of 20stim/min for 3 hours after the initial 10 minutes of baseline recording; thus, the nerves were stimulated at least 3800 times during the study time (190 minutes). This condition was labelled as “after stimulation” throughout this study. The purpose of the second set of experiment was to put an extra load on the astrocytes and examine how they were affected while their metabolism was halted by the toxin. In addition, the ability of axons to conduct AP was also recorded during higher frequency of stimulation.

4.6.3.1 Effects of FC on glial cells

Ultrastructural changes in both astrocytes and oligodendrocytes in three different stages of development were investigated after treatment with 0.125 mM FC for 3 hours in glucose-free aCSF that contained 10 mM L-lactate as an energy substrate. Astrocytes showed different features of injury which were common to all the ages, however, the severity of injury was different between different age groups. The commonest signs of injury were involving mitochondria and ER; changes in other cytoplasmic organelles and cytoplasm itself seemed to follow in later stages. After injury, astrocytes appeared globular with a clear decrease in the number of their processes before signs of death became apparent which included loss of cell and /or nuclear membrane with widespread loss of cytoplasmic organelles among others.
4.6.3.1.1 Astrocytes

In P0 nerves before stimulation, astrocytes were least affected by FC treatment. When looked at under low magnification, astrocytes had their typical star shaped morphology with few main processes radiating from the soma. In almost all the cells, an intact cell membrane which could be tracked all the way around the cell was preserved, which indicated that the astrocytes were still alive in this age after FC treatment. Nuclei were largely similar to the nuclei of control cells; they had their characteristic multiple, irregular lobes with chromatin dispersed uniformly throughout the nucleus. The nucleus normally retained its nuclear envelope with no observable chromatin condensation. A more detail examination of the astrocytes revealed signs of injury within both cytoplasm and cytoplasmic organelles. Signs of injury were most often found in mitochondria and ER. Mitochondria were swollen and the mitochondrial matrix seemed lighter than controls, although this was difficult to quantify. Occasionally, complete electron-lucent patches appeared in the mitochondria which did not have clearly defined boundaries. The mitochondrial crests were fragmented, dissociated or completely disappeared. In some cases, the mitochondrial membrane was broken. ER, when injured, were swollen and/or fragmented. On visual inspection, ER number appeared to be less compared to control cells although a quantitative analysis on the numbers of ER was not conducted. The cytoplasm itself was usually brighter in injured cells and it seemed to change proportionally with the degree of injury, i.e. the greater the cell injury, the brighter it looked. In addition, cytoplasm was sometimes vacuolated, different amounts of vacuoles and debris could be found. Some features of astrocyte injury before stimulation are shown in Figure 4-10.
Figure 4-10: astrocyte injury in P0 RON before stimulation

A: Astrocytes in P0 nerves did not show any observable decrease in the number of their processes or change in the nuclear morphology; in addition cell membrane integrity remained intact. The white rectangular area is shown in detail in C; scale bar is 4 µm. B: when looked at under low magnification, astrocytes show their star shaped morphology; no process loss is apparent; scale bar is 10 µm. C: a detailed view of the rectangle in A. Astrocytes usually suffer a low degree of injury. Three mitochondria are shown which are labelled from 1 to 3; all of them show some degree of injury. In the first one (m1), majority of the mitochondrial crests are damaged; however, a few of them can still be seen. In (m2), all the crests appear to have fragmented; they appear as small bubbles distributed throughout the matrix. In the third one (m3), all the crests are completely dissociated, leaving behind a cloud of debris in the matrix. All of them are swollen, but their membranes are still intact. ER is also greatly damaged; most of them are fragmented to small pieces, while others are hugely swollen (black arrow head), only very few ER (white arrow head) appear to have survived the insult. The nucleus (N) has typical wide spread chromatin distribution throughout the nucleus; the nuclear envelope (white arrows) are also intact. In addition, the cell membrane has retained its integrity along the entire border of the cell (black arrows). Scale bar is 1 µm.
After stimulation, astrocytes in P0 nerves showed considerably more injury features than before stimulation. At low magnification, astrocytes seemed to be smaller with a more spherical shape; they appeared to have shed their processes. Even when a cell had a process, often the process appeared shorter than usual. However, the cell membranes of most of the cells were intact. It seemed, therefore, that shedding of the processes had happened while the cell was healthy enough to seal the place with a membrane. Some nuclei also appeared much rounder than their normal irregular shape; but the most striking difference was the change in chromatin distribution. Chromatin condensation was a ubiquitous feature; it appeared as clumps of matter under the nuclear envelope and within the deeper portions of the nucleus. A cell with normal, evenly distributed chromatin within the nucleus was never encountered in the samples. The nuclear envelope, on the other hand, was rarely damaged. Mitochondria and ER showed the same features of injury as described for P0 nerves before stimulation, albeit on a wider scale. In particular, loss of mitochondrial membrane was very common, and many cells had only few mitochondria. Masses of unrecognizable debris were scattered throughout the cytoplasm, which sometimes gave the appearance of severely damaged mitochondria. Cytoplasm was much lighter compared to either control or before stimulation conditions; various amounts of debris, vesicles and unidentified bodies were also common. Occasionally, threads of intermediate filament, possibly of GFAP, were seen to be lacerated. Some features of astrocyte injury after stimulation are shown in Figure 4-11.
Figure 4-11: astrocyte injury in P0 RON after stimulation

A: after stimulation, astrocytes appeared to be severely damaged. The cell appears smaller with reduced number of processes; however, the integrity of cell membrane is preserved. The nucleus is more spherical with clear chromatin condensation under the nuclear envelope. The number of cytoplasmic organelles is greatly reduced. The white rectangular area is shown in detail in C. Scale bar is 2µm. B: under low magnification, astrocytes have lost their star shaped morphology with a great reduction in the number of processes. Scale bar is 10µm. C: a detailed view of the rectangle in A. This astrocyte has suffered from severe degree of injury. The mitochondrion (m) is highly swollen; the crests were all damaged and fragmented; the mitochondrial membrane is broken in upper and lower parts. Most of the ER are already fragmented, or swollen as a sign of onset of injury (arrow head). The cytoplasm is much lighter than in control cells and has few organelles. In addition, many vesicles (v) and debris (D) of various sizes are distributed throughout it. In closer look, many IFs can be seen above and below the upper part of the mitochondrion which appear to be lacerated. Chromatin condensation (cc) in the nucleus (N) is clear; however, the nuclear envelope (white arrows) is still intact. Many normal appearing axons (Ax) can also be seen. Scale bar is 0.5 µm.
In P10 nerves before stimulation, astrocytes generally appeared to be rounder than normal star-like shape; the number of processes arising from a single cell was clearly less than those of a control cell. In some cases where an astrocyte process looked intact, a more detailed examination revealed that the process was in fact torn somewhere along its length. Most often, this process of shedding was happening in the vicinity of the processes leaving the soma of the cell alone; thus, making the cell to appear rounder and losing its normal morphology. This phenomenon of process shedding or shortening was in a striking contrast to what was seen in P0 nerves before stimulation where no evidence of process loss was visible (Figure 4-10 B). However, the cell membrane integrity in most cells was preserved; even in places of process breakage, the injury site seemed to be sealed by a membrane which was contiguous with the cell membrane and hence preserved the continuity of the cell membrane. Occasionally some cells were severely damaged and showed features of necrosis including cell membrane break down (Figure 4-13).

Many cells had normal appearing nuclei with no visible abnormalities regarding their shape, chromatin condensation or nuclear envelope. Nonetheless, some of them appeared more spherical than nuclei of control cells and had chromatin condensation under the nuclear envelope and inside the nucleus. Except the cells which were severely damaged and showed other features of necrosis, the nuclear envelopes remained undamaged. Cytoplasm was lighter with various amount and various sizes of vesicle and debris in it; however, the amount of cytoplasmic organelles appeared to be less than control cells. In addition, dense bodies which appeared as membrane bound, hyper-dense organelles, and dense inclusions which appeared as amorphous, hyper-dense, membrane-unbound inclusions were also common. Mitochondria and ER were the most commonly affected organelles. Very rarely they had normal morphological appearances. Their injury pattern was very similar to those in P0 nerves. Other cytoplasmic organelles such as Golgi apparatus were also sometimes injured, but the rate of injury was less than mitochondria and ER. Features of astrocyte injury before stimulation are shown in (Figure 4-12).
Figure 4-12: astrocyte injury in P10 RON before stimulation

A: in P10 nerves before stimulation, the most common finding was the reduction in the number of astrocyte processes. The nuclear shape and structure, and cell membrane integrity were largely undistributed. The two boxes are shown in detail in C. scale bar is 5 µm. B: low magnification; most of the cells are rounder than control cells and have very few processes, scale bar is 10 µm. C: a detailed view of the rectangle in A. This astrocyte has suffered a moderate degree of injury. The mitochondria (m) are hugely swollen; the crests are lost and the matrix is full of debris. The ER is either swollen or fragmented (arrow). The cytoplasm has many vacuoles and debris, distributed throughout it. In addition, few dense bodies (db) are also present. Two examples of Golgi apparatus (GA) are also visible. Some of their cisternae appear to be broken. However, the cell membrane integrity is well preserved. The nucleus (N) and nuclear envelope (arrow heads) are undisturbed. Scale bar is 1 µm. The inset: is the enlarged view of the square in A; it shows a process which appears to be intact, but in fact it is broken down (arrows) and both parts are membrane bound.
Figure 4-13: features of necrosis

A necrotic cell from a P10 nerve before stimulation. The nucleus (N) has a characteristic glassy appearance with loss of nuclear membrane (arrow heads); the nuclear content has spilled out and mixed with cytoplasm. The cytoplasm has lost most of the organelles; mitochondria (m) are swollen and vacuolated. The cell membrane is also ruptured (arrows). Scale bar is 2 µm.

After stimulation, features of astrocyte injury were very similar to those seen in the before stimulation condition; astrocytes were more spherical and the number of processes was greatly reduced. However, the cell membrane integrity was preserved in majority of the cells. Morphological changes in nuclei were also similar to the before stimulation condition, but with one major exception. Chromatin condensation was an apparent feature in all the astrocytes; not a single cell was found without chromatin condensation. Cytoplasm was lighter compared to control cells, with vesicles, debris, dense inclusions and dense bodies in it; mitochondria and ER were also swollen, fragmented or dissociated. Features of astrocyte injury after stimulation are shown in Figure 4-14.
Figure 4-14: astrocyte injury in P10 RON after stimulation

A: features of astrocyte injury in P10 nerves after stimulation were similar to before stimulation condition with the exception of chromatin condensation (arrows) inside the nucleus (N) which was observed in all the astrocytes. The integrity of cell membrane was preserved in most of the cells (arrow heads). The rectangular area is shown in C. Scale bar is 2 µm. B: low magnification; most of the cells are rounder than control cells and have very few processes; in addition, chromatin condensation is a prominent feature. Scale bar is 10 µm C: a detail view of the rectangle in A. The astrocyte has suffered from severe degree of injury. All mitochondria are damaged; they are swollen and have lost their crests with holes in their matrices. Some of them still have an intact mitochondrial membrane (m2) while others have lost it (m1). All ER are lost except one (arrow) in the field. Cytoplasm appears lighter than cytoplasm of a control cell (compare to Figure 4-9), with fewer organelles; instead, it has some vesicles (v) and debris (d) in it. In addition, many small, dense inclusions (ellipse) are also distributed throughout the cytoplasm. Golgi apparatus (GA) is also seen. Chromatin condensation under the nuclear envelope and within the nucleus (N) is very clear; however, both the nuclear (white arrow heads) and cell membranes (black arrow heads) are still intact. Scale bar is 0.5 µm.
In adults before stimulation, astrocytes were injured most severely compared to other ages after FC treatment. Features of injury were ubiquitous in all the astrocytes. Most of the glial cells were damaged so severely that it was difficult to recognize them. Cells with necrotic features such as the one shown in (Figure 4-13) were commonly encountered.

Even at low magnification, the changes in cell morphology were easily recognizable. Almost all the astrocytes were oval or spherical in shape; the number of processes was greatly reduced. The majority of the cells did not have any processes at all; cells with two processes were extremely rare. Furthermore, the glial element within the nerves was less than control nerves. The processes which had detached form the soma had suffered from injury too; features of injury were similar to cytoplasmic injury. Surprisingly, the majority of astrocytes had intact cell membrane; even in places where astrocyte processes were detached, both the proximal cytoplasmic part and the distal process part were sealed with a membrane; thus, ensuring the continuity of the cell membrane.

Nuclear transformation was also dramatic; they were much rounder than nuclei of control cells with chromatin condensation as a recognized feature in every single nucleus. Although chromatin condensation was also observed in some control cells, it was not present in all the cells. In addition, the nuclear membrane was occasionally bubbly (the two layers of the nuclear membrane separated occasionally along its length to form small bubbles in between the layers). Invariably, the cytoplasm was lighter and had fewer organelles than control. Most of the time, the cytoplasm was severely disturbed and untidy; various amounts of debris, vesicles and even large cysts were scattered through it. Mitochondria, ER and other organelles were severely injured too. Mostly, they were already dissociated and disappeared, leaving behind a swarm of vesicles and debris, or they were hugely swollen and fragmented. Features of astrocyte injury before stimulation are shown in Figure 4-15.
Figure 4-15: astrocyte injury in adult RON before stimulation

A&B: Compared to control condition (A), astrocytes before stimulation (B) showed a more spherical morphology in their cellular and nuclear shape (compare the arrows in both images), and a great reduction in the number of their processes. Scale bars are 10 µm. C: an astrocyte which has suffered from a severe degree of injury. All the cytoplasmic organelles are dissociated and lost. One mitochondrion (arrow) can be seen which is swollen and has lost its crests. The cytoplasm is considerably lighter compared to control cells with some small vesicles, debris, a large cyst (c) and GFAP (arrow heads) in it. The nucleus has lost its characteristic irregular shape and has gained a much more oval shape. However, the nuclear and cell membranes are still intact. Scale bar is 1 µm.
Figure 4-16: astrocyte injury in adult RON after stimulation

A: After stimulation, features of astrocyte injury were very similar to before stimulation condition. The cell and the nucleus are more spherical with chromatin condensation easily visible under the nuclear membrane and inside the nucleus. In addition, the cell appears to have lost its processes. The black square is shown in detail in B. scale bar is 2µm. B: the cell has suffered from a sever degree of injury. Two mitochondria are labelled (m1&2) which are hugely swollen and damaged. All ER are also swollen and fragmented (arrow). Cytoplasm is disturbed and filled with vesicles, debris and even a large cyst (c). The cell membrane is still intact, but the nuclear membrane is swollen in some places (arrow head). In addition, chromatin condensation (cc) is also prominent. Scale bar is 1µm.
After stimulation, astrocytes in adult nerves appeared very similar to before stimulation condition. Therefore, the morphological changes of astrocytes after stimulation have not been described again (see before and Figure 4-15). A sample of astrocyte injury after stimulation is shown in Figure 4-16.

4.6.3.1.2 Oligodendrocytes

Oligodendrocytes in P10 nerves before stimulation appeared mostly intact with minimal signs of injury. Nonetheless, some cells appeared to have suffered from low degree of injury which, in general, injury features resembled those features that are described for astrocyte injury. Mitochondria and ER were the two organelles that were affected most frequently and possibly before other changes in cell morphology, since other morphological changes were always accompanied by injury to mitochondria and ER. The most common sign of mitochondrial injury was swelling, in which the mitochondrial appearance was ball-like. Furthermore, mitochondrial crests were sometimes reduced or disappeared completely, and the mitochondrial matrix was replaced by a cloud of debris. In this stage, most of the mitochondrial membranes were still intact. Like in astrocytes, injury to ER resulted in swelling, fragmentation and dissociation. Sometimes, intact ER could also be found inside the cytoplasm. Compared to control cells, cytoplasm occasionally appeared lighter; in addition, vesicles and debris were not uncommon. The cell membrane remained uninjured in majority of oligodendrocytes. No change in nuclear morphology was observed; it appeared with a characteristic oval shape nucleus with evenly distributed chromatin and an intact nuclear membrane. Features of OL injury before stimulation are shown in Figure 4-17.
Figure 4-17: oligodendrocyte injury in P10 RON before stimulation

A: low magnification of an OL before stimulation. The general characteristics of the cell is preserved, such as an oval nucleus, evenly distributed charomatin inside the nucleus and many mitochondria inside the cytoplasm. Black square is shown in detail in B. scale bar is $5 \mu m$. B: an OL which has suffereed from mild degree of injury. Most mitochondria (m) are swollen, and have a ball-like appeareance, some crests can still be seen, although their number is reduced. The mitochondrial membrane is intact in majority of them. Only few ER are still intact (arrows): the majority of them are fragmented and can be seen as small pieces distributed throughout the cytoplasm. The cytoplasm is light in color, but it is a normal feature in oligodendrocytes; however, presence of some debris (d) is pathologoical. The nucleus (N) and nuclear membrane (arrow heads) are undisturbed. Scale bar is $1 \mu m$. 
After stimulation, oligodendrocytes displayed the same features of injury as oligodendrocytes in before stimulation with one striking difference. Nuclear chromatin condensation was ubiquitous among oligodendrocytes, no cell could be found with no chromatin condensation. The features of oligodendrocyte injury in P10 nerves after stimulation are shown in Figure 4-18.

In adult nerves before stimulation, features of injury in oligodendrocytes were more prominent than in P10 nerve oligodendrocytes. Features of injury were variable among oligodendrocytes; some cells had only few injury signs while others had many. Similar to astrocytes, mitochondria and ER were the most commonly injured organelles. Mitochondria were swollen, the number of crests reduced and the matrix was filled with debris. ER was swollen, fragmented and dissociated. Mostly, cell membrane integrity was preserved, except in few cases. Various amounts of debris, vesicles, cysts, dense inclusions and dense bodies were also encountered. Nuclear transformation was an obvious sign compared to control cells; invariably among oligodendrocytes, chromatin was condensed inside the nucleus and below the nuclear envelope. Although chromatin was also condensed in some control cells, it was not in every cell as after FC treatment. In addition, chromatin condensation was more intense and appeared more electron dense compared to control cells. Although nuclear membrane was sometimes bubbly or occasionally ruptured, it was undisturbed in majority of the cells. Features of OL injury before stimulation in adult nerves are shown in Figure 4-19.

After stimulation, the general features of OL injury were very similar to before stimulation (see above) and hence it has not been described in detail again. The only difference was that the features of injury were encountered more frequently. An example of an OL after stimulation is shown in Figure 4-20.
Figure 4-18: oligodendrocyte injury in P10 RON after stimulation

A: low magnification of an OL after stimulation. The cell membrane integrity is well preserved with many mitochondria distributed throughout the cytoplasm. Chromatin condensation (arrows) inside the nuleus (N) is clear. Black square is shown in detail in B. scale bar is 2µm. B: the enlarged view of the square in (A), showing many mitochondria which are swollen and have lost their crests (m1) or have few crests (m2). Few ER are recognisable (arrow head), others have fragmented. The cytoplasm has some vesicles in it (black arrow), but the plasm membrane is still intact (arrows). The nucleus (N) has chromatin condensation (cc) under the nuclear membrane. Scale bar is 0.5µm.
Figure 4-19: oligodendrocyte injury in adult RON before stimulation

A: low magnification of an OL before stimulation. Chromatin condensation is a prominent feature under the nuclear membrane and inside the nucleus. The nucleolus (n) is also shown. Black square is shown in detail in B. Scale bar is 2 µm. B: an OL which shows many mitochondria (m), some of them are swollen with reduced crest number. Few ER are still intact (arrow head), while others are fragmented or dissociated. The cytoplasm is lighter than normal control cell and has some vesicles (arrow) and debris in it; however, the cell membrane integrity is well preserved. Inside the nucleus (N), chromatin condensation (cc) is a prominent feature. Scale bar is 1 µm.
Figure 4-20: oligodendrocyte injury in adult RON after stimulation

A: low magnification of an OL after stimulation. Chromatin condensation is a prominent feature under the nuclear membrane and inside the nucleus. The nucleolus (n) is also shown. Black square is shown in detail in B. Scale bar is 2 µm. B: an OL which has suffered from severe degree of injury. All mitochondria (m) are swollen with reduced crest number. All ER (ER) are also hugely swollen or fragmented. The cytoplasm is much lighter than normal control cell and has vesicles (arrow), cysts (c), dense inclusion (arrow head) and debris in it; however, the cell membrane integrity is well preserved. Inside the nucleus (N), chromatin condensation (cc) is a prominent feature. In addition, the nuclear membrane (rectangle) is bubbly. Scale bar is 0.5 µm.
4.6.3.2 Effects of FC on axons

Before stimulation, many of the axons in P0 nerves appeared very similar to control axons with no obvious signs of abnormalities. However, some axons showed signs of injury which included a reduction or complete disappearance of microtubules and the presence of debris inside the cytoplasm which usually appeared as an electron-dense material. Occasionally, large cysts were also observed within the cytoplasm. In addition, the axolemmal integrity was sometimes disrupted. Some axons also had very dense cytoplasm. Features of axons before stimulation are shown in Figure 4-21.

After stimulation, the same features were also present in some axons, although the frequency of their appearance was less than before stimulation. One striking difference after stimulation was that the axons were more closely packed together and the extracellular space was much narrower. Features of axons after stimulation are shown in Figure 4-22.

![Figure 4-21: effects of FC on P0 axons before stimulation](image)

Most of the axons have normal appearance; however, few axons show signs of injury which include losing of the microtubules (Ax), disruption of the continuity of the axolemma (arrow), presence of the debris (arrow head) or large vesicle within the cytoplasm (v). A nearby astrocyte (Ast) is also seen which has hugely swollen mitochondria (m). Scale bar is 0.5µm.
Figure 4-22: effects of FC on P0 axons after stimulation

Most of the axons have normal appearance; however, a few axons show signs of injury which include losing of the microtubules (Ax), presence of the debris (arrow) or large vesicles within the cytoplasm (black arrow head). Some axons also appear to have a very dense cytoplasm (white arrow head). An astrocyte nucleus (N) is also seen which has chromatin condensation (cc). The cytoplasm of the astrocyte is lighter with few organelles in it, the mitochondria (m) are also swollen; but the processes (AstP) are still attached to the soma. Scale bar is 0.5µm.

In P10 nerves, features of injury to axons were very similar to what is described for P0 nerves. Likewise, after stimulation, axons appeared to be packed more closely compared to before stimulation with narrower extracellular space. Because of the similarities with P0 nerves, no further detail is discusses here. Features of injury to axons are shown in (Figure 4-23 and Figure 4-24) for before stimulation and after stimulation respectively.
Most of the axons have normal appearance; however, some axons show signs of injury which include losing of the microtubules (Ax), disruption of the continuity of the axolemma (arrow), presence of the debris (arrow head). Some axons are filled with dense cytoplasm (*) and appear darker. The extracellular space appears wide compared to after stimulation (compare it to Figure 4-24). Scale bar is 0.5µm.
Figure 4-24: effects of FC on P10 axons after stimulation

Most of the axons have normal appearance; however, some axons show signs of injury which include losing of the microtubules either partially (arrow) or totally (Ax), presence of the debris (black arrow head), small vesicles, and even large cyst within the cytoplasm (c). An inner tongue process of an oligodendrocyte is also injured and contains debris (white arrow head). A nearby astrocyte process (AstP) is also seen which shows GFAP filaments, but considerably lighter cytoplasm compared to a normal process. The extracellular space appears narrow compared to before stimulation (compare it to Figure 4-23). Scale bar is 0.5µm.
In adults, axons were more severely affected compared to other ages both before and after stimulation. The changes were wide spread among the axons, making them easy to recognize in cross and longitudinal sections. The changes ranged from a minimal disturbance in the structural integrity to the complete degeneration and disappearance of the axons.

Before stimulation, some axons, especially small sizes, showed only a minor disturbance in their cytoplasm with reduction in the number of recognizable microtubules. In other axons with more severe injury, the same features became more pronounced; in addition, debris and vesicles were also common. The appearance of vesicular bodies was an extremely common finding; the vesicles were most commonly observed between the axon and its myelin sheath; however, sometimes it was within the axoplasm as well. Although sometimes a solitary vesicle was observed, mostly vesicles appeared in groups and filled either a part or the entire nerve fiber. In some cases, the groups of vesicles were seen to compress the axon on one hand and cause bulging out of the myelin on the other hand. Other changes inside the axon were the appearance of dense cytoplasm in which the entire cytoplasm of the axons was replaced by a dark, electron dense cytoplasm. Furthermore, some axons seemed to have degenerated completely, leaving behind a myelin sheath with no content or a cloud of electron-lucent debris (Figure 4-25). When looked at low magnification, many axons appeared to be larger and had rounder profile compared to control axons.

Myelin sheath shearing (fixation artefact) as described for control nerves was also common; in addition other features became apparent. The most common finding was the presence of large round or ovoid cavities called myelin balloons (Figure 4-26A). Balloons usually appeared within the layers of myelin sheath or inside the cytoplasm of the axons; they varied greatly in size, some of them were few microns in diameter. The contents of balloons can appear as electron lucent matrix or completely clear. It has been suggested that these balloons are fluid filled spheres (Peters, 2002), but the source of the fluid is yet to be fully elucidated. When they were large, balloons could press the axon to the periphery of the nerve fibre and flattened the axon in the process. In addition, they may bulge out the myelin sheath that covers them and even
pressurize the nearby axons. Balloons appear to be localized, with changes confined to a limited length of the fibre.

Another feature of the myelin sheath was the formation of redundant myelin (Figure 4-26B) which usually appeared as myelin sheath that was much bigger for the enclosed axon, and as a result, the myelin was folded from one end to the surrounding area leaving the axon in the other end of the sheath. The folded end of the myelin was either fully obliterated i.e. the myelin layers were attached completely or was forming a bulb and contained cytoplasm-like material. Redundant myelin was a feature of small diameter axons; mostly the axons had a diameter of less than 1 micron, an axon with a diameter of 1 micron was rarely encountered. Other features that appeared less frequently were the splitting of the myelin sheath; when the myelin was split along the entire circumference of the axon, it appeared as double myelin i.e. two circles of myelin one inside the other (Figure 4-26C). Sometimes, the space between two myelin circles was filled with dark cytoplasm. However, not all splitting was complete along the entire circumference; instead, the myelin sheath divided locally along a short distance of the circumference. Like complete splitting, mostly the space was filled with dark cytoplasm, or sometimes by multiple vesicular bodies. Finally, some abnormalities for which an explanation could not be found were encountered, such as the one shown in (Figure 4-26D), in which a ring of myelin sheath was embedded inside the axon, dividing the axon into two separate parts.

After stimulation, the same features of injuries which are described for “before stimulation axons” were also common in both axons and their myelin sheaths. Surprisingly, some axons appeared to be healthier compared to before stimulation condition. In general, the smaller axons looked better that larger ones. Feature of axons after stimulation are shown in Figure 4-27.
Figure 4-25: effects of FC on adult axons before stimulation

Signs of injury are widespread among the axons. Only few axons, especially ones with small diameters, look healthy with minimal signs of injury (Ax1). Other axons have lost some of their microtubules, and their cytoplasm contains debris (Ax2). Furthermore, some axons (Ax3) have dense inclusions (arrows) in addition to debris (arrow head), and many vesicular bodies (v) have filled one side of the nerve fibre, bulging out the covering myelin to the surrounding area. This vesicular body formation is sometimes more prominent and fills the entire nerve fibre (Ax4). Some other axons are filled with dense cytoplasm (Ax5) and appear much darker than healthy axons. Lastly, some axons appear to have degenerated completely (Ax6), leaving behind an empty myelin sheath. Scale bar is 2µm.
Figure 4-26: myelin changes before and after stimulation in adult RONs.

A: a large balloon (balloon) with a diameter of nearly 3 microns that has occupied most of the nerve fibre and pushed the axon (Ax) to one side, which in turn, has bulged out the covering myelin to the surrounding area. In addition, a small axon (white arrow) has a redundant myelin (arrow heads); the other end of the redundant myelin sheath has formed a bulb (black arrow). Scale bar is 1µm. B: redundant myelin; an axon (Ax) has a myelin sheath which is much bigger than the axon, and thus the myelin sheath has folded to form a section of redundant myelin. The other side of the myelin sheath is fully obliterated (arrow). Scale bar is 1µm. C: double myelin; an axon (Ax) appear to have two concentric layers of myelin sheaths one within the other. The space between the two myelin rings is filled with a rim of dense cytoplasm with some vesicles in it. The inner ring also has a localized myelin split (arrow). A nearby glial cell is dead, chromatin is condensed (cc) and the nuclear envelope is ruptured (arrow head). Scale bar is 1µm. D: a longitudinal section of an adult nerve, showing an unidentified abnormality in the myelin sheath in which a ring of myelin is embedded within an axon. Scale bar is 0.5µm.
Figure 4.27: effects of FC on adult axons after stimulation

Signs of injury are widespread among the axons. Only few axons, especially small diameters, look healthy with minimal signs of injury (Ax1). Other axons have lost some of their microtubules, and their cytoplasm contains debris (Ax2). Furthermore, some axons (Ax3) have multiple vesicles within their axoplasm; while others (Ax4) have large cavities which filled the fibre and pressurizing the axon (arrow) to the periphery. Some other axons are filled with dense cytoplasm (Ax5) and appear much darker than healthy axons. Lastly, some axons appear to have degenerated completely (Ax6), leaving behind an empty myelin sheath filled with a cloud of debris. Scale bar is 1µm.
4.6.4 Quantitative analysis

For the quantitative analysis, the images were collected in groups and given separate codes for analysis by two people independently (Miss Natalie Allcock and myself) with the scorer blind to the age and treatment condition.

4.6.4.1 Astrocytes

In control (untreated) RONs, astrocytes in all ages were largely intact and scored near the ideal score of healthy cells (ideal healthy cells will have the maximum score of “4” in the scoring system), (P0: 3.77 ± 0.07, N=31; P10: 3.74 ± 0.07, N=39; adult: 3.71 ± 0.07, N=49; N represents the number of analysed cells). Comparing untreated with FC treated cells, there was a significant main effect of treatment in all age groups (P0: F (2, 84) = 100.8, P < 0.0001; P10: F (2, 121) = 136.6, P < 0.0001; adult: F (2, 105) = 178.1, P < 0.0001; one way ANOVA in all cases; comparing untreated to FC treated, before and after stimulation conditions).

In FC treated RONs, before stimulation, astrocyte score decreased significantly in all ages (P0: 2.64 ± 0.23, N=25, P < 0.0001; P10: 1.52 ± 0.13, N=46, P < 0.0001; adult: 1.24 ± 0.16, N=29, P < 0.0001; post-hoc Tukey’s test, compared to controls) (Figure 4-28).

After stimulation, no significant changes occurred in either P10 or adults compared to before stimulation condition (P10: 1.2 ± 0.11, N=39; P > 0.5, adult: 1.43 ± 0.1, N=30, P > 0.5, post-hoc Tukey’s test, compared to before stimulation). However, P0 astrocytes showed a significant decrease in their injury score after stimulation compared to before stimulation condition (0.9 ± 0.13, N=31, P < 0.0001, post-hoc Tukey’s test, compared to before stimulation) (Figure 4-28).
Figure 4-28: astrocyte score in all ages

Astrocytes in control (untreated) condition were largely intact. In FC treated nerves, before stimulation, astrocyte injury score decreased significantly in all ages. However, after stimulation, astrocyte score decreased significantly only in P0 nerves. The numbers in the bottom of the bars are the number of analysed astrocyte cells. *** P < 0.001, significant difference from control; ††† P < 0.001 significant difference between before and after stimulation; post-hoc Tukey’s test, based on significant main effect of ANOVA.

A second measure was to count the number of processes from individual astrocytes. The number of processes as expressed here represents the number of recognizable processes in cross section; however, astrocytes have a complex morphology and send processes in all the directions, which inevitably results in some processes not appearing in cross section, and this in turn, results in an obvious underestimation of the true number of processes.

In control (untreated) nerves, the average number of processes of astrocytes were slightly higher in adult nerves compared to younger ones (P0: 1.79 ± 0.25, N=29; P10: 1.75 ± 0.21, N=33; adult: 2.21 ± 0.19, N=52). Comparing untreated with FC treated cells; there was a significant main effect of treatment in all age groups (P0: F (2, 89) = 19.21, P < 0.0001; P10: F (2, 96) = 18.48, P < 0.0001; adult: F (2, 134) = 42.90, P < 0.0001; one way ANOVA in all cases; comparing untreated to FC treated, before and after stimulation conditions).
In FC treated RONs, before stimulation, the number of astrocyte processes decreased significantly in only P10 and adult nerves, while P0 astrocytes did not show any significant reduction in the number of their processes (P0: 2.08 ± 0.27, N=25, P > 0.05; P10: 0.64 ± 0.16, N=25, P < 0.0001; adult: 0.52 ± 0.13, N=46, P < 0.0001; post-hoc Tukey’s test, compared to controls) (Figure 4-29).

After stimulation, no significant changes occurred in either P10 or adult astrocyte processes number compared to before stimulation condition (P10: 0.43 ± 0.12, N=41; P > 0.5, adult: 0.38 ± 0.1, N=39, P > 0.5, post-hoc Tukey’s test, compared to before stimulation). However, P0 astrocytes showed a significant decrease in the number of their processes after stimulation compared to before stimulation condition (0.39 ± 0.13, N=38, P < 0.0001, post-hoc Tukey’s test, compared to before stimulation) (Figure 4-29).

**Figure 4-29: astrocyte process number**

Astrocytes show slightly higher numbers of processes in adults in control condition. In FC treated nerves, before stimulation, astrocyte processes are lost significantly only in P10 and adult nerves. However, after stimulation, astrocyte processes decreased significantly only in P0 nerves; no further injury happened in other ages. The numbers in the bottom of the bars are the number of analysed astrocyte cells. *** P < 0.001, significant difference from control; ††† P < 0.001 significant difference between before and after stimulation; post-hoc Tukey’s test, based on significant main effect of ANOVA.
A third measure was the length of astrocyte processes. Like measuring astrocyte processes number, measuring the length of the processes is not an easy task. Astrocyte processes have a tortuous pathway through the nerve, which makes it very difficult to follow it completely, especially in tissue fixed for electron microscope analysis. For this analysis, however, distance between the soma and the most distant point of the process was measured.

In control (untreated) nerves, the average length of astrocyte processes appeared very similar in P0 and P10 astrocytes (P0: 6.91 ± 0.51, N=51; P10: 7.05 ± 0.39, N=58), while adult astrocytes showed much longer processes (adult: 12.10 ± 0.5101, N=115). Comparing untreated with FC treated cells; there was a significant main effect of treatment in only P0 astrocytes, while P10 and adult did not show any effects (P0: F (2, 115) = 6.305, P = 0.0025; P10: F (2, 89) = 1.780, P = 0.1746; adult: F (2, 151) = 0.4271, P = 0.6532; one way ANOVA in all cases; comparing untreated to FC treated, before and after stimulation conditions).

In FC treated RONs, before stimulation, astrocyte processes length did not decrease significantly in any age groups (P0: 7.16 ± 0.42, N=52, P > 0.05; P10: 7.13 ± 1.04, N=16; adult: 11.87 ± 1.1, N=24, P > 0.05) (Figure 4-30). After stimulation, no significant changes occurred in either P10 or adult astrocyte processes length compared to before stimulation condition (P10: 5.5 ± 0.61, N=18; P > 0.5, adult: 10.75 ± 0.92, N=15, P > 0.5). However, P0 astrocytes showed a significant decrease in the length of their processes after stimulation compared to before stimulation condition (3.87 ± 0.48, N=15, P < 0.01, post-hoc Tukey’s test, compared to before stimulation) (Figure 4-30).
4.6.4.2 **Astrocytes**

In control condition, astrocytes show much longer processes in adults compared to other ages. In FC treated nerves, before or after stimulation, astrocyte processes were not shortened in either P10 or adult nerves. However, after stimulation, the length of astrocyte processes decreased significantly in P0 nerves compared to either control or before stimulation condition. The numbers in the bottom of the bars are the number of analysed astrocyte cells. **P < 0.01, significant difference from control; †† P < 0.01 significant difference between before and after stimulation; post-hoc Tukey’s test, based on significant main effect of ANOVA.**

**Figure 4-30: Astrocyte process length**

4.6.4.2 **Axons**

In control (untreated) RONs, axons in all ages were largely intact and scored near the ideal score of healthy axons (ideal healthy axons will have the maximum score of “3” in the scoring system), (P0: 2.72 ± 0.02, N=19; P10: 2.6 ± 0.04, N=20; adult: 2.83 ± 0.01, N=49; N represents the number of sections analysed, not the number of axons). Comparing untreated with FC treated axons, there was a significant main effect of treatment in all age groups (P0: F (2, 62) = 10.67, P < 0.0001; P10: F (2, 72) = 5.072, P = 0.0087; adult: F (2, 118) = 195.1, P < 0.0001; one way ANOVA in all cases; comparing untreated to FC treated, before and after stimulation conditions).
In FC treated RONs, before stimulation, axon score decreased significantly in all ages (P0: 2.14 ± 0.07, N=28, P < 0.0001; P10: 2.42 ± 0.05, N=26, P < 0.05; adult: 1.52 ± 0.05, N=42, P < 0.0001; post-hoc Tukey’s test, compared to controls) (Figure 4-31).

Contrary to expectation, after stimulation, axon score improved significantly in all age groups (P0: 2.5 ± 0.15, N=18, P < 0.05; P10: 2.59 ± 0.03, N=29; P < 0.05, adult: 1.86 ± 0.09, N=30, P < 0.0001, post-hoc Tukey’s test, compared to before stimulation). As a result of this improvement in axon score, no statistical difference was detected in comparison between control and after stimulation in either P0 or P10 nerves; however, adult nerves remained significantly injured in post-stimulation nerves even after the improved scores (Figure 4-31).

**Figure 4-31: axon score in all ages**

Axons in control (untreated) condition were largely intact in all ages. In FC treated nerves, before stimulation, axon injury score decreased significantly in all ages. Surprisingly, after stimulation, axon score increased significantly in all age groups compared to before stimulation condition; as a result, axon scores were no longer statistically different from control nerves in P0 and P10 nerves, but this effect did not happen in adult nerves. The numbers in the bottom of the bars are the number of sections analysed; the numbers of axons analysed are (P0: 2917, 3403, 2454; P10: 1820, 1195, 1322; adult 1820, 1195, 1322 in control, before and after stimulation respectively. In all cases, 3 nerves were used, except adult control and before stimulation in which 4 nerves were used). * P < 0.05; *** P < 0.001, significant difference from control; † P < 0.05, ††† P < 0.001 significant difference between before and after stimulation; post-hoc Tukey’s test, based on significant main effect of ANOVA.
Then the percentage of each score in all the ages was calculated. As could be expected from the average score of the axons, the number of axons with the score of 0 (which indicates severe injury) was very low in control nerves in all ages (mostly between 0.5-1.5 percent) (Figure 4-32A). FC treatment alone without stimulation increased the number of axons with the score of 0 in all ages; especially in adults. Conversely, the number of axons with the score of 3 (which indicates healthy axons) was high in control nerves in all ages (approximately 80-90% of the axons) (Figure 4-32D). FC treatment without stimulation decreased the number of healthy axons (score of 3) significantly in all ages; again, adult nerves were affected the most compared to other ages. The changes in the percentage of axons with the score of 1 and 2 are shown in Figure 4-32 B and C respectively. Furthermore, after stimulation, the average score of the axons improved significantly in all ages (Figure 4-31) and the changes in the percentage of each score is shown in (Figure 4-32 A-D).

**Figure 4-32: detailed analysis of axon injury**

The percentage of axons which have scored 0, 1, 2 and 3 is shown in A, B, C and D respectively. Note that the adult nerves have the highest rate of injured axons compared to other ages. * P < 0.05; ** P < 0.01; *** P < 0.001, significant difference from control; post-hoc Tukey’s test, based on significant main effect of ANOVA.
In order to assess the changes in axon structure in greater depth, the number of microtubules was used as a measure of axon integrity. The numbers of microtubules were calculated in P0 and P10 nerves, but not adults, owing to the large number of microtubules in adult nerves and technical difficulty in recognizing them.

In control (untreated) RONs, P10 axons had more microtubules than P0 axons (P0: 5.18 ± 0.15, N=17; P10: 6.89 ± 0.25, N=20; N represents the number of sections analysed, not axons). This increase in the number of microtubules in P10 axons is to be expected, as some of the axons in this stage of development had already expanded and were showing a clear increase in the number of their microtubules. Comparing untreated with FC treated axons, there was a significant main effect of treatment in both age groups (P0: F (2, 59) = 10.67, P < 0.001; P10: F (2, 72) = 5.833, P = 0.0045; one way ANOVA in both cases; comparing untreated to FC treated, before and after stimulation conditions).

In FC treated RONs, before stimulation, the number of microtubules decreased significantly in both ages (P0: 3.88 ± 0.15, N=25, P < 0.001; P10: 5.92 ± 0.21, N=26, P < 0.05; post-hoc Tukey’s test, compared to controls) (Figure 4-33).

Contrary to expectation, after stimulation, microtubule number increased significantly in both age groups (P0: 5.12 ± 0.16, N=18, P < 0.05; P10: 6.82 ± 0.2, N=29; P < 0.05, post-hoc Tukey’s test, compared to before stimulation). As a result of this increase in the number of microtubules, no statistical difference was detected in comparison between control and after stimulation in either P0 or P10 nerves (Figure 4-33). These results are in agreement with improvement in axon score that was observed after stimulation (see Figure 4-31).
Figure 4-33: microtubules numbers in axons

Axons in control (untreated) condition had more microtubules in P10 nerves. In FC treated nerves, before stimulation, microtubule numbers decreased significantly in both ages. Surprisingly, after stimulation, microtubule number increased significantly in both age groups compared to before stimulation condition; as a result, microtubules numbers were no longer statistically different from control nerves. The numbers at the bottom of the bars are the number of sections analysed; the numbers of axons analysed are (P0: 2740, 2046, 2454; adult: 1820, 1195, 1322; in control, before and after stimulation respectively. In all cases, 3 nerves are used). * P < 0.05; *** P < 0.001, significant difference from control; † P < 0.05; significant difference between before and after stimulation; post-hoc Tukey’s test, based on significant main effect of ANOVA.

The changes in axon areas in cross sections were also calculated. To determine area, the circumference of individual axons was selected by hand in ImageJ software and the area was obtained by the software from its circumference.

In control (untreated) RONs, as expected, the average area was the smallest in P0 axons, slightly larger in P10 axons and considerably larger in adult axons (P0: 0.052 ± 0.003, N=29; P10: 0.085 ± 0.003, N=20; adult: 0.45 ± 0.017, N=49; N represents the number of sections analysed, not axons; all the values are in µm²). Comparing untreated with FC treated axons, there was a significant main effect of treatment in P10 and adults, but not P0 nerves (P0: F (2, 82) = 2.237, P = 0.1133; P10: F (2, 72) =
7.831, P = 0.0008; adult: F (2, 124) = 17.22, P < 0.0001; one way ANOVA in all cases; comparing untreated to FC treated, before and after stimulation conditions).

In FC treated RONs, before stimulation, axon area changed differently in each age group. As indicated by the ANOVA, there was no significant difference in the area of the axons in P0 before (0.055 ± 0.006, N = 38) or after (0.036 ± 0.003; N = 18) stimulation.

Figure 4-34: axon areas in all ages
Axons in control (untreated) condition have the smallest average area in P0 nerves, slightly larger area in P10 and considerably larger areas in adult nerves. In FC treated nerves, before stimulation, changes in area are age dependent. In both P0 and adult nerves, area increased, but reached significant level only in adult. In contrast, area decreased significantly in P10 nerves. After stimulation, however, axon area decreased in all ages and reached significant level only in P10 nerves when compared to controls. The numbers in the bottom of the bars are the number of sections analysed; the numbers of axons analysed are (P0: 2917, 3403, 2454; P10: 1820, 1195, 1322; adult 1820, 1195, 1322 in control, before and after stimulation respectively. In all cases, 3 nerves are used, except adult control and before stimulation in which 4 nerves are used). * P < 0.05; *** P < 0.001, significant difference from control; ††† P < 0.001 significant difference between before and after stimulation; post-hoc Tukey’s test, based on significant main effect of ANOVA.
In P10 axons, there was a significant decrease in area before stimulation (0.071 ± 0.002, N = 26, P < 0.0001), while in adult there was a significant increase (0.71 ± 0.03, N=48, P < 0.0001) (post-hoc Tukey’s test compared to control). After stimulation, there was no change in area in P10 (0.065 ± 0.004, N=29; P > 0.05) but a significant decrease in area in adults 0.48 ± 0.064, N=30, P < 0.001 (post-hoc Tukey’s test compared to before stimulation) (Figure 34)

Next, whether the changes in axon area were also shown in axon density was measured. To measure density, all the axons in a field of an image was counted and divided by the general area of the field. Glial component was not included in the general area to avoid unnecessary changes in axon density.

In control (untreated) RONs, P0 axons were most densely packed per square microns, P10 axons less densely and adult axons considerably less (P0: 11.48 ± 0.56, N=29; P10: 4.99 ± 0.28, N=20; adult: 0.85 ± 0.02, N=49; N represents the number of sections analysed, not axons; all the values are axons/µm²). This is not surprising, as axons are considerably smaller in P0 nerves, and thus, more of them can be packed in any given space (Figure 4-34). Comparing untreated with FC treated axons, there was a significant main effect of treatment in all age groups (P0: F (2, 82) = 77.45, P < 0.0001; P10: F (2, 72) = 5.189, P=0.0078; adult: F (2, 124) = 27.47; P < 0.0001; one way ANOVA in all cases; comparing untreated to FC treated, before and after stimulation conditions).

In FC treated RONs, before stimulation, axon density decreased in both P0 and adult nerves; however, the decrease was only significant in adult nerves (P0: 10.61 ± 0.32, N=38, P > 0.05; adult: 0.62 ± 0.018, N=48, P < 0.0001). Contrary to other ages, axon density in P10 nerves showed no significant change (P10: 6.44 ± 0.41, N=26, P > 0.05) (post-hoc Tukey’s test, compared to controls in all ages) (Figure 4-35).

After stimulation, axon density increased in all age groups, however, this increase reached significant level only in P0 and adult axons, not P10 (P0: 18.79 ± 0.43, N=18, P < 0.0001; P10: 7.58 ± 0.71, N=29, P > 0.05; adult: 0.98 ± 0.06, N=30, P < 0.0001, post-hoc Tukey’s test, compared to before stimulation) (Figure 4-35). These
results are in agreement with the results of change in axon area, as the decrease in area is expected to result in an increase in axon density (Figure 4-34).

**Figure 4-35: axon density in all ages**

Axons in control (untreated) condition have the highest density in P0 nerves, slightly less dense in P10 and considerably less dense in adult nerves. In FC treated nerves, before stimulation, axon density in both P0 and adult nerves is reduced, but reached significant level only in adult. In contrast, axon density increased, although not significantly, in P10 nerves. After stimulation, however, axon density increased in all ages and reached significant level in both P0 and adult nerves when compared to before stimulation. Furthermore, after stimulation, axon density increased significantly in all ages when compared to controls. The numbers in the bottom of the bars are the number of sections analysed; the numbers of axons analysed are (P0: 2917, 3403, 2454; P10: 1820, 1195, 1322; adult 1820, 1195, 1322 in control, before and after stimulation respectively. In all cases, 3 nerves are used, except adult control and before stimulation in which 4 nerves are used). * P < 0.05; ** P < 0.01; *** P < 0.001, significant difference from control; ††† P < .001 significant difference between before and after stimulation; post-hoc Tukey’s test, based on significant main effect of ANOVA.
Finally, the diameters of the axons were measured to determine whether FC treatment causes any change. As most of the axons did not have a circular profile, a simple measurement of the diameters was not possible. Instead, the diameters were calculated mathematically from its area \(A=\pi r^2\), where \(A=\text{area of the axon}, r=\text{radius of the axon}; \text{diameter } = 2r\).

In control (untreated) RONs, as expected, the average diameters of the axons were the shortest in P0 nerves, slightly longer in P10 axons and considerably longer in adult axons (P0: 0.3 ± 0.009, N=29; P10: 0.38 ± 0.006, N=20; adult: 0.83 ± 0.013, N=49; N represents the number of sections analysed, not axons; all the values are in µm). Comparing untreated with FC treated axons, there was a significant main effect of treatment in all age groups (P0: \(F (2, 82) = 16.55, P < 0.0001\); P10: \(F (2, 72) = 10.86, P < 0.0001\); adult: \(F (2, 124) = 18.99, P < 0.0001\); one way ANOVA in all cases; comparing untreated to FC treated, before and after stimulation conditions).

In FC treated RONs, before stimulation, changes in axon diameters were age dependent. Average axon diameter decreased in both P0 and P10 nerves; however, the decrease reached significant level only in P10 nerves (P0: 0.29 ± 0.004, N=38, \(P > 0.05\); P10: 0.34 ± 0.005, N=26, \(P < 0.05\)). Contrary to other ages, diameter of adult axons increased significantly (adult: 0.97 ± 0.021, N=48, \(P < 0.0001\); post-hoc Tukey’s test, compared to controls in all ages) (Figure 4-36).

After stimulation, axon area decreased in all age groups; however, this decrease reached significant level only in P0 and adult axons (P0: 0.24 ± 0.003, N=18, \(P < 0.0001\); P10: 0.32 ± 0.009, N=29, \(P > 0.05\), adult: 0.79 ± 0.03, N=30, \(P < 0.0001\), post-hoc Tukey’s test, compared to before stimulation) (Figure 4-36).
Figure 4-36: axon diameters in all ages

Axons in control (untreated) condition are the smallest in P0 nerves, slightly larger in P10 and considerably larger in adult nerves. In FC treated nerves, before stimulation, axon diameter decreased in both P0 and P10 nerves, but reached significant level only in P10. In contrast, axon diameters increased significantly in Adult nerves. After stimulation, however, axon diameter decreased in all ages and reached significant level in both P0 and adult nerves when compared to before stimulation. Furthermore, after stimulation, axon diameters were decreased significantly in P0 and P10 nerves when compared to controls, but not in adults. The numbers in the bottom of the bars are the number of sections analysed; the numbers of axons analysed are (P0: 2917, 3403, 2454; P10: 1820, 1195, 1322; adult 1820, 1195, 1322 in control, before and after stimulation respectively. In all cases, 3 nerves are used, except adult control and before stimulation in which 4 nerves are used). * P < 0.05; *** P < .001, significant difference from control; ††† P < 0.001 significant difference between before and after stimulation; post-hoc Tukey’s test, based on significant main effect of ANOVA.
4.6.4.3 Oligodendrocytes

Since oligodendrocytes did not appear in P0 nerves, oligodendrocytes were analysed only in P10 and adult nerves. In control (untreated) RONs, oligodendrocytes were largely intact and scored near the ideal score of healthy cells (ideal healthy cells will have the maximum score of “4” in the scoring system), (P10: 3.93 ± 0.26, N=82; adult: 3.78 ± 0.48, N=83; N represents the number of analysed cells). Comparing untreated with FC treated cells, there was a significant main effect of treatment in both age groups (P10: F (2, 193) = 17.91, P < 0.0001; adult: F (2, 202) = 80.57, P < 0.0001; one way ANOVA in both cases; comparing untreated to FC treated, before and after stimulation conditions).

In FC treated RONs, before stimulation, oligodendrocyte score decreased significantly only in adults (P10: 3.87 ± 0.03, N=58, P > 0.05; adult: 3.21 ± 0.07, N=71, P < 0.0001; post-hoc Tukey’s test, compared to controls) (Figure 4-37).

After stimulation, oligodendrocyte score decreased significantly in both ages compared to before stimulation condition (P10: 3.55 ± 0.06, N=56, P < 0.0001; adult: 2.33 ± 0.12, N=71, P < 0.0001; post-hoc Tukey’s test, compared to before stimulation). In addition, after stimulation, oligodendrocyte score was significantly lower in both ages when compared to control cells (Figure 4-37).
Oligodendrocytes in control (untreated) condition were largely intact. In FC treated nerves, before stimulation, oligodendrocytes injury score decreased significantly only in adult cells. However, after stimulation, oligodendrocyte score decreased significantly in both ages when compared to either control or before stimulation condition. The numbers in the bottom of the bars are the number of analysed oligodendrocyte cells. *** P < 0.001, significant difference from control; ††† P < 0.001 significant difference between before and after stimulation; post-hoc Tukey’s test, based on significant main effect of ANOVA.

4.6.5 CAP recording at different frequencies

As discussed earlier, prior to the fixation of some of the nerves for electron microscope analysis, the nerves were stimulated at a higher rate (20stim/min) compared to the normal (2stim/min) rate that was used for routine CAP recording. By stimulating the nerves at higher rate, an extra load was put on the astrocytes and the consequences were investigated ultra-structurally. Furthermore, the rates of CAP decline in both frequencies have been compared.

Contrary to expectation, stimulation at a higher rate reduced the rate of CAPs decline by approximately 10% in all ages (Table 2). However, this improvement in the
CAPs measurement did not reach significant level in any time points or any ages; unpaired Student t test was used to determine significance in all cases. In P0 nerves, P values were (0.6731, 0.702, 0.525 and 0.6399), P10 (0.6608, 0.8799, 0.8931 and 0.7784) and adult (0.3283, 0.1311, 0.2119 and 0.2491) in 1, 2, 3 and 100 minutes respectively in all ages. The rates of CAP decline in different ages at different frequencies in different time points is shown in Table 2, also in Figure 4-38, Figure 4-39 and Figure 4-40.

<table>
<thead>
<tr>
<th>Age</th>
<th>1st hour</th>
<th>2nd hour</th>
<th>3rd hour</th>
<th>100 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2stim/min</td>
<td>84.49 ± 11.00</td>
<td>84.62 ± 12.00</td>
<td>69.98 ± 6.00</td>
<td>84.26 ± 12.00</td>
</tr>
<tr>
<td>20stim/min</td>
<td>93.05 ± 15.00</td>
<td>92.44 ± 9.00</td>
<td>79.22 ± 17.00</td>
<td>94.12 ± 12.00</td>
</tr>
<tr>
<td>P10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2stim/min</td>
<td>72.02 ± 9.00</td>
<td>65.59 ± 10.00</td>
<td>57.06 ± 8.00</td>
<td>73.90 ± 10.00</td>
</tr>
<tr>
<td>20stim/min</td>
<td>80.27 ± 15.00</td>
<td>68.51 ± 6.00</td>
<td>55.00 ± 3.00</td>
<td>79.29 ± 3.00</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2stim/min</td>
<td>93.63 ± 11.00</td>
<td>51.82 ± 7.00</td>
<td>38.19 ± 3.00</td>
<td>59.79 ± 9.00</td>
</tr>
<tr>
<td>20stim/min</td>
<td>112.2 ± 11.00</td>
<td>73.90 ± 12.00</td>
<td>47.58 ± 8.00</td>
<td>79.50 ± 13.00</td>
</tr>
</tbody>
</table>

Table 2: rates of CAP decline in different ages at different frequencies.
**Figure 4-38: rate of CAP decline in P0 RONs.**

Comparing the rate of CAPs decline in P0 RONs after stimulation at a rate of 2stim/min vs. 20stim/min; note that in all time points the rates of CAPs decline were higher in lower frequency of stimulation. However, no significant change was determined; unpaired Student t test. N= 6 and 3 nerves in 2 and 20stim/min respectively.

**Figure 4-39: rate of CAP decline in P10 RONs.**

Comparing the rate of CAPs decline in P10 RONs after stimulation at a rate of 2stim/min vs. 20stim/min; note that in all time points the rates of CAPs decline were higher in lower frequency of stimulation. However, no significant change was determined; unpaired Student t test. N= 6 and 2 nerves in 2 and 20stim/min respectively.
Figure 4-40: rate of CAP decline in adult RONs.

Comparing the rate of CAPs decline in adult RONs after stimulation at a rate of 2stim/min vs. 20stim/min; note that in all time points the rates of CAPs decline were higher in lower frequency of stimulation. However, no significant change was determined; unpaired Student t test. N= 6 and 3 nerves in 2 and 20stim/min respectively.

4.7 Discussion

Normal ultrastructural features of all the major cellular components of WM i.e. astrocytes, axons and oligodendrocytes were well preserved and appeared normal in control nerves of all the ages that were tested. FC treatment produced widespread, age-depandant damage to all the cellular components. Astrocytes in particular were most commonly affected after FC treatment. Most of the astrocytes showed signs of injury which included swelling, fragmentation or complete dissociation of ER and mitochondria. Other cytoplasmic organelles were sometimes affected and the cytoplasm itself appeared brighter. With more severe injury, the cytoplasmic membrane, nucleus and nuclear membrane appeared to be damaged. The axons also showed signs of injury which included reduction or disappearance of the microtubules, debris in the cytoplasm or axolemmal disruption. In myelinated nerves, the myelin sheaths showed signs of injury similar to those that are usually seen in old age sheaths. In general, the features of injury
were age-dependant and most commonly seen in adult nerves. These results are comparable to the results which were obtained in immunohistochemical staining and electrophysiological recording which showed that adult nerves are most sensitive to FC-induced injury. In addition, in P10 and adult nerves where oligodendrocytes have appeared, oligodendrocytes were sometimes injured. Injury features were similar to those of astrocytes and appeared to happen secondarily to other cellular injury rather than primarily as a result of FC toxicity. Finally, the nerves were put under an extra-load by stimulating them at a higher rate before examining them under electron microscopy. Surprisingly, stimulating the nerves at higher rate improved the injury features of the axons although the astrocytes suffered more injury. This counter-intuitive feature could be due to better utilization of lactate by the axons in periods of high demands.

4.7.1 Features of astrocyte injury are age dependant

It is well known that severe forms of ischemic insults can lead to astrocytic cell death by apoptosis or necrosis (Salter and Fern, 2008, Thomas et al., 2004, Fern, 2001, Fern, 1998), while less severe forms can initiate reactive changes (section: 1.10 Reactive astrocytosis, page 18). Furthermore, there are many lines of evidence to suggest that astrocytes in vivo are equally or even more sensitive than neurones to ischemia (Zhao et al., 2003). In the current study, FC treatment produced signs of injury in astrocytes of all the ages. Compared to each other between different experimental ages that have been used, features of injury were most common in adult astrocytes (Figure 4-10, Figure 4-12 and Figure 4-15). Likewise, quantitative analysis revealed the same pattern of injury at different ages (Figure 4-28). FC treatment induces a cellular decrease in ATP level, which in turn, is associated with Ca\(^{2+}\) sequestration in mitochondria and triggers mitochondrial dysfunction (Morley et al., 1994). Mitochondrial dysfunction will ultimately disturb cellular homeostasis and causes cell death. However, during FC treatment it has been observed that astrocytes hydrolyse any residual ATP that is left to maintain mitochondrial membrane potential
(Voloboueva et al., 2007). This hydrolysis is essential in keeping the astrocytes alive, since the disturbance of mitochondrial membrane potential eventually results in cell death and release of pro-apoptotic factors such as cytochrome c and pro-caspases (Susin et al., 1999). In fact, Voloboueva et al. (2007) have shown that up to 70% of mitochondrial membrane potential might be lost while the astrocyte is still alive. In addition, Largo et al. (1996) have shown that after FC treatment pH level decreases significantly. Although the origin of H\(^+\) ions is difficult to be determined precisely, it is known that astrocytes contribute to pH regulation. Likewise, ischemic depletion of cellular ATP is associated with cellular acidosis which amplifies astrocyte death (Swanson et al., 1997).

The reasons that renders adult astrocytes specifically more prone to FC induced injury has not been further examined in the current study, but traditionally it is thought that adult astrocytes have low passive resistance due to the existence of an extensive network of gap junctions between the cells (Orthmann-Murphy et al., 2008) which can help in propagating injurious molecules between them.

To investigate whether putting an energy load accelerates astrocytes death, the nerves were stimulated at the rate of 20stim/min for 3 hours before being fixed for electron microscope processing. Surprisingly, stimulation only induced more injury in P0 astrocytes, while P10 and adult astrocytes did not show more signs of injury (Figure 4-28) (also Figure 4-11, Figure 4-14 and Figure 4-16 for features of injury). Similar results, where P0 nerves responds differently than adult nerves to FC treatment have been observed with electrophysiological recording (See Chapter 3). It is long known that neonatal astrocyte have different injury mechanism compared to astrocytes of older ages. For instance, neonatal astrocytes suffers from high intracellular Ca\(^{2+}\) concentration by L and T type voltage gated Ca\(^{2+}\) channels with the T type responsible for most of the intracellular Ca\(^{2+}\) increase during initial face of ischemia followed by a more prominent role of L type channels at later stages (Fern, 1998). Furthermore, neither Ca\(^{2+}\) release from intracellular store nor Ca\(^{2+}\) influx from glutamate gated channels contribute to rise of intracellular Ca\(^{2+}\) concentration. Surprisingly, Na\(^+\)-Ca\(^{2+}\) exchanger, which imports Ca\(^{2+}\) in other cell types during ischemic (DiPolo and Beauge, 2006), was acting to remove Ca\(^{2+}\) in neonatal RON astrocytes (Fern, 1998). Collectively,
the above mechanisms which have been observed only in P0 astrocytes can explain why P0 astrocytes respond differently to FC treatment. These mechanism, however, have not been further investigated in the current study.

4.7.2 Clasmatodendrosis

FC treatment in P10 and adult nerves resulted in a clear change in the morphology of astrocytes, even when the nerves were fixed for electron microscopic observation without stimulation. However, P0 astrocytes appeared to be more resistant to injury and showed similar signs of injury as older nerves only after the nerves were stimulated prior to electron microscopic fixation. The changes were mostly presented as rounding of the cell bodies and simplification of the astrocyte processes architecture, with a clear decrease in the number of astrocyte processes, a process known as clasmatodendrosis. In fact, reduction in the number of astrocyte processes has long been observed to be associated with ischemic changes and tissue acidosis (Butt and Colquhoun, 1996, Friede and Van Houten, 1961). Furthermore, it is known that astrocytes in an attempt to regulate interstitial glutamate level may become acidotic to a level that can damage their processes with subsequent tendency to loss them (Hulse et al., 2001). In addition, FC treatment is also associated with decrease in pH (Largo et al., 1996), a condition which also favours clasmatodendrosis. Inevitably, the changes in astrocyte morphology results in alteration in astrocyte relations with neighbouring elements. Changes that can be expected to occur as a result of shedding of astrocyte processes include losing contact with synapses, detachment of astrocytic end-feet processes from the blood vessels or losing of the gap-junctional coupling between the astrocytes.

Pathological changes in brain that are associated with inflammation or RA are usually accompanied by modification of connexin 43 expression, a major gap-junction forming protein between the astrocytes (section: 1.7 Astrocytes, page 13). For example, some pro-inflammatory cytokines such as IL-1β, TNF and β-amyloid are associated with down-regulation of connexin 43 (John et al., 1999). Although the
functional consequences of such down-regulation is not fully understood yet, it is suggested that such down-regulation can limit the passage of injury induced molecules between the cells, thereby, isolating the primary lesion site. Clasmatodenrosis can have similar consequences where the intercellular communication can be limited by losing the gap-junction communication as a result of shedding of astrocyte processes. However, the beneficial effects of losing contact with blood vessels can be harder to explain, as the intact communication between the astrocytes and blood vessels are essential for astrocyte survival.

4.7.3 Changes in the axons and myelin sheath

In control nerves, axonal diameters were similar to what have been documented previously in RONs (Foster et al., 1982) and the microscopic features were well preserved. FC treatment, however, produced wide spread injuries inside the axons, especially in the adult nerves (Figure 4-31). Adult axons are known to have low tolerance to ischemia compared to younger ages. Oxygen-glucose deprivation, for example, produced robust axonal structural injury in adult and P21 mice nerves, but not in P7 or P3 nerves (McCarran and Goldberg, 2007). This study, however, has noticed delayed axonal degeneration which is significant axonal fragmentation and fibre loss after 9 hours of reperfusion. In the current study, fixation of the samples was carried out at the end of experiments (3 hours) and hence, the possibility of axons to undergo delayed axonal degeneration cannot be excluded. In agreement with the previous study, Fern et al. (1998) found no permanent loss of CAPs in RONs younger than 10 days, while older ages suffered significantly after oxygen-glucose withdrawal. One possible explanation for heightened sensitivity of adult axons to injury is their intrinsically high vulnerability to excitotoxicity as a result of enhanced toxicity to glutamate (Baltan et al., 2008). In addition, the astrocyte population is established early in development (Vaughn, 1969) while the neurons continue to grow, and thus, the same number of astrocytes have to spread over a much larger area of the nerve, rendering the astrocyte density much lower in adults compared to younger nerves. Since astrocyte contain glycogen almost exclusively (section: 1.18 Glycogen in brain,
and glycogen can be utilized as a source of energy during energy crisis, this can render the axons of adult nerves deprived of energy sooner that younger ages. In fact, a recent observation by (Fern, 2015) has confirmed this hypothesis, in which axons in zones around the astrocytes were spared when extrinsic energy supply was withdrawn.

The important question will be, were the axons affected primarily as a result of excitotoxicity after increased glutamate concentration or secondarily to other causes? Traditionally, it was believed that glutamate causes axonal injury secondarily as a result of damage to glial cells or myelin sheath (Li and Stys, 2000). Recently, however, there are reports to suggest that axons also express functional glutamate receptors and glutamate can injure the axons directly. Although Li and Stys (2000) found GluR 2/3 and GluR4 subunit immunoreactivity within axoplasm, they suggested that these receptors are merely transported with the axoplasm to be inserted to the nerve terminals. Furthermore, the authors suggested that these receptors are either expressed in a very low concentration and their activation is not sufficient to induce axonal damage or they are not functional at all. In contrast, Ouardouz et al. (2009a), Ouardouz et al. (2009b) have detected functional AMPA and kainate receptors on axons of dorsal columns. In addition, Fowler et al. (2003) have shown that intracerebral injection of AMPA causes axonal damage in vivo. Interestingly, a recent report Pitt et al. (2010) suggested that myelin sheath may even protect the axons from AMPA induced excitotoxicity and the absence of myelin increases axon vulnerability to glutamate. Although Huria et al. (2015) have shown the presence of functional NMDA receptors on pre-myelinated axons, the expression of NMDA receptors on adult axons is less clear and currently there is no report on the presence of NMDA receptors on adult axons cylinders. Furthermore, even MK-801, a NMDA receptors antagonist, was shown to have no effect on CAPs decline when adult axons were exposed to glutamate (Ouardouz et al., 2009b).

Alternatively, axonal injury can be secondary to glutamate receptor activation on myelin, oligodendrocytes or astrocytes (Ouardouz et al., 2009b, Alix and Fern, 2009, Tekkok et al., 2007, Back, 2006). In addition, microglia, which appeared to be activated, based on their amoeboid shape and reduced processes length (Kettenmann
et al.), was observed in the current study, and the activated microglia are known to secrete injurious substances that can affect the axons (Volpe (2009b), Liberto et al. (2004), and section: 1.13 Microglia, page 25). Finally, the possibility that the axons can suffer both primarily and secondarily to other cellular injury cannot be excluded with the current results.

In addition to the axonal changes, myelin sheaths, especially in adult nerves where the axons are fully myelinated, showed different kinds of injuries. The injuries were ranged from myelin ballooning, redundant myelin, myelin splitting and even injuries that were difficult to explain (Figure 4-26). These features have been described previously as normal ageing processes (Peters et al., 2000). Since these features were very common in FC treated nerves, it can be speculated that FC treatment and subsequent death of astrocytes provide similar micro-environment around the axons as normally aged nerves. Furthermore, it raises the possibility that the astrocytes function may be below optimal in old brains and subsequently lead to axonal damage which further reinforces the role of astrocytes in normal brain functioning.

In regard to the myelin sheath, myelin shears, which are fixation artefacts were very common feature in control nerves (Figure 4-6), but it is not surprising since in the current study a diffusion-fixation has been used. Even when perfusion-fixation is used, it is very difficult to obtain a good fixation of the myelin especially in WM as it has a poor blood supply compared to grey matter axons, as shown by (Peters, 2002).

To investigate how different cellular elements of RONs will be affected when stimulated at higher rates and thereby putting an extra energy load, RONs were stimulated at a rate of 20 stim/min. Unexpectedly, stimulation at higher rate decreased axonal structural injury and improved the CAPs decline in all the ages (Figure 4-31). These results are counter-intuitive especially considering that the astrocytes appeared to be damaged even more when RONs were stimulated at higher rates (Figure 4-11, Figure 4-14 and Figure 4-16). However, there are studies to suggest that stimulation improves lactate flux to the axons (reviewed in Pellerin and Magistretti (2012)). Since in the current study lactate has been applied concomitantly
with FC an as energy source, it is likely that the stimulated axons have taken up lactate more avidly and hence better preserved their structural and functional integrity as have been determined by electron microscopy and electrophysiological data. Furthermore, Ivanov et al. (2011) have shown that lactate, under normal oxygen conditions, is even better than glucose in preserving neuronal activity. In the current study, oxygen supply was maintained in a controlled manner and hence axons possibly have used lactate efficiently. The current results do not agree with results of Largo et al. (1996) in which repeated spreading depression only damages the neurons if the glial cells were injured with FC previously. It is important to notice that in that study lactate was not utilized and the astrocytes, which normally supply lactate as an energy substrate to the neurons during periods of energy crisis (section: 461.20 Astrocyte-neuron lactate shuttle, page 46), were also injured by FC, leaving the neurons devoid of energy.

Injury to the axons was further examined by measuring different parameters of their size and the number of microtubules in P0 and P10 nerves. The number of microtubules in adult was not measured for its technical difficulty. Previously, it has been shown that axons which were exposed to AMPA were oedematous, lacked organelles and cytoskeleton components and occasionally loss axolemma (Pitt et al., 2010). Likewise, neurons exposed to glutamate exhibited irreversible Na⁺ induced swelling (Arundine and Tymianski, 2015). In agreement with these results, FC treatment increased axons diameter in adult nerves, which consequently increased axons area and decreased axons density. In adult, most axons lacked their normal organelles and cytoskeleton structure. Some axons showed disrupted axolemma as well. Surprisingly, P0 and P10 nerves behaved reversely to adult axons (i.e. smaller diameter and area but higher density).

After stimulation, however, axon parameters returned toward normal values, which, similar to CAPs measurement, indicate better axonal survival after stimulation. Similarly, the number of microtubules in P0 and P10 nerves, which were decreased significantly in “before stimulation” condition, returned toward normal values and were no more significantly lower compared to control nerves (Figure 4-33). In P0 and P10 nerves, in “after stimulation” condition, axon diameter and area further
decreased and axon density further increased. The causes of such discrepancy in axon behaviour in different ages are not clear and have not been further tackled in this study. In addition, the pathophysiological mechanism of axonal swelling after glutamate exposure is not currently clear, but it has been shown that there are highly localized clusters of signalling molecules on the inter-nodal axolemma which can increase intra-cytoplasmic $\text{Ca}^{2+}$ ions and induce axonal injury after different kinds of insults which are associated with glutamate release (Ouardouz et al., 2009a, Ouardouz et al., 2009b).

4.7.4 Oligodendrocytes injury

In addition to astrocyte and axons, oligodendrocytes were also examined for structural injuries. Since oligodendrocytes first appear 6 days postnatally (Hildebrand and Waxman, 1984), oligodendrocytes injury analysis has been performed in only P10 and adult nerves. After FC treatment in P10 and adult nerves, oligodendrocytes showed signs of injury (Figure 4-17 and Figure 4-19). Likewise, quantitative analysis revealed a low score for cell integrity after FC treatment which decreased even further in “after stimulation” condition (Figure 4-37). In general, adult oligodendrocytes showed more signs of injury compared to P10 oligodendrocytes. In the current study, there are different reasons as to why oligodendrocytes were injured. Although the presence of gap junctions between the oligodendrocytes is still questionable, there is an extensive network of gap junctions between the adjacent astrocytes and between the astrocyte and oligodendrocytes (Orthmann-Murphy et al., 2008) which allows the passage of many ions and small molecules between the adjacent cells (section: 1.7 Astrocytes, page 13). Since FC is an astrocyte specific toxin and preferentially taken up by astrocytes (section: 1.14.3 Support for glial specificity of fluorotoxins, page 35), oligodendrocytes injury can be the result of FC discharge from the astrocyte to oligodendrocytes. Alternatively, astrocyte injury can release a large amount of glutamate and ATP to the extracellular space. Oligodendrocytes express a large array of different kind of ATP and glutamate receptors including ionotropic P2X and metabotropic P2Y, NMDA and non-NMDA receptors (Domercq et al., 2010, Bergles et
al., 2000, Back et al., 2007a, Salter and Fern, 2005, Fern and Moller, 2000) which can get activated as a result of exposure to ATP and glutamate and induce oligodendrocytes injury. In agreement with that, ATP and glutamate receptors blockers are shown to be protective for oligodendrocytes injury (Matute, 2011, Wilke et al., 2004). In addition, astrocyte injury can release TNF, interferon and other cytotoxic molecules which can injure the oligodendrocytes (Orthmann-Murphy et al., 2008, Volpe, 2009a). Oligodendrocytes in particular are sensitive to ROS attack which can be released as a result of FC-induced astrocyte injury (Volpe, 2001, Back et al., 1998). Another possibility is dying back oligodendropathy, in which injury to oligodendrocytes processes as a result of axonal dysfunction may induce oligodendrocytes injury as has been observed in case of myelin associated glycoprotein deficiency (Lassmann et al., 1997). Lastly, oligodendrocytes injury primarily as a result of FC exposure, although unlikely, cannot be excluded. Similar to the current results Paulsen et al. (1987) found slight ultra-structural changes in oligodendrocytes after FC treatment. Similarly, the authors suggested that oligodendrocytes injury is more likely to be a by-product of astrocyte injury rather than primarily as a result of FC exposure.
5. Discussion

Traditionally, WM was thought to be a passive tissue which operates to modulate the distribution of APs, thereby, acting to co-ordinate the brain processes between different regions of the grey matter. However, recent data have shown that WM is an active participant in brain functions and WM actively determines how the brain learns and functions. Furthermore, WM can be injured in a wide array of insults to brain and WM injury can have serious consequences on brain functions. The severity and subsequent long term sequelae of WM damage is usually dependant on the site of injury. CP, which is the most common form of chronic debilitating disease in neonates and is associated with WM damage, can present with muscle stiffness, weakness, uncontrolled body movement, seizure, learning difficulties, speech and hearing defects. Likewise, WM is mostly involved in adult patients with stroke and WM injury can have similar symptoms in adults as in neonates.

WM is composed of a compacted tissue of neuronal axons with neighbouring glial cells with few neuronal cell bodies. Thus, these components are in close proximity to each other and a strict cooperation between them is essential in providing an ideal environment for their functions and survival. Both the axons and the glial cells have been shown to express a wide variety of receptors, release neuro-active substances, provide trophic supports and secrete injurious substances that affect the health of each other. The full extent of such interaction, however, appears to be complex and remain largely elusive. This study investigated the role of astrocytes in white matter on the neighbouring elements by inactivating the astrocytes' Krebs cycle through an astrocyte specific toxin called FC. RON, which is a white matter tract and has been extensively studied and used in the past to study WM physiology and pathology, has been used as a model to conduct this research.

Since the mechanism of injury and WM tolerance to different ischemic insults changes dramatically with age, 3 different critical time points during development were studied to determine how WM at different age responds to FC-induced astrocyte injury. The ages were P0: an age when the axons are fully unmyelinated, P10: the age of active myelination and adults: when the axons are fully myelinated.
Firstly, the WM astrocytes and axons were stained immunohistochemically to identify the effects of FC on them. In the presence of glucose in the preparation, FC treatment failed to have any significant effects on astrocytes and axons. WM astrocytes are known to be able to survive on glycolysis alone. Furthermore, astrocytes may lose as much as 70% of their membrane potential but still remain viable. To tackle this issue, glucose was removed from the preparation and replaced with lactate. Lactate is an avid energy substrate for the neurons and hence supports the axonal functions when the astrocytes are inactivated by FC. These alterations caused a significant astrocyte injury as determined by GFAP staining. These changes in astrocytes were further confirmed by nuclear double staining with PI, a nuclear specific dye. The axonal staining by anti-NF remained unaffected. In terms of axonal structural integrity, these results were interpreted to mean that the axons were spared while the astrocytes were damaged. However, further investigation later confirmed that in fact the axons were non-functional and they were functionally compromised and structurally damaged as evidenced by electrophysiological recording and electron microscopic analysis respectively. Furthermore, these results show that the immunohistochemical results should be interpreted with caution in regards to cell viability.

Later, under the same experimental conditions as immunohistochemistry, the functionality of RONs was investigated by electrically inducing CAPs and measuring CAP changes over time. CAPs remained stable for at least 5 hours in aCSF containing glucose. Likewise, replacing glucose with lactate did not affect CAPs, which suggests that lactate can be as effective as glucose in supporting axonal functions. In fact, there is a wealth of data which suggest that lactate is released from the astrocytes and avidly taken up by the neurons as a source of energy. The current results are in agreement with those observations and reinforce the hypothesis that lactate is an important energy substrate for the neurons. Although the axons were stained normally immunohistochemically, as discussed before, they were unable to conduct CAPs normally. In all the ages which were tested, CAPs were restricted and decreased significantly after FC treatment. Previously, members of our labs have shown that GFAP staining alone can be misleading in assessing astrocyte viability. The current
results raise serious doubts about the dependability of axonal staining alone to assess axonal viability. In conclusion, even when the axons are stained normally, they may not be able to conduct APs. The possibility that these non-functional axons undergo delayed degeneration can be excluded.

RONs at different ages responded differently to FC treatment. Axons of adult nerves were the most sensitive to injury. Surprisingly, P0 axons behaved uniquely and differently from the other ages. CAPs in P0 failed to stabilize when the toxin was washed out after 100 minutes of FC treatment, whereas in P10 and adult nerves, the CAPs did stabilize. This difference is attributable to fundamentally different pathway of injury in P0 nerves. Since glutamate is the major excitatory neurotransmitter in brain and FC treatment is associated with elevated level of glutamate, glutamate receptor blockers were used to determine the role of glutamate. NMDA or non-NMDA glutamate receptor blockers alone or in combination did not increase CAPs compared to FC treatment alone. However, partial recovery was observed, although this was not statistically significant. These results suggest that glutamate is not the only cause of decreased CAPs after FC treatment. In fact, it has been shown previously that the death of astrocytes is associated with release of other injurious substances such as cytochrome c, TNF and ILs which can adversely affect the well-being of the axons. Additionally, ATP is a potent neurotoxin when present in high concentration. Although the level of ATP has not been measured directly in the current study, results from other studies that have used FC, together with the astrocytic cell death that has been observed in the current study, suggest that ATP level will be high, possibly causing a decline in CAPs.

To further examine the roles of FC on different cellular elements of WM, electron microscopy was used to investigate the ultra-structural changes of astrocytes, oligodendrocytes and axons. In control condition, all the cellular elements appeared normal with intact cellular, nuclear and cytoplasmic organelles. FC treatment, however, produced robust signs of injury in astrocyte and axons. Oligodendrocytes, which are absent in P0 nerves, also showed some signs of injury in P10 and adult nerves. In agreement with the results of immunohistochemistry and electrophysiological recording, adult nerves were most sensitive to injury. Generally,
the signs of injury were similar in all the ages, but it was most common in adult cells and least common in P0 cells. Cytoplasmic organelles were initially affected and injury to them was ubiquitous in the injured cells, followed by changed in nuclear morphology and ultimately cytoplasmic membrane disruption. One significant feature of injury in the astrocytes was rounding of the cell body and shedding of the processes, a process known as clasmatodendrosis. Clasmatodendrosis is suggested to be a preservative measure taken by the astrocytes to restrict the distribution of the injurious substances and reduce the lesion size. However, this hypothesis needs further study.

Like astrocytes, adult axons were most sensitive to FC-induced injury. Cytoplasmic organelles were initially affected followed by cytoskeleton dissolution and sometimes axolemmal disruption. Myelin sheaths showed signs of injury that previously have been observed in old-age nerves. These observations suggest that FC-induced astrocytic cell death provides a microenvironment around the axons similar to old age.

Stroke is most common in old age brains (see section: 1.4 Stroke, page 7) and since it has been shown in the current study that FC treatment produced myelin sheath changes similar to those that happen normally in aged brains (Figure 4-26), it raises the possibility that FC treatment produces similar micro-environment as normally aged brains. Thus, preserving the axons in FC treated WM can provide a method to protect the axons in stroke patients. One surprising finding of this study is the improvement of the CAPs and ultrastructural integrity of the axons when stimulated at higher rates. Stimulation at a higher rate puts an energy load on the nerves, and in turn, it was expected to worsen injury. Although the astrocytes and oligodendrocytes suffered more injury, as expected, axons showed no such exacerbation of injury, and indeed showed significant signs of improvement. This observation leads to the idea that the early stimulation of the parts of the body that have been affected after stroke may stimulate the axons in the affected area and increase the chance of their survival. In addition, stroke usually diminishes the blood flow to the affected part of the brain with subsequent decrease in glucose supply. However, lactate might still be released from the astrocytes (see section: 1.19 Role of
lactate in brain metabolism, page 44), and as shown by the current study, lactate might be better utilized by the stimulated axons, which can further support the axonal survival. The results therefore suggest a novel therapeutic strategy which may be relevant for clinical practice, although clearly further validation experiments would be required first.

Although excitotoxic injury to the axons is a major factor in determining brain injury after stroke, this study has also provided evidence that glutamate release alone is not responsible for subsequent axonal and oligodendrocyte injury. This, in turn, can be one reason why in the past glutamate receptors antagonists alone have failed to provide a successful treatment for stroke patients (Hazell, 2007). Therefore, revealing the reactions that happen during early phases of ischemic, and later on during recovery, represents a first step toward discovering new medications to treat stroke.

In conclusion, we suggest that, since FC is shown to affect only the astrocytes in the concentration that has been used in this study, the axonal and oligodendroglial damage is secondarily to the consequences of astrocyte injury. In this context astroglial handling of glutamate and K$^+$ are of particular importance. Astrocytes are fundamental in nourishing, supporting and regulating the micro-environment around the neurons and failure of astrocytes to function properly can result in uncontrolled concentration of glutamate, K$^+$ ions and pH with subsequent decline in CAPs and ultimately ultrastructural damage.

**Conclusions**

- RON preparations in vitro provide a viable model for studying the role of astrocytes in neural function after injury, although GFAP staining of the astrocyte or NF staining of the axons alone can be misleading in assessing the cell viability, since this study has shown that normally stained axons were unable to conduct APs.
• Lactate has been shown to be as effective as glucose as a source of energy for the axons, as determined by immunohistochemical staining, electrophysiological recording and electron microscopic examination.

• Results from a wide range of studies have shown that FC is an astrocyte specific toxin in the range that has been used in this study. Axonal and oligodendrocytes injury, therefore, appear to be secondary to astrocyte injury.

• Clasmatodendrosis, which is defined as shedding of the astrocyte processes, has been observed in the current study. The full extent of effects of clasmatodenrosis in astrocyte injury is not clear yet.

• Age-related differences in injury processes were observed: P0 nerves responded differently from the other ages to FC treatment. CAPs failed to stabilize after the toxin was washed out. In addition, after putting an energy load on the nerves, P0 astrocytes were the only age to show a lower injury score (i.e. more injured) and show lower number of processes. On the other hand, adult astrocytes showed severe decline of GFAP staining. In addition, CAPs declined fastest in adult nerves. Electron microscopic analysis also showed that adult astrocytes and axons were most sensitive to FC treatment. Collectively, these results suggest that adult nerves are more prone to FC induced injury compared to other ages.

• This study has shown that myelin sheath injury after FC treatment is similar to the myelin changes that normally occur in old age brains. This suggests that astrocytes injury produce the same changes as in normal ageing brain.

• Glutamate receptor blockers only partially recovered CAPs during FC treatment. This result suggests that glutamate induced excitotoxicity is not the only injury pathway after astrocyte injury. There are other mechanisms which may operate to induce axonal injury and CAPs decline such as increased ATP concentration and secretion of injurious substances.
References


GEISLER, N. & WEBER, K. 1983. Amino acid sequence data on glial fibrillary acidic protein (GFA); implications for the subdivision of intermediate filaments into epithelial and non-epithelial members. EMBO Journal, 2, 2059-63.


activation of the lipoxygenase pathway mediate oxidative astrocyte death by reversed glutamate transport. GLIA, 54, 47-57.


