

The role of GABA, glycine, nicotinic, and adrenergic receptors in developing central white matter ischaemic injury and the features of NG2 expressing cells in this tissue

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Abstract

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Prolonged perinatal ischaemia is an important factor in the development of periventricular leukomalacia (PVL), which is the most common white matter pathology associated with cerebral palsy (CP): approximately 1.5 to 2.5 per 1,000 live births per year suffer from CP. During central nervous system (CNS) ischaemia, there is an excessive accumulation of extracellular neurotransmitters. Ischaemia affects axons and glial cells of white matter, which occupy approximately 50% of the human brain. The mechanisms of ischaemic-induced injury in astrocytes vary at different ages. Ischaemic injury in postnatal day 0 (P0) of rat optic nerve (RON) astrocytes is Ca^{2+} -dependent and mediated by voltage-gated Ca^{2+} channels (VGCCs). Astrocytes are replete with neurotransmitter receptors. Using an *ex vivo* model of ischaemia (oxygen-glucose deprivation: OGD) and Ca^{2+} imaging, I investigated the role of gamma-aminobutyric acid (GABA), glycine, nicotine and norepinephrine in P0 RON during ischaemia. OGD produced a rapid and significant increase in cell death. A GABA antagonist (picrotoxin), a glycine antagonist (strychnine) and their combination protected P0 RON astrocytes from ischaemic injury. They reduced the total percentage of cell death and postponed the initial cell death. Immunohistochemistry revealed that GABA and glycine receptors are expressed by astrocytes and axons in P0 RON. On the other hand, blocking nicotinic acetylcholine receptors (nAChRs: mecamylamine or α -bungarotoxin) or blocking adrenergic receptors (combination of propranolol and phentolamine) had no effect. Using a glial injury scoring system, ultrastructural studies confirmed the protective action of picrotoxin and strychnine against ischaemia, which cannot be achieved by mecamylamine or a combination of propranolol and phentolamine.

Chondroitin sulphate proteoglycans (NG2) (+) cells are a distinct type of glial cell which, over time, have come to be known as the fourth type of glia. These cells are distributed throughout the developing and adult CNS, and are known to be mitotically active even in the adult CNS. The astrocyte fate of NG2 (+) cells is a matter of debate. In the current study, the focus was on studying the morphological features of NG2 (+) cells at the ultrastructural level and under confocal microscope, and more precisely their relation to astrocytes in nRON following post-embedding immunolabelling. Immuno-electron microscope (I-EM) revealed NG2 immunoreactivity in nRON astrocytes. Double immunolabelling showed an overlap between NG2 (+) and (glial fibrillary acidic protein) GFAP (+) populations in nRON, adult RON and cortical grey matter.

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This thesis is dedicated
to my mother, my father and my husband

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Abbreviations:

AAT	Aspartate aminotransferase.
ADP	Adenosine diphosphate.
AEP	Anterior entopeduncular eminence.
AM	Acetoxymethyl esters.
AMP	Adenosine monophosphate.
AEP	Anterior entopeduncular eminence.
ARL67156	6- <i>N,N</i> -Diethyl-D- β - γ -dibromomethylene adenosine triphosphate.
α-Bgt	α -bungarotoxin.
AChR	Acetyl Choline Receptor.
aCSF	Artificial cerebrospinal fluid.
ADRs	Adrenergic receptors.
α-KG	α -ketoglutarate.
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.
ANOVA	Analysis of variance.
ATP	Adenosine triphosphate.
APC	Adenomatous polyposis coli.
BBB	Blood brain barrier.
bFGF	Basic fibroblast growth factor.
BrdU	Bromodeoxyuridine.
Ca²⁺	Calcium ion.
CAP	Compound action potential.
CC	Corpus callosum.
CFA	Cell-free area.
ChAT	Choline acetyltransferase.
CHT	Choline transporter.
CNS	Central Nervous system.
CP	Cerebral Palsy.
DOPA	3,4-dihydroxyphenylalanine.
DDW	Distilled deionised water.
DIDS	4, 4-Diisothiocyanostilbene-2, 2-disulfonic acid
DER 736	Diglycidyl ether of polypropylene glycol flexibiliser.
DMAE	Accelerator dimethylaminoethanol.
DMSO	Dimethyl sulfoxide.
DNA	Deoxyribonucleic acid.
DRG	Dorsal root ganglion.
DlgA	Drosophila disc large tumour suppressor.
EAAT	Excitatory amino acid transporter.
ECT	Electroconvulsive therapy.
ECS	Electroconvulsive shock.
ecto-NTDase	Ecto-nucleoside triphosphate diphosphohydrolase.
EM	Electron microscope.
EME	Experimental autoimmune encephalitis.
ERL 4221D	Vinyl cyclohexene dioxide.
EYFP	Enhanced yellow fluorescence protein.
G	Glial specific antigen.
GABA	γ aminobutyric acid.
GAD	Glutamic acid decarboxylase.
GAT	GABA transporter.

GFAP	Glial fibrillary acidic protein.
GFP	Green fluorescent protein.
GLT	Glutamate transporter.
GLAST	Glutamate Aspartate Transporter.
GLYT	Glycine transporter.
GLY	Glycine.
GluR	Glutamate Receptor.
GSA I-B4	Griffonia simplicifolia isolectin B4.
GST Y_b	Glutathione-S-Transferase Y _b .
GS	Glutamine synthetase.
5-HT₃R	5 hydroxytryptamine.
HAIR	Hypoxic, acidic, and ion-shifted Ringer.
HCl	Hydrochloric acid.
HCO₃⁻	Bicarbonate.
IHC	Immunohistochemistry.
HPLC	High-pressure liquid chromatography.
ISO	Isoproterenol.
I-EM	Immuno-electron microscope.
IHC	Immunohistochemistry.
K⁺	Potassium ion.
LAT	L-type amino acid transporter.
LGIC	Ligand-gated ion channel.
LRD	Lysinated rhodamine dextran.
M or MT	Transmembrane segment.
MAP	Microtubule-associated protein.
MCAO	Middle cerebral artery occlusion.
MGE	Median ganglionic eminence.
Mg²⁺	Magnesium ion.
MK	Dizocilpine.
MAG-CHO	Chinese hamster ovary cells expressing myelin-associated glycoprotein.
MGE	Median ganglionic eminence.
mRNA	Messenger RNA.
MLA	Methyllycaconitine.
MON	Mouse optic nerve.
MS	Multiple sclerosis.
N	Neuronal specific antigen.
Na⁺	Sodium ion.
NACHRs	Nicotinic acetylcholine receptors.
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione.
NE	Norepinephrine.
NEM	N-ethylmaleimide.
NF023	8,8'-[carbonylbis(imino-3,1-phenylenecarbonylimino)]bis-1,3,5-naphthalene-trisulphonic acid, hexasodium salt; a broad-spectrum P2X receptor blocker.
NF-L	Neurofilaments light chain.
NG2	Chondroitin sulphate proteoglycans.
NKCC	Na ⁺ -K ⁺ -Cl ⁻ cotransporter.
NMDA	N-methyl-D-aspartate.
NO	Nitric oxide.

NSA	Nonenyl succinic anhydride.
NPA	Nipecotate, a GABA uptake inhibitor.
OGD	Oxygen glucose deprivation.
OPC	Oligodendrocytes precursor cells.
P	Post-natal day.
P2X, and P2Y	Purinergic receptors subtypes.
PCR	Polymerase chain reaction.
PDS	Post-synaptic density protein.
PDGFαR	Platelet derived growth factor alpha receptor.
PBS	Phosphate buffer saline.
pH	Power of hydrogen (decimal logarithm of the reciprocal of the H ⁺ activity).
PEEP	Promoter-expressing NG2 progenitors.
Pf	Fractional current.
PHI	Periventricular haemorrhagic infarction.
PNS	Peripheral Nervous System.
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid; a broad-spectrum P2X/2Y receptor blocker.
Pre-OLs	Pre-myelinating Oligodendrocytes.
PLP	Proteolipid promoter.
PVL	Periventricular Leukomalacia.
RON	Rat optic nerve.
ROS	Reactive oxygen specious.
sec	Second.
SERCA	Sarcoendoplasmic reticulum (SR) calcium transport ATPase.
SVZ	Subventricular zone.
SNT	Spinal nucleus of the trigeminal nerve.
SDEV	Standard deviation.
SEM	Standard error of the mean.
TCA cycle	Tricarboxylic acid cycle (Krebs cycle).
TLE	Temporal lobe epilepsy.
TST	Spinal tract of trigeminal nerve.
TTX	Tetrodotoxin.
TMT	Trimethyltin.
VACHT	Vesicular acetylcholine transporter.
VCD	Vinyl cyclohexene dioxide.
VD	Voltage-dependent.
VGCC	Voltage-gated calcium channel.
VIAAT	Vesicular inhibitory amino acid transporter.
[X]_i	Intracellular concentration of substance X.
[X]_e	Extracellular concentration of substance X.
Zo-1	Zonula occludens-1 protein.

Part I: The features of NG2 expressing cells in RON

Section 1:

Features of glial cells in RON

CHAPTER 1

1.1 Introduction:

1.1.1 Components of White Matter:

White matter occupies half of the human brain and contains glial cells and the axons that connect neurons together and grey matter to other parts of the body. Myelin sheath is the predominant part of white matter which is found to compromise around 50% of its weight and that was the reason for the characteristic glistening white appearance of WM (Brady S. T., 2012). On the other hand, gray matter is compacted with neuronal cell body and extensive dendrites branches.

1.1.2 Axons and Myelin Sheath:

Axons are long, slender projections of neurons that form axon bundles which transmit information from neurons to the other parts of nervous system. Axons are extended from the axon hillock, the part of neuron responsible for generating the action potential, and terminate to form a synapse or neuromuscular junction. The speed of action potential conduction along an axon depends on several factors, such as the presence of a myelin sheath and axon diameter.

Myelin sheath is high in lipids in which cholesterol is the main component and that helps myelin sheath to work as an insulator for nerve fibres (Sherman and Brophy, 2005). It wraps the nerve axons leaving only small areas uncovered (node of Ranvier) to facilitate saltatory conduction which allows rapid current transmission (Squire L.R., 2008, Fields, 2008). The myelin sheath increases electrical resistance and reduces conductance, resulting in a much more rapid speed of nerve conduction (Brady S. T., 2012). It is estimated that the speed of impulse is 100 times faster in the myelinated axons (~100 m/sec) than in the non myelinated ones (~ 1m/sec) (Squire L.R., 2008).

CNS myelin sheath is structurally and antigenically different from that of PNS. In PNS, Schwann cells form myelin sheath which wraps the nerve fibre and leaves a small amount of cytoplasm in the inner surface of myelin in areas called “Schmidt-Lanterman incisures” which is not present in the CNS. Multiple myelin specific lipid has been identified in which CNS myelin express more cerebroside and sulfatide but less sphingomyelin than PNS. Both CNS and PNS express similar myelin proteins such as myelin basic protein and myelin-associated glycoprotein. However, proteolipid protein (PLP), myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) are uniquely expressed in CNS myelin while P₀ glycoprotein, peripheral myelin protein-22 and P₂ protein are expressed in PNS myelin (Brady S. T., 2012).

Node of Ranvier has several domains known as node, paranode, juxtaparanode, and internode. Each domain has different ion channels, cell adhesion molecules and cytoplasmic adaptor protein. Nodal domain expresses high level of Na⁺ channels which plays important role in action potential generation (Waxman and Ritchie, 1993). Moreover, nodal domain expresses adhesion molecules such as immunoglobulin (IgG) Nrcam and neurofascin-186; and cytoplasmic adaptors such as cytoskeletal adaptor ankyrin G and actin binding protein spectrin β IV (Davis et al., 1996, Berghs et al., 2000, Kordeli et al., 1990, Kordeli et al., 1995). Paranodal domain is located just next to nodal domain and contains the axoglial junction which express contactin-associated protein (Caspr) and contactin (Einheber et al., 1997, Menegoz et al., 1997, Rios et al., 2000). Juxtaparanodal domain expresses K⁺ channels, which play a role in preventing re-entrant excitation and maintaining the intermodal membrane potential (Chiu and Ritchie, 1984, Vabnick et al., 1999). In addition, juxtaparanodal domain has some proteins such as Caspr2, transient axonal glycoprotein (Tag1), and connexin (Cx29) (Altevogt et al., 2002, Li et al., 2002, Poliak et al., 1999, Traka et al., 2002). Intermodal

domain expresses low level of K^+ channels, Caspr and contactin proteins (Poliak and Peles, 2003).

1.1.3 Glial Cells:

It was thought for a long time that glial cells are brain glue holding the sections of the brain together (Squire L.R., 2008). In fact, glial cells play many essential roles other than providing physical support to the brain. Oligodendrocytes provide myelination; microglia provide immune responses, and astrocytes establish homeostatic control for the nervous system. More recent findings indicate further astrocyte functions, such as roles in signal integration and development (Wang and Bordey, 2008). In the human brain, glial cells are estimated to occupy 90% of the brain volume, while in a mouse they occupy around 65% (Allen and Barres, 2009).

1.1.3.1 Astrocytes:

Astrocytes are the most abundant non-neuronal cell type in the CNS and occupy 20–50% of most brain regions (Tower and Young, 1973, Squire L.R., 2008). Astrocytes are stellate in morphology and they are of two types; protoplasmic astrocytes in gray matter and fibrous astrocytes in white matter in which the latter have more extensive processes. However, human astrocytes are 2.6 times bigger than the rodent astrocyte and extend 10 times more processes (Oberheim et al., 2009). Astrocytes are connected to each other via gap junction, allowing rapid transmission of Ca^{2+} waves (Allen and Barres, 2009, Wang and Bordey, 2008). Astrocytes play multiple roles in the nervous system including neurotransmitters homeostasis, extracellular pH regulation and K^+ buffering, energy metabolism, blood brain barrier (BBB) formation, and synaptic remodelling (Dienel and Hertz, 2005, Barres, 2008, Li et al., 2008, Squire L.R., 2008).

1.1.3.2 Oligodendrocytes and Schwann Cells:

Oligodendrocytes and Schwann cells are the myelinated cells of the CNS and PNS, respectively (Squire L.R., 2008, Fields, 2008). Comparing these two cells revealed that oligodendrocytes send their processes to contact several internodes (~50) while Schwann cells contact only one internode and remain in intimate contact with its internode (Sherman and Brophy, 2005, Baumann and Pham-Dinh, 2001). That gives oligodendrocytes an important role during ischemic injury as damaging one oligodendrocyte can damage around 50 axons (Brady S. T., 2012).

1.1.3.3 Microglia:

These cells occupy around 5–20% of the total cells in a mouse brain (Squire L.R., 2008). While no immune cells are allowed to pass the BBB, microglia work as an immune defence for the nervous system by engulfing degenerating cells and debris (Allen and Barres, 2009). In addition, these cells may have a role in synaptic remodelling during early developmental stages and are responsible for cytokines and growth factors secretion (Squire L.R., 2008).

1.2 Materials and Methods:

1.1 Animals:

Wister rats aged P8—P10 were provided by the animal house of Leicester University. They were culled by cervical dislocation following schedule 1, in accordance with the regulations of the British Home Office. The brain and the two optic nerves were immediately washed in Sorensen's buffer, and under a microscope, both optic nerves were dissected and submerged in the fixatives.

1.2 Immuno-Electron Microscope:

1.2.1 Buffer and Fixatives:

Phosphate buffer solution (PBS) and sodium cacodylate buffer were used in this technique. Sodium cacodylate buffer is found to be a good alternative to Sorensen's buffer, as it prevents the phosphate overexposure produced by PBS (Bozzola and Russell, 1999). It is made by mixing cacodylic acid with DDW and adjusting the pH to 7.2 with concentrated hydrochloric acid (HCl). Two types of primary fixatives were used. The first was 0.1 M Sorensen's with 3% glutaraldehyde, prepared by mixing 2.5 ml of 0.2 M Sorensen's with 0.6 ml of 25% glutaraldehyde and 1.9 ml of distilled deionised water. The second one was 0.1 M Sorensen's with 3% glutaraldehyde and 2% paraformaldehyde, made by mixing 2.5 ml of 0.2 M Sorensen's with 0.6 ml of 25% glutaraldehyde, 0.95 ml of 8% paraformaldehyde and 0.95 ml of distilled deionised water. A secondary fixation, 1% osmium tetroxide in 0.05 M buffer, was used in addition to the primary fixation.

1.2.2 Embedding Protocol:

This protocol is used to embed P10 RON based on a common previously used protocol for RON (Constantinou and Fern, 2009, Salter and Fern, 2008a, Thomas et al., 2004, Wilke et al., 2004). Table 1-1 shows the details of protocol 1, in which the sample is passed through four main steps: fixation, dehydration, embedding and polymerisation. Samples were primarily fixed by 3% glutaraldehyde and then secondarily fixed by 1% osmium tetroxide. A gradual dehydration was introduced by using 70%, 90% and 100% ethanol. Samples were then embedded in Spurr's resin, a low viscosity epoxy resin, and polymerised in an oven. As a result, the tissue was fixed well and looked good under the electron microscope.

Table 1-1: Embedding protocol.

Steps	Description	Duration
Primary fixation	3% glutaraldehyde in 0.1 M Sorensen's buffer at pH 7.4	90 minutes
Wash	Three washes in 0.1 M buffer	10 minutes each
Secondary fixation	1% osmium tetroxide in 0.1 M buffer	2 hours
Wash	Two washes in buffer	20 minutes each
	One wash with DDW	20 minutes
Dehydration	70% ethanol	40 minutes
	70% ethanol	Overnight
	90% ethanol	30 minutes
	100% ethanol twice	30 minutes each
Embedding	Propylene oxide twice	10 minutes each
	50:50 propylene oxide/Spurr's resin	4 hours
	Lid was pierced to allow propylene oxide evaporation	Overnight
	Resin was replaced with fresh Spurr's resin and left in mixer	5 hours
Polymerisation	Samples were embedded and polymerised in a 60 °C oven	16 hours

1.3 Results and discussion:

1.3.1 Identification of Cell Type at the Ultrastructural Level:

Cell types in the P0–P2 RON were distinguished at the ultrastructural level, following James Vaughn's classification of glial cells in developing rat optic nerve, with confirmation from other studies (Peters and Vaughn, 1967, Skoff et al., 1976a, Skoff et al., 1976b, Vaughn, 1969). Cells in neonatal RON pass through several stages before maturation. As shown in Figure 1-1, a mitotic cell divides into two daughter cells, which later become small glioblasts and then large glioblasts. Some large glioblasts continue to participate in cell division, while others become large precursor cells. These differentiate into either oligodendrocytes or astrocytes, or continue as precursor cells into adulthood. Both oligodendrocytes and astrocytes have immature stages before they become fully mature. Each stage has its own morphology and ultrastructural features (Vaughn, 1969).

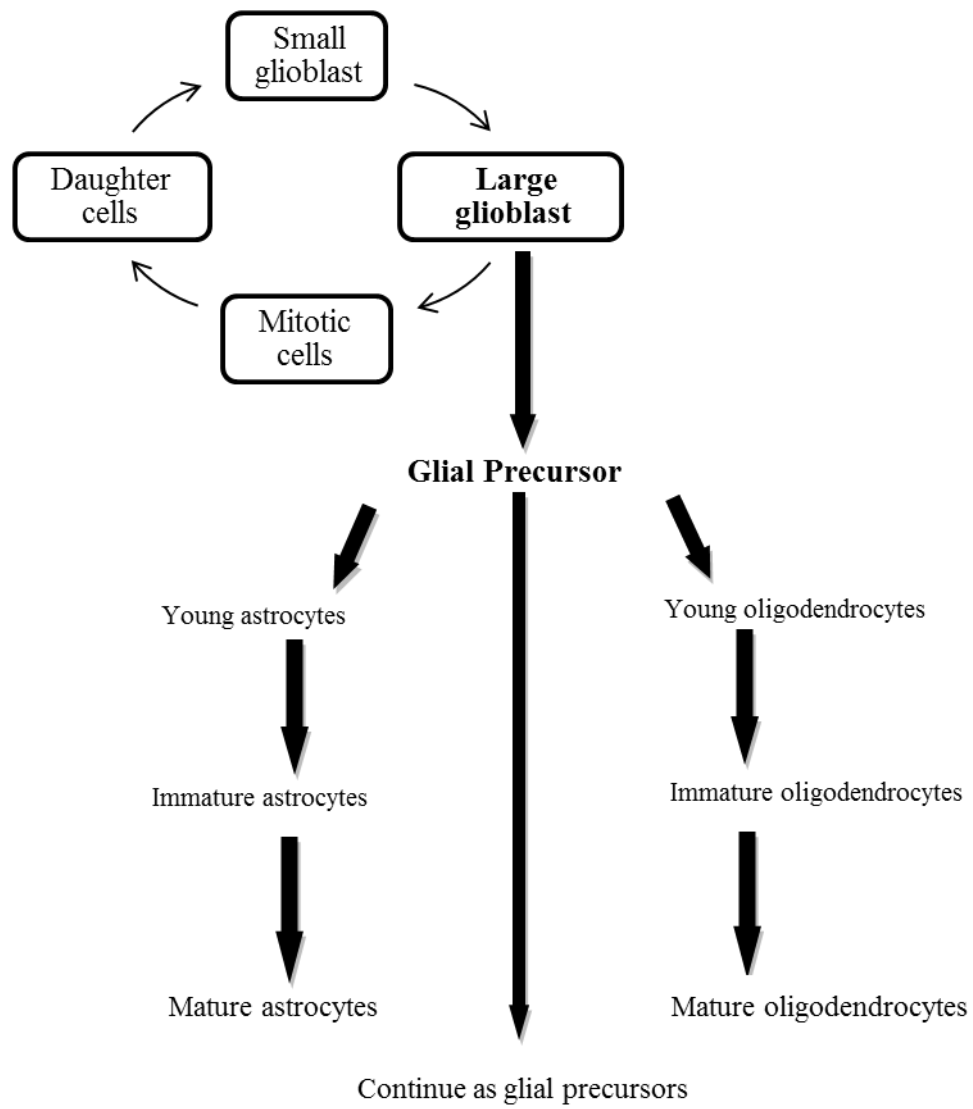


Figure 1-1: Glial cell differentiation.

Mitotic and Daughter Cells:

In general, mitotic cells are simple non-branching round cells. There are some ultrastructural features which help to identify this type of cell, such as an irregular arrangement of chromosomes, lack of a nuclear envelope, equal distribution of small groups of ribosomes, fat droplets, coated vesicles, dense matrices of small, rounded mitochondria, and less commonly, a narrow pore ER at the soma periphery; see Figure 1-2. These cells are usually located at the periphery of the nerve or around blood vessels. Between P1 to P14, mitotic cells represent around 3% to 6% of all glial cells. After each mitotic cell divides into two daughter cells, the latter usually are smaller and more oval in shape. However, the ultrastructural features of daughter cells look similar to the mitotic ones (Vaughn, 1969).

Glioblasts:

Small Glioblasts:

Small glioblasts are similar to the daughter cells in shape and size in addition to some small protrusions emanating from cell bodies. However, there are some differences in cytoplasmic organelles. Small glioblasts usually have a long, winding, narrow ER, a paranuclear Golgi apparatus and dense bodies in cytoplasm; see Figure 1-3. The cytoplasm usually looks less dense than that of the daughter cells due to the lower level of free ribosomes. An obvious aggregation of chromatin under the nuclear membrane is usually seen in the nucleus. During the early postnatal period, these cells represent around 10% of all glial cells (Vaughn, 1969).

Large Glioblasts:

These cells are usually similar to the small glioblasts but are larger in size. In general, they are round in shape, with few or no cell processes, and they are commonly

present in perivascular or submeningeal sites. The nucleus of such cells has a clumpy chromatin under the nuclear envelope and scattered throughout the nucleus. The free ribosomes are dispersed all over the cytoplasm and give it a dense appearance. The ER of a large glioblast looks shorter than the ER in small glioblasts. Large glioblasts lack coated vesicles, fat droplets, dense laminar bodies, microtubules and Golgi apparatus (Vaughn, 1969).

Large Glial Precursors:

This type of cell—precursor cells—can develop into either astrocytes or oligodendrocytes, and for that reason, these cells are shown to share some features of astrocytes or oligodendrocytes. The obvious nucleolus inside the nucleus is a characteristic feature of this type of cell; see Figure 1-4. Large glial precursors have more organelles than large glioblasts, especially the Golgi apparatus (Vaughn, 1969).

Astrocytes:

Young Astrocytes:

This kind of cell shares some features of both large glial precursors and immature astrocytes and is thought to be a transitional form.

Immature and Mature Astrocytes:

Both immature and mature astrocytes have a stellate morphology, with multiple processes emanating from the soma at right angles to the axons, as shown in Figure 1-5A. Cell processes commonly attach to the nerve periphery, forming glia limitans and surrounding the pre-myelinated axons with small finger-like processes, as shown in Figure 1-5C and D. It is important to notice that the number of microtubules is higher in immature astrocytes, while the number of glial filaments is higher in the

mature astrocytes; see Figure 1-5A (Peters and Vaughn, 1967, Vaughn, 1969). The nucleus is usually homogenous, with a thin layer of chromatin under the nuclear membrane. There are usually plenty of ribosomes that present in clusters or rosettes and cause the cytoplasm to be dense. Cytoplasmic organelles give astrocytes some characteristic features that help in differentiating them from other types of glial cells such as the regular, wide ER filled with dense matrix, opaque bodies, and long, plump mitochondria, as shown in Figure 1-5B (Vaughn, 1969). Astrocytes send their finger-like processes to ensheath axons as shown in Figure 1-6.

Oligodendrocytes:

Young Oligodendrocytes:

This kind of cell shares some features of both large glial precursors and the immature oligodendrocytes into which they are transforming.

Active Oligodendrocytes:

These cells start to appear at P5 and represent around 20% of glial cells between P7 and P9 when myelination starts and continue to generate between P30-P35 when myelination has been completed (Skoff et al., 1976b, Tennekoon et al., 1977). Active oligodendrocytes are larger than the mature oligodendrocytes, and they extend many processes. Nucleoli are usually obvious inside the nuclei, which have a homogenous scattering of chromatin. Cisternae of both ER and Golgi apparatus are more obvious in active oligodendrocytes than in the mature ones; see Figure 1-8 (Vaughn, 1969).

Mature Oligodendrocytes:

Mature oligodendrocytes represent around 60% of adult glial cells and are known to have an oval shape and a dense nucleus and cytoplasm, more than the other

glial cells, as shown in Figure 1-7. A mature oligodendrocyte has a nucleus which is present at the periphery of the cell, with a rim of chromatin under the nuclear membrane. Usually it does not extend many processes, some of which can be traced out to myelin sheaths around axons. These cells usually have a prominent Golgi apparatus, short, regular, narrow ER, and microtubules; see Figure 1-7 (Skoff et al., 1976b, Vaughn, 1969).

Microglia:

Microglial cells represent 4% to 5% of glial cells. The nucleus is usually oval in shape, and the chromatin is aggregated under the nuclear envelope. The cytoplasm is scanty and contains a tortuous, long, narrow ER, which is a characteristic feature for this type of cell. A Golgi apparatus, dense laminar bodies, homogenous droplets, lysosomes, and lipofuscin are usually seen in cytoplasm; see Figure 1-9 (Vaughn, 1969).

Glial Processes:

Glial processes can often be identified at the ultrastructural level without reference to somata. When a glial process expresses glial filaments or a wide-pore ER, it is more likely to be astrocytic (see Figure 1-10). On the other hand, an oligodendrocyte process does not express glial filaments, and it is usually extended to wrap axons multiple times for myelination. Interestingly, astrocyte processes send finger-like processes to ensheath axons (see Figure 1-10).

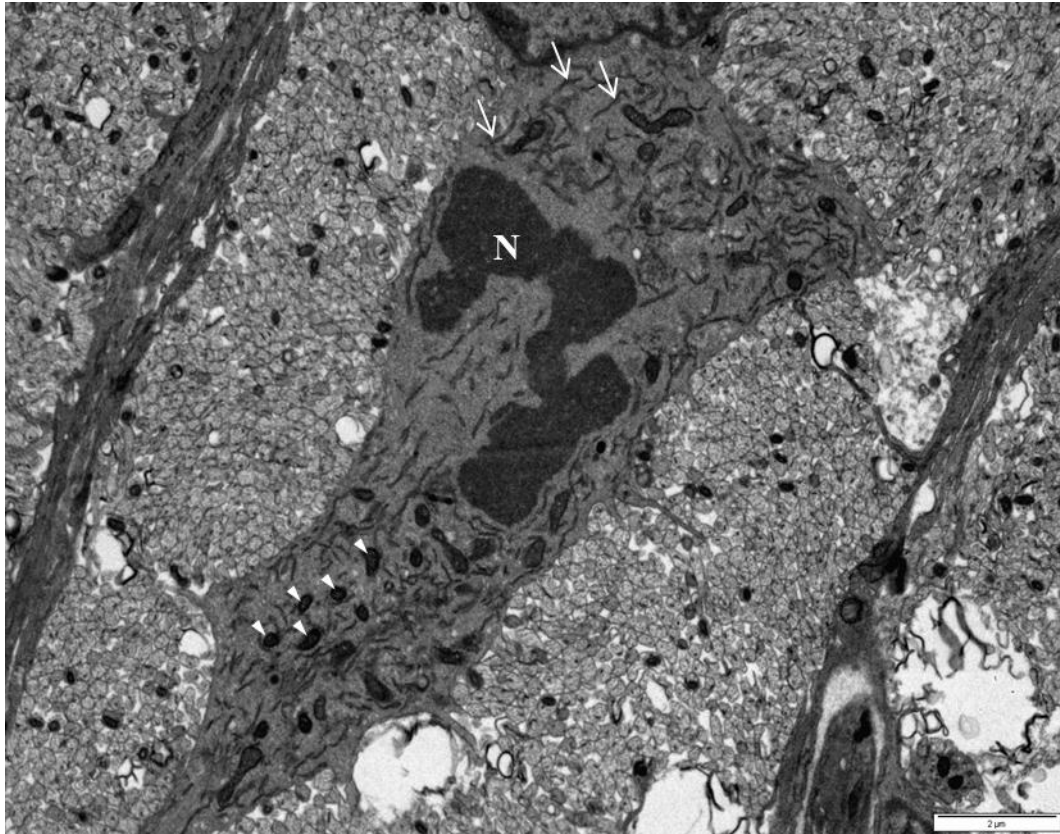


Figure 1-2: Ultrastructural features of a mitotic cell in P0 RON.

Micrograph shows irregular shaped nucleus (N), which lacks a nuclear envelope and has an irregular arrangement of chromosomes (arrows) and dense matrices of small, rounded mitochondria (arrowheads).

Scale bar = 2 μ m.

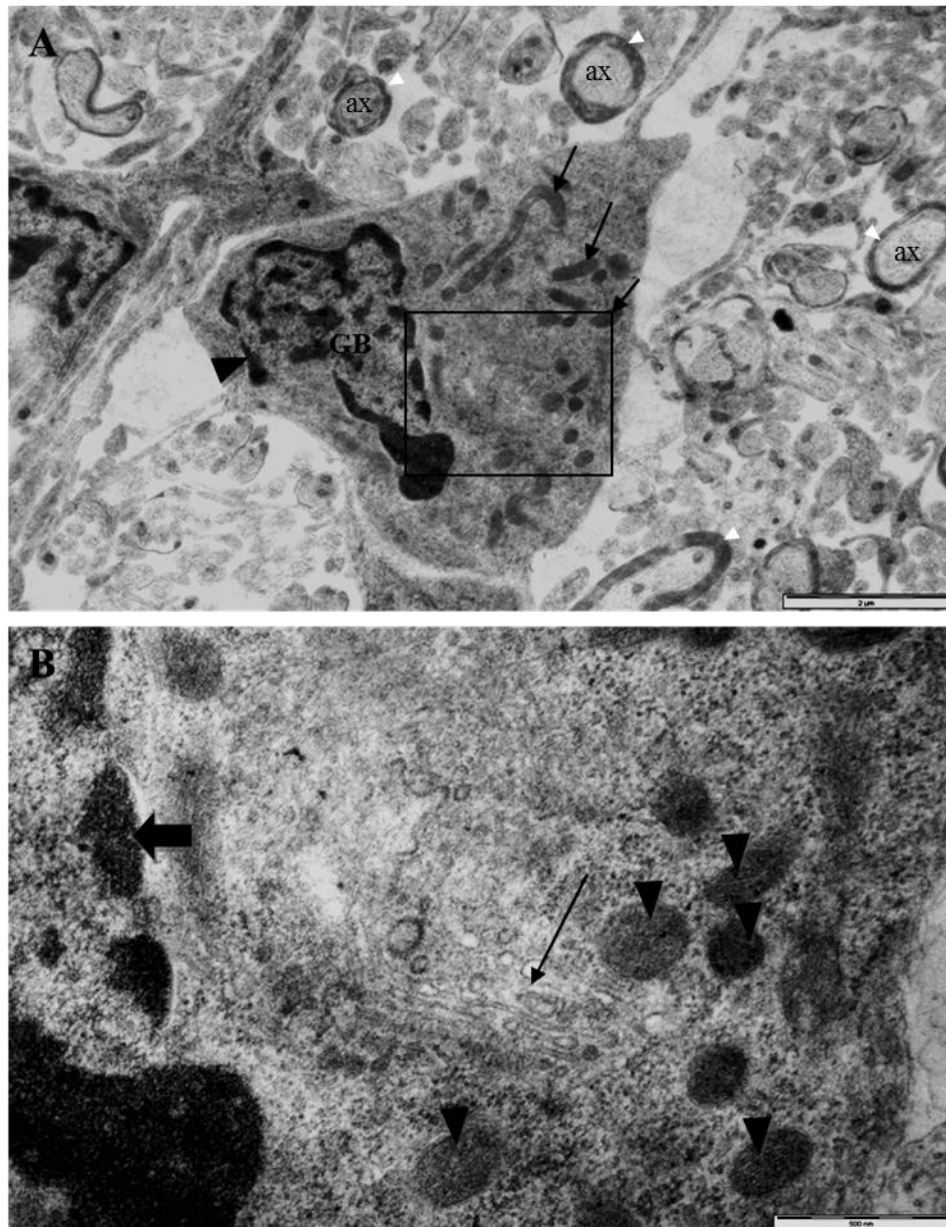


Figure 1-3: Glioblast (GB) ultrastructural features in P10 RON.

(A) Micrograph shows glioblast morphology with dense elliptical mitochondria (arrows). The nucleus (arrowhead) looks irregular in shape, with chromatin clumped under the nuclear envelope. Note the absence of cell processes. The box is shown in higher magnification in (B). Note that some axons (ax) started to be wrapped by myelin sheath (white arrowhead).

(B) A magnified micrograph shows a paranuclear Golgi apparatus (arrow) and multiple round mitochondria (arrowheads). The bold arrow points to the nucleus site.

Scale bar = 2 μ m in (A) and 500 nm in (B).

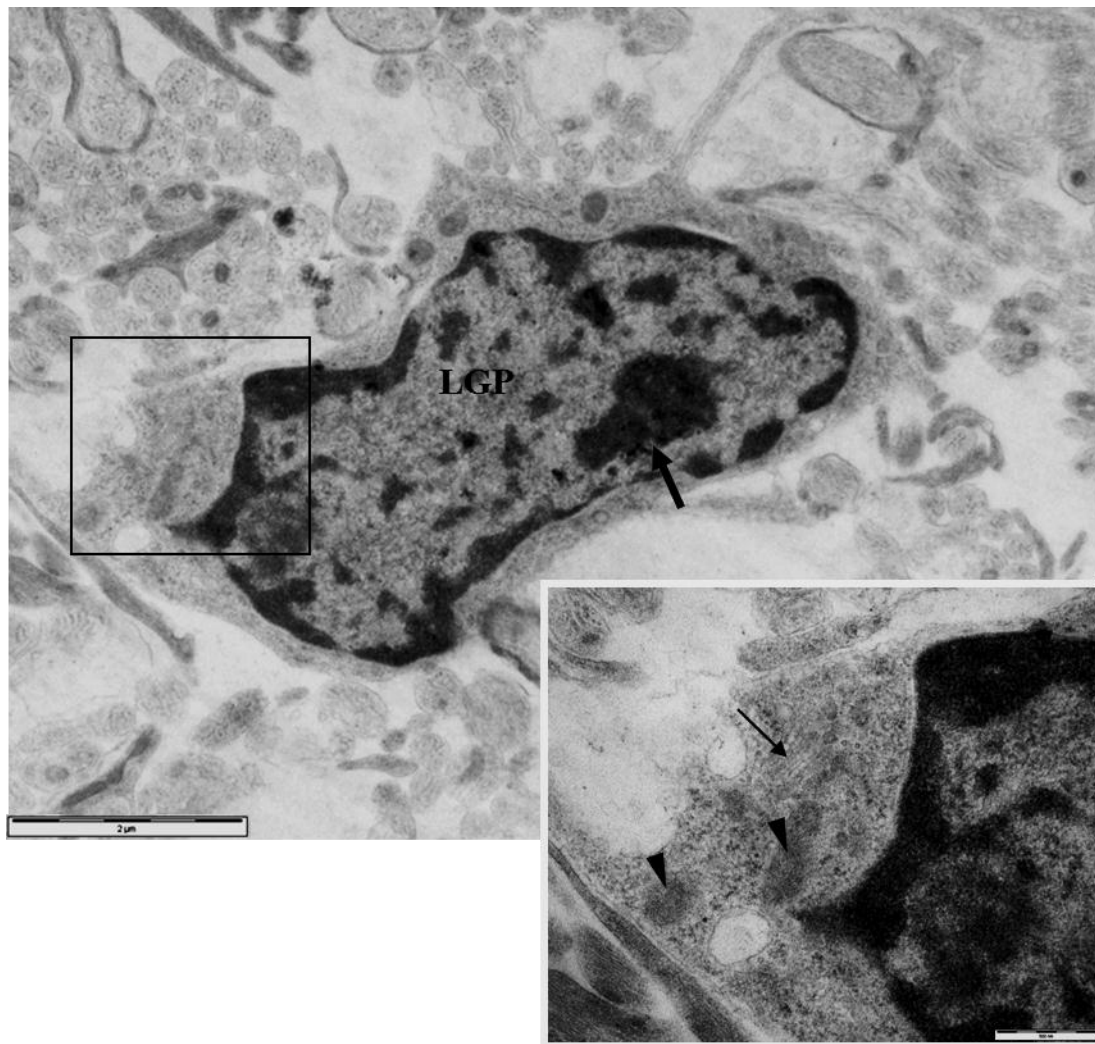


Figure 1-4: Ultrastructural features of large glial precursor cell in P10 RON.

Micrograph shows a large glial precursor cell with a prominent nucleolus (bold arrow) inside an irregularly shaped nucleus with chromatin aggregation beneath nuclear envelope. The box is shown in a higher magnification in the inset, in which the arrow points to the Golgi apparatus. Note the few rudimentary processes. Arrowheads point to mitochondria.

Scale bar = 2 μ m and 500 nm in inset.

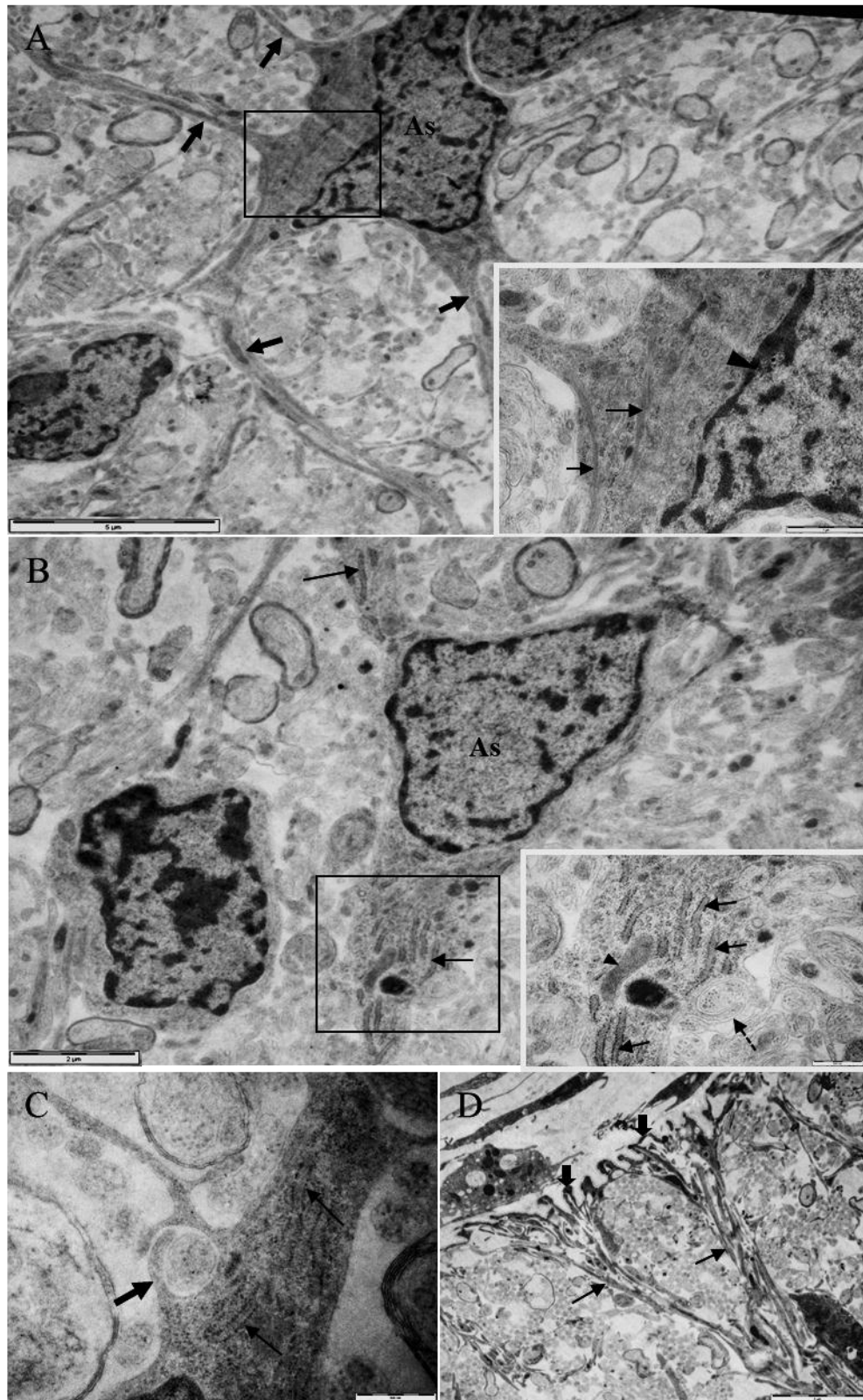


Figure 1-5: Astrocyte ultrastructural features in P10 RON.

(A) Micrograph shows the stellate morphology of astrocytes with multiple cell processes (bold arrows) extended from the cell body and perpendicular to the axons. The nucleus looks irregular in shape, with a thin rim of chromatin under its envelope. Boxed area is shown in a higher magnification in the inset. The inset shows some obvious glial filaments (arrows). Arrowhead points to the nucleus.

(B) Micrograph shows obvious wide ER which is filled with dense debris (arrows), which is a characteristic feature for astrocytes. Notice the homogenous distribution of chromatin inside the nucleus, which also aggregates in a thin rim under the nuclear envelope. Boxed area is shown in a higher magnification in the inset and shows wide, rough ERs filled with dense debris (arrows), an elongated mitochondria (arrowhead), and a finger-like projection surrounding an unmyelinated axon (dotted arrow).

(C) A high power micrograph shows an astrocyte cell process with finger-like projections surrounding unmyelinated axons. Notice the wide rough ER.

(D) A low power micrograph shows the astrocytes' cell processes (arrows) directed towards glia limitans (bold arrows).

Scale bar = 5 μm in (A) and (D), 2 μm in (B), 1 μm in (A: inset), 500 nm in (B: inset) and (C).

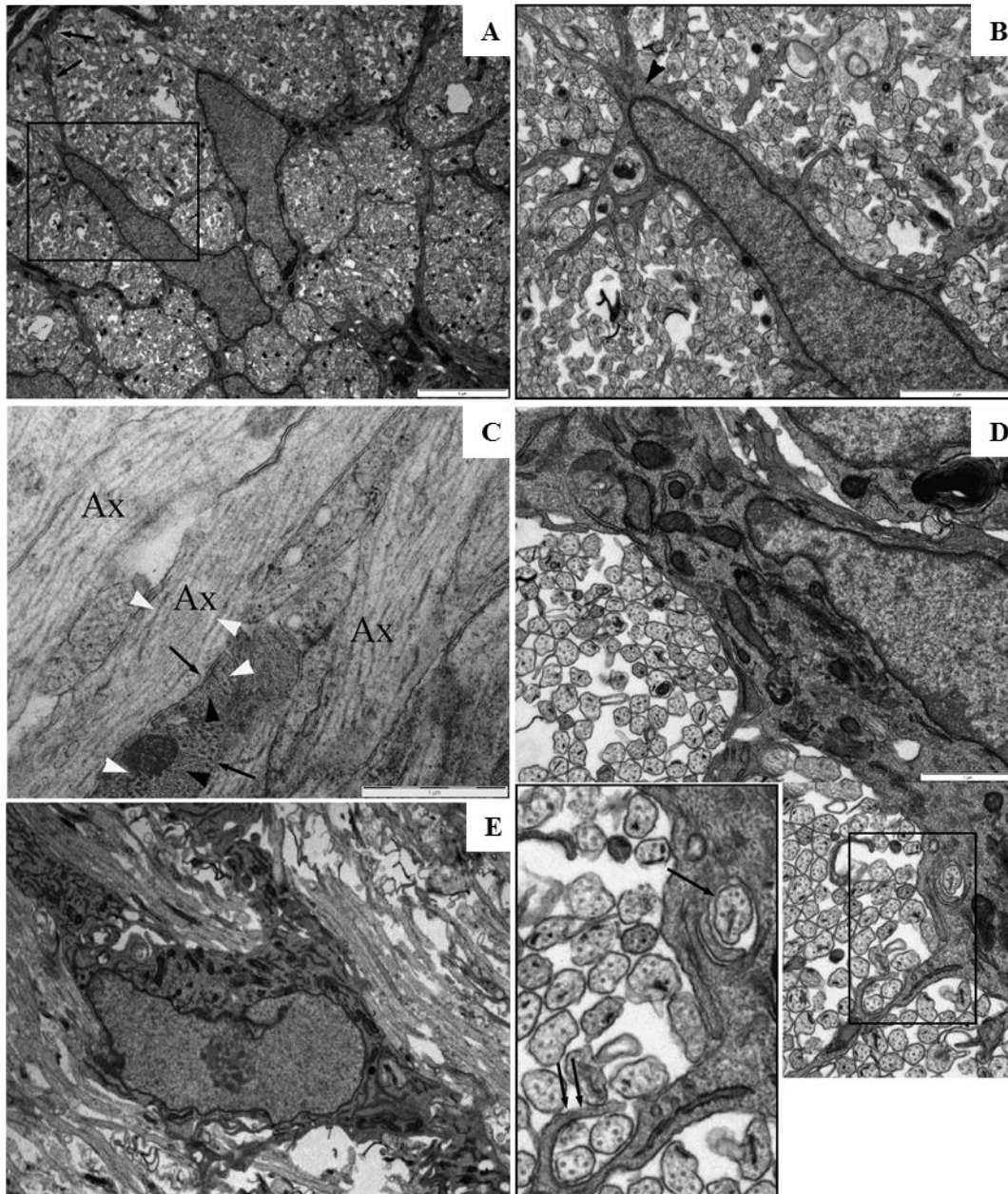


Figure 1-6: Astrocytes ensheath axons.

(A) Low magnification micrograph showing two neighbouring astrocytes in cross section P10 RON. The boxed area is shown at higher power in (B), revealing the highly ramified nature of the many fine processes that extend from the soma and main branches of the cell. The cell can be identified as an astrocyte due to occasional GFAP fibres (arrowhead) and the contribution the cell makes to the glial limitans (arrows).

(C) Longitudinal section of P10 RON at high gain, showing the close apposition of premyelinated axons ("Ax") and astrocyte processes (arrows). Microtubules (white arrowheads) and neurofilaments are arranged longitudinally in axons, while GFAP filaments (black arrowheads) are arranged generally transversely in astrocyte processes.

(D) Montage showing an astrocyte ensheathing a single axon (arrow) and extending a finger process around two axons (double arrow). Boxed area is shown at higher magnification in the inset.

(F) An astrocyte shown in longitudinal section extending thick processes parallel to pre-myelinated axons.

Scale bar = 5 μm in (A), 2 μm in (B, and F), 1 μm in (C, and D).

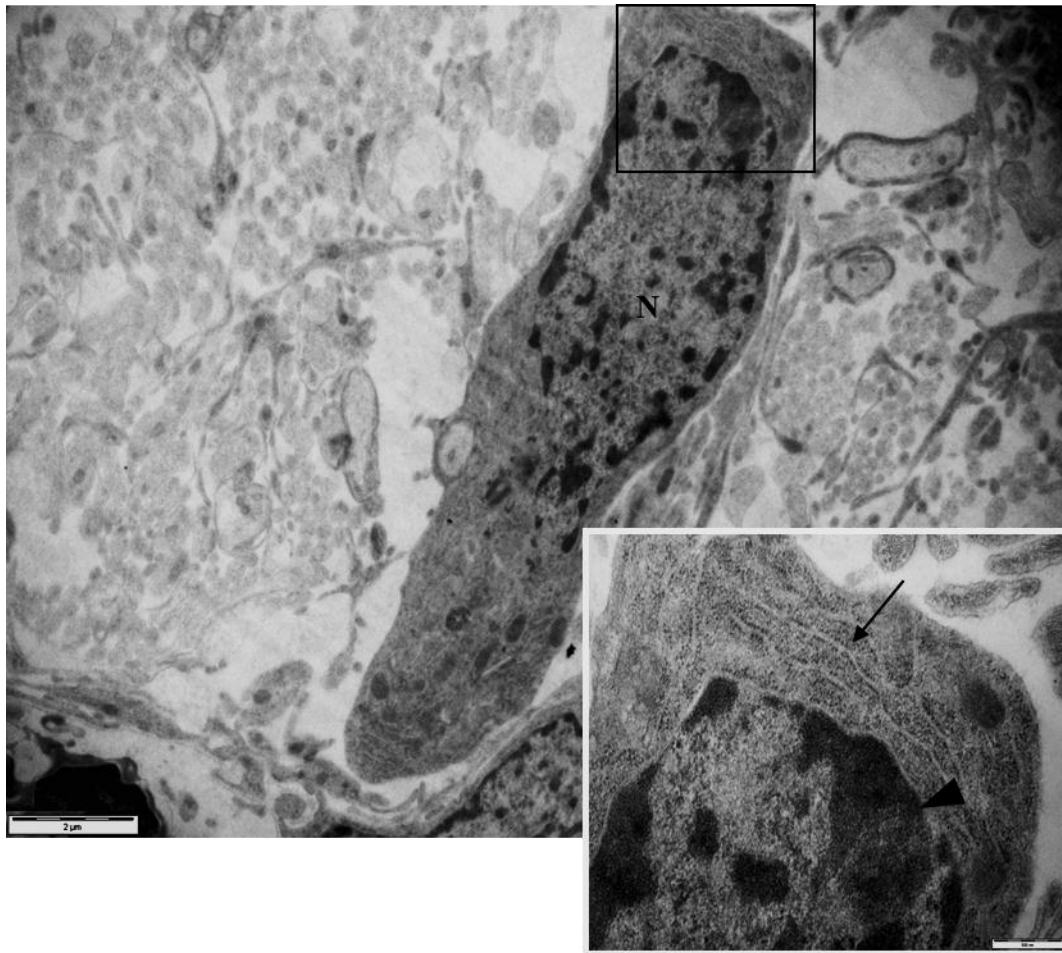


Figure 1-7: Mature oligodendrocyte in P10 RON.

A low power micrograph shows the oval morphology of oligodendrocyte. Notice the high density of the cytoplasm and the nucleoplasm. The nucleus (N) is present near one pole of the cell and the chromatin aggregate beneath nuclear envelope. Boxed area is shown in higher magnification in the inset, which shows long, narrow pore ER (arrow) near the nucleus (arrowhead).

Scale bar = 2 μm and 500 nm in the inset.

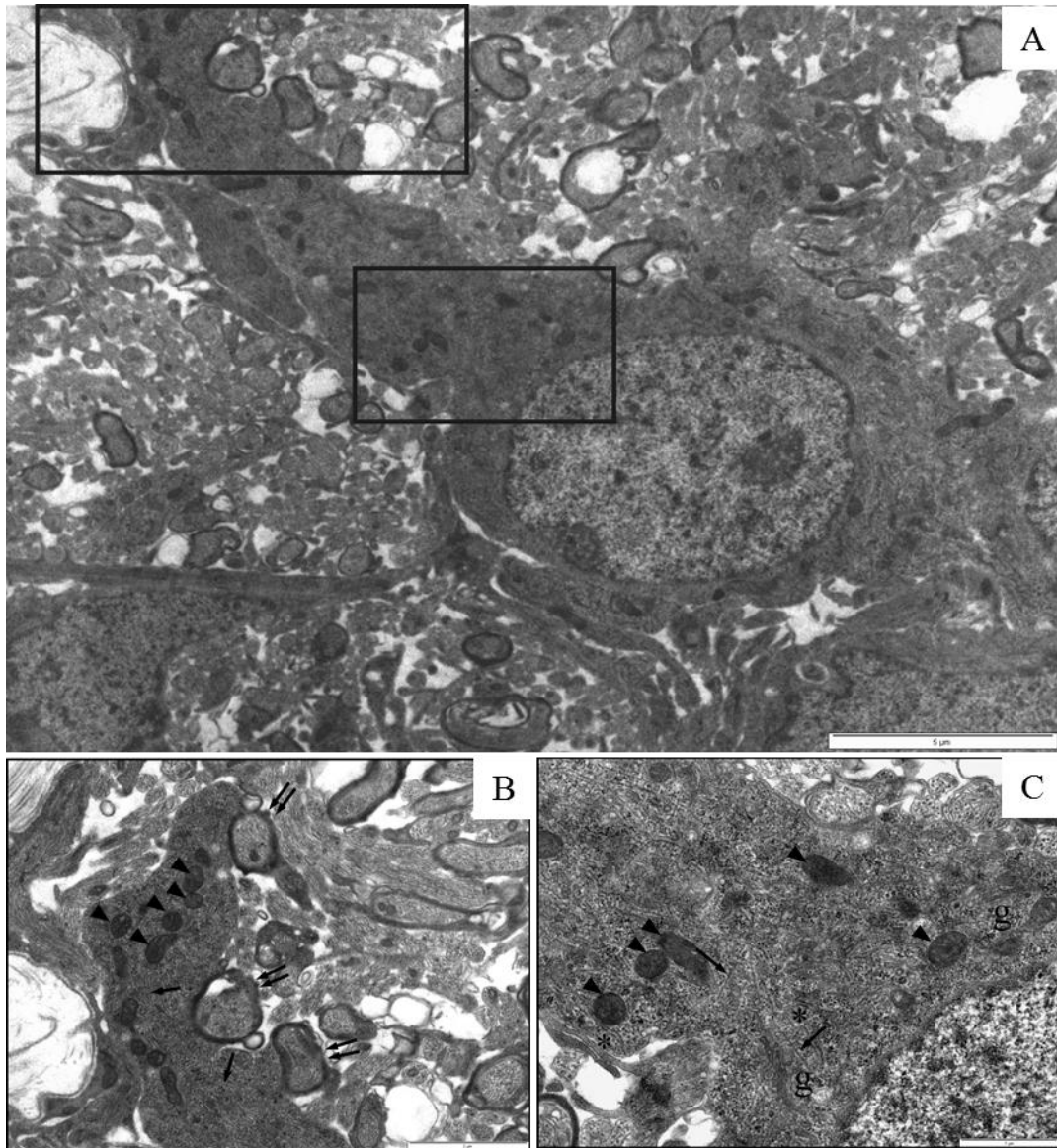


Figure 1-8: Active oligodendrocyte ultrastructural morphology in P10 RON.

(A–C) Typical oligodendrocyte (boxed areas in (A) shown at higher gain in (B) and (C)). This cell contains numerous mitochondria (arrowheads), Golgi apparatus (“g”), narrow-bore ER (“*”) and microtubules (arrows). A large process is actively myelinating several large-diameter axons (double arrows).

Scale bar = 5 μm in (A); 2 μm in (B); 1 μm in (C).

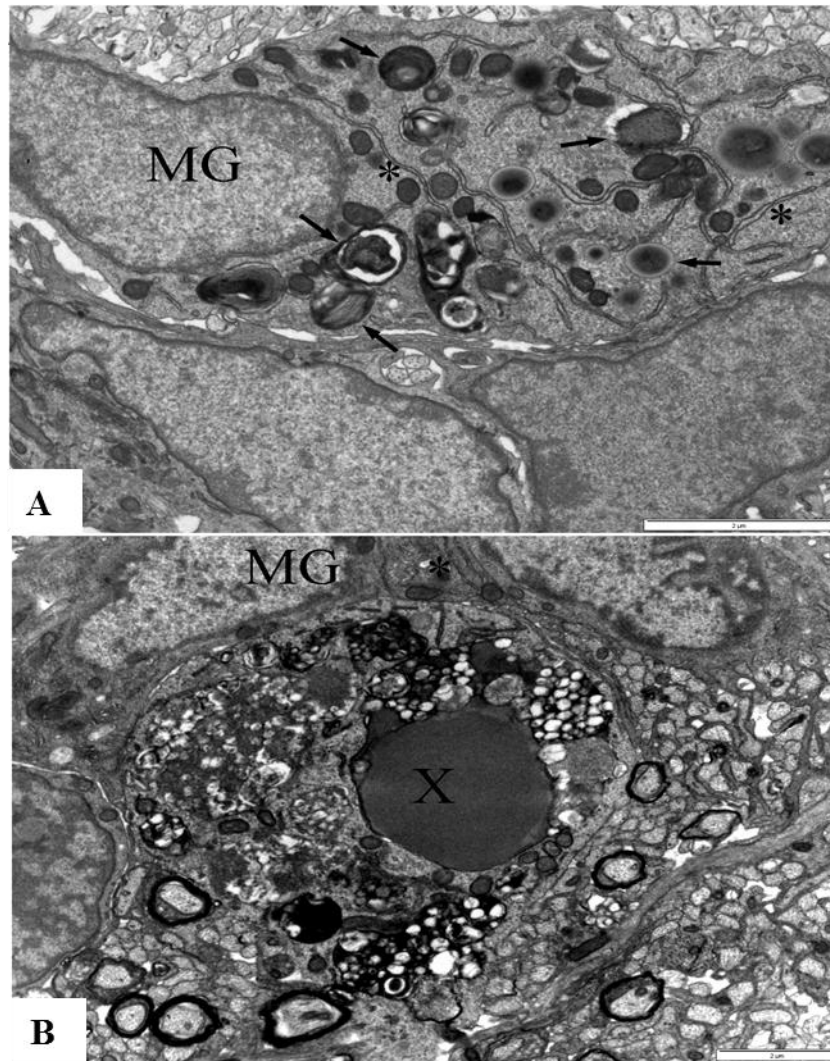


Figure 1-9: Microglia in P10 RON.

(A) Typical microglial cell (“MG”), with a nuclear morphology similar to that of other glia but containing extensive elongated ER (“*”) and various types of dark body and vesicular structures (arrowheads). Two neighbouring glia appear normal.

(B) Apoptotic cell (“X”) with a neighbouring microglial cell (“MG”) containing elongated ER (“*”).

Scale bar = 2 μ m.

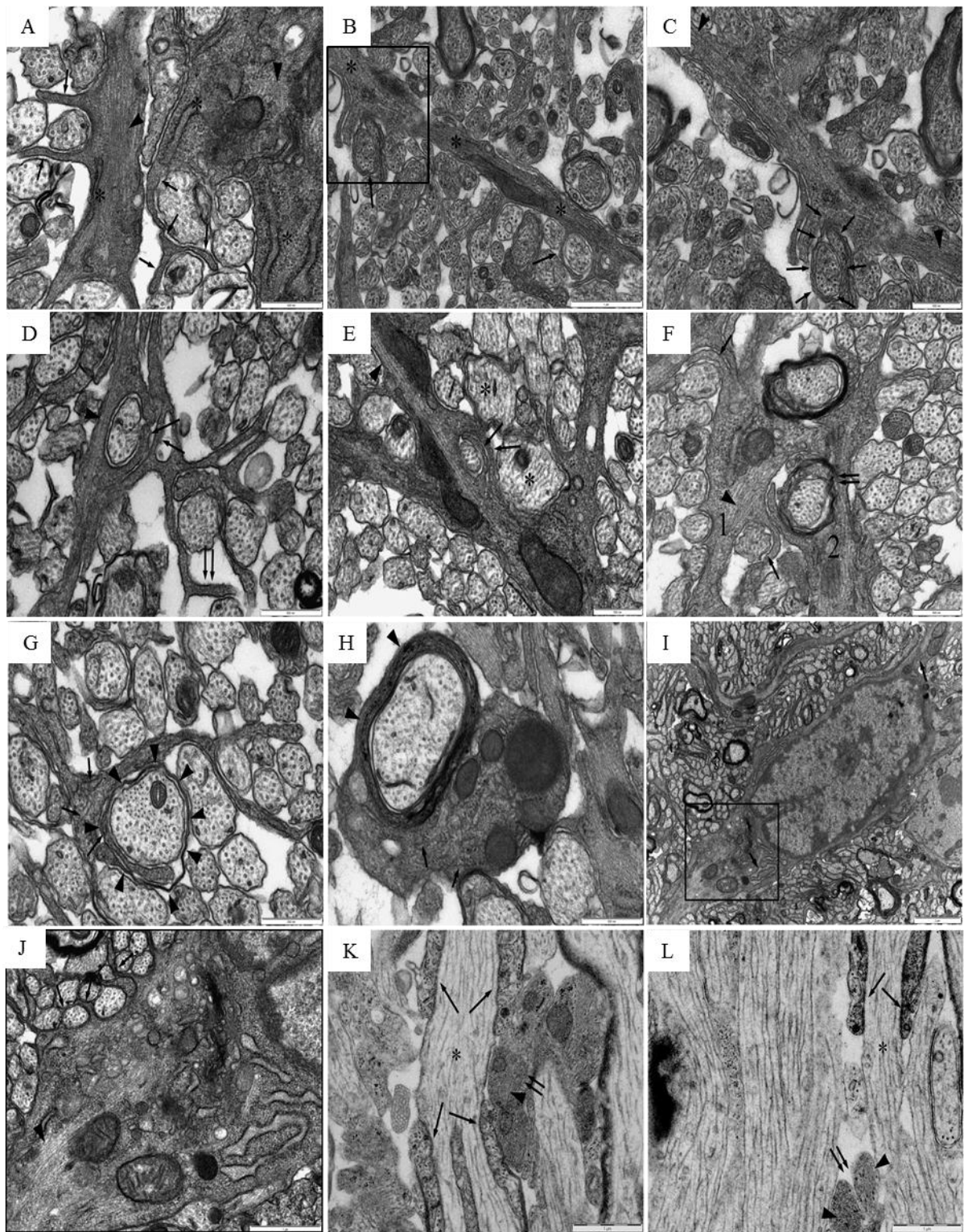


Figure 1-10: Glial processes in P10 RON.

A-I Micrographs of nerve cross sections.

(A) Two astrocyte processes identified by the presence of GFAP filaments (dark arrowheads), also containing wide-bore ER ("*"), extend finger-like processes between small-diameter pre-myelinated axons (arrows).

(B–C) An astrocyte process oriented radially (“*”) navigates between pre-myelinated axons. Ensheathing processes either partially or completely surround several neighbouring small-diameter axons (arrows). Boxed area in (B) is shown at higher gain in (C), revealing extensive GFAP filaments within the main astrocyte processes (arrowheads) and cytoplasmic continuity of an ensheathing process (arrows).

(D–E) Examples of double-wrapping of small-diameter pre-myelinated axons (dark arrows) by GFAP-containing (arrowheads) processes. Note the presence of neighbouring larger-diameter pre-myelinating axons that are not ensheathed (e.g., “*”), and the *en passant* nature of the ensheathment, with processes continuing on to navigate between neighbouring axons (e.g., double arrows).

(F) Two glial processes. Process 1 contains GFAP filaments (dark arrowhead) and has partially ensheathed several small axons (e.g., dark arrows). Process 2 appears to have wrapped several layers around a larger axon (double arrows) and looks similar to 1, other than containing no obvious filaments.

(G) A glial process containing neurofilaments, some oriented transversely (arrows), but not GFAP filaments, navigates between small-diameter axons and has initiated wrapping of a large axon (arrowheads).

(H) Oligodendrocyte process showing multiple layers of myelin (arrowhead) contains neurofilaments (arrows) but no GFAP filaments. Note the presence of GFAP filaments in neighbouring glial processes.

(I) Astrocyte somata; note the wide-bore ER (arrows) and the characteristic heterochromatin. The boxed area is shown at higher gain in “J”.

(J) Note the GFAP filaments (arrowhead) and the finger processes originating directly from the soma ensheathing small-diameter axons (e.g., arrows).

(K–L) Examples of longitudinal-section micrographs showing early myelination of a node of Ranvier in “K” and a hemi-node in “L” (“*”). Oligodendroglial processes navigate along the axon (arrows), and GFAP-filled (arrowheads) astrocyte processes cluster adjacent to the putative nodal membrane but do not wrap around it (double arrows).

Scale bar = 500 nm except in (B), (J), (K), and (L) = 1 μm , and in (I) = 2 μm .

Section 2:

The features of NG2 expressing cells
in RON

CHAPTER 2 : INTRODUCTION

2.1 Classification of Nervous System Cells:

In the early 1970s, cells of the nervous system were categorised into four groups, depending on their electrophysiological properties and specific cell markers: neurons, glia, pseudoneurons, or pseudoglia (Wilson et al., 1981, Schubert et al., 1974, Bulloch et al., 1977, Stallcup and Cohn, 1976, Arner and Stallcup, 1981). For example, any cell with the ability to generate an action potential, with voltage-dependent Na^+ and K^+ channels, and that expresses any of the neuronal specific antigens (N1, N2 and N3) was considered to be a neuron. If a cell had Na^+ and K^+ channels similar to neurons, but failed to generate an action potential or express neuronal markers, it was called a pseudoneuron. On the other hand, any non-excitabile cell which expressed glial specific antigens (G1 and G2) and which lacked voltage-dependent Na^+ and K^+ channels was classified as glia. However, if a cell shared the same properties of a glia, in addition to the presence of K^+ channels, it was considered to be a pseudoglia (Arner and Stallcup, 1981, Bulloch et al., 1977, Schubert et al., 1974, Stallcup and Cohn, 1976, Wilson et al., 1981). This was the widely known classification of the time, until Dr Stallcup and his co-workers identified the NG2 antigen as a shared surface antigen for both pseudoglia and pseudoneurons, by applying the specific antisera, anti-B49 serum, to the neuronal cell lines B103 and B65 (1981). Broadly speaking, NG2 expressing cells usually exhibit some characteristics of both neurons (N) and glial cells (G), and this was the reason for the name (NG2).

2.2 NG2 Antigen Background:

The NG2 antigen is in the class of chondroitin sulphate proteoglycans and consists of three main parts: extracellular, intracellular and transmembrane (Stallcup, 2002). The short, 76 amino acid, intracellular part contains a PDZ (post-synaptic

density) protein (PSD95), drosophila disc large tumour suppressor (DlgA), and zonula occludens-1 protein (ZO-1) binding site, a proline-rich segment and a threonine phosphorylation binding site (Songyang et al., 1997, Barritt et al., 2000, Nishiyama et al., 1991a). The intracellular region is separated from the extracellular region by a 25-residue transmembrane chain, and for this reason, NG2 proteoglycan is classified as a membrane spanning molecule (Stallcup, 2002). The third part of the NG2 proteoglycan molecule is the large 2225 residue extracellular component which is divided into three subdomains: a membrane proximal globular domain that has a proteolytic site, a central domain with a single chondroitin sulphate chain and type V and VI collagen binding sites, and an N-terminal globular domain which is stabilised by disulphide bonds (Burg et al., 1997, Nishiyama et al., 1995, Stallcup, 2002, Stallcup and Dahlin-Huppe, 2001, Tillet et al., 1997). The rotatory shadowing technique has shown the morphology of the purified NG2 proteoglycan molecule under the electron microscope, which shows its arrangement in pairs of globules (Tillet et al., 1997).

NG2 is not only expressed in the CNS but is also found in immature chondroblasts (Nishiyama et al., 1991b), developing skeletal muscles and bone (Fukushi et al., 2003, Petrini et al., 2003), pericytes (Ozerdem et al., 2001) and Schwann cells (Schneider et al., 2001). Moreover, NG2 proteoglycan is also expressed by some types of tumours such as melanomas (Real et al., 1985, Burg et al., 1998), glioblastomas (Chekenya et al., 1999, Schrappe et al., 1991), chondrosarcomas (Leger et al., 1994) and lymphoid leukaemia (Smith et al., 1996). Based on the fact that NG2 is expressed by immature progenitor cells and some types of tumour cells, which are known to proliferate and migrate, NG2 is suggested to contribute to cell proliferation and motility (Stallcup, 2002).

2.3 NG2 Expressing Cells:

Since the identification of the NG2 antigen in the early 1980s, intensive studies of NG2 expressing cells have been carried out, creating disagreements and debates which have been stimulating this field of research for several years now. William Stallcup was the first to investigate the localisation of the NG2 antigen in rat CNS cultures by applying IHC, which revealed NG2 expression by multiple process-bearing cells (Stallcup et al., 1983). Several studies followed, giving rise to multiple names for the NG2 expressing cells, such as OPC (Ong and Levine, 1999), O2A (Raff et al., 1983), polydendrocytes (Nishiyama et al., 2002), synantocytes (Butt et al., 2002b), β astrocytes (Reyners et al., 1982) and β neuroglia (Peters, 2004).

2.3.1 Origin of NG2 (+) Cells:

The origin of platelet derived growth factor alpha receptor (PDGF α R) (+) cells in the E13.5 rat forebrain has been suggested to be the anterior entopeduncular eminence (AEP), between the median ganglionic eminence (MGE) and anterior hypothalamus (Pringle et al., 1992, Tekki-Kessaris et al., 2001). The first appearance of NG2 (+) cells was detected near E17 in the rat CNS, which was 2 days after the first appearance of PDGF α R (+) cells (Nishiyama et al., 1996a). Both PDGF α R (+) and NG2 (+) cells were shown to be distributed throughout the rat CNS, with a high level of NG2 and PDGF α R overlap (Nishiyama et al., 1996a). NG2 and PDGF α R co-expression increases and reaches a peak during late embryonic development, with some exceptions in the subventricular zone (SVZ) where there are PDGF α R (+) / NG2 (-) cells (Nishiyama et al., 1996a, Tekki-Kessaris et al., 2001). Some studies suggest the role of SVZ in NG2 (+) cell generation during postnatal and adult periods. Marking the perinatal SVZ by retroviral injection revealed that NG2 (+) cells of the corpus callosum

and neocortex are generated from the SVZ (Levison et al., 1999). GFP retroviral injection into the adult SVZ revealed that GFAP (+) astrocytes (type B cells) can generate NG2 (+) cells in the corpus callosum (Gonzalez-Perez et al., 2009, Menn et al., 2006). Moreover, fate mapping studies reported that NG2 (+) cells and oligodendrocytes are generated from radial glial cells in the striatum (Malatesta et al., 2003, Malatesta et al., 2000). Consistent with this, a study of dorsal radial glial cells injected with adenovirus expressing Cre recombinase revealed detectable Cre in NG2 (+) cells, suggesting the ability of radial cells to produce NG2 (+) cells of the neonatal neocortex and subcortical white matter (Ventura and Goldman, 2007). In the visual pathway, progenitor cells were spread from the chiasma into the retinal end of the optic nerve without penetrating the retina (Colello et al., 1995, French-Constant et al., 1988).

2.3.2 Proliferation and Distribution:

One of the significant features of NG2 (+) cells is that they are capable of cell division in the developing and mature CNS. Previous studies have reported high bromodeoxyuridine (BrdU) uptake in NG2 (+) cells, reaching around 65% (various ages), in both the white and grey matter of the adult rat brain and spinal cord (Horner et al., 2000, Lasiene et al., 2009). It is suggested that NG2 (+) cells divide symmetrically, and the cell cycle of these cells was estimated to be less than 2 days at P6, around 9 days at P60, and 70 days at P240 or P540 in the corpus callosum, while in the cortex, it was 48 hours at P6, 78 hours at P9, 18–37 days at P60 and 170 days at P540 (Kukley et al., 2008, Psachoulia et al., 2009, Simon et al., 2011). It has also been suggested that the extended cell-cycle time in grey matter is likely due to the increased length of the G1 phase (Simon et al., 2011). As was previously demonstrated, NG2 (+) cell proliferation reaches a peak in the first postnatal week and declines after that; however, there is still

a population of NG2 (+) cells in the adult CNS that is shown to be mitotically active (Clarke et al., 2012, Nishiyama et al., 1996a).

Plenty of NG2 glial cells are distributed throughout the adult CNS, making up 8–9% of the total white matter cell population and 2–3% of the total grey matter population (Dawson et al., 2003). A comparison between NG2 cell densities in different white and grey matter regions shows a slight increase in the total NG2 cell count density in white matter when compared to grey matter (Dawson et al., 2003). In the spinal cord, NG2 cells represent around 2.5% of all white matter glial cells and 2.3% of all grey matter glial cells (Bjugn, 1993, Bjugn and Gundersen, 1993). However, different regions of the spinal cord show different densities of NG2 cells, with a higher density found in the ventro-medial side of the spinal cord (Horner et al., 2002). In the brain stem, there is no significant difference in NG2 cell densities in white matter regions, such as the spinal tract of the trigeminal nerve (TST), or grey matter regions, such as the spinal nucleus of the trigeminal nerve (SNT) (Levine and Reynolds, 1999). NG2 immunopositive cells were found to be distributed throughout the hippocampus and cerebellar layers, and they delineate the CA1, CA2 and CA3 structures of hippocampus (Levine and Card, 1987). These cells are also spread over the cerebral cortex to the dentate gyrus (Ong and Levine, 1999). However, it is obvious that some layers have lower NG2 immunopositive cell densities than others, such as the stratum pyramidale and stratum granulosum, which could be due to the presence of a high level of neuronal cell bodies in these layers (Ong and Levine, 1999).

2.3.3 Importance:

NG2 is a member of the chondroitin sulphate proteoglycan group, which was previously thought to inhibit axon growth (Snow et al., 1990, Brittis et al., 1992, Oakley and Tosney, 1991). More recently, thoughts about the impermissive behaviour of NG2 (+) cells towards axonal growth have changed. NG2 knockout and wild type mice examined after spinal cord transactions revealed no difference in the extent of axon regeneration (de Castro et al., 2005). Yang and co-workers studied the relationship between NG2 (+) cells, instead of NG2 antigens, and the growing axons of rat hippocampal and neocortical neurons, and their study revealed that NG2 expressing cells facilitate axonal growth (Yang et al., 2006). Following spinal cord injury, successfully regenerated axons were found in NG2 (+) cell dense areas (Jones et al., 2003). Using time-lapse microscopy, they found that growth cones preferred contact with NG2 expressing cells rather than myelin-associated glycoprotein (MAG-CHO) cells (Yang et al., 2006). Interestingly, it has been shown that NG2 expressing cells have the ability to penetrate the lesion site to create a favourable environment for regenerating axons in adult DRG (dorsal root ganglion) neurons (Busch et al., 2010).

NG2 (+) cells are shown to contribute to many CNS injuries. Focal ischaemia following MCAO in NG2CreBAC:ZEG double transgenic mice showed a sudden increase in the EGFP (+) NG2 (+) levels at the site of injury when compared to controls (Honsa et al., 2012). Moreover, NG2 (+) cells increased in the case of global ischaemia following bilateral common carotid artery occlusion with decreased oxygen intake, and showed an increased proliferation rate (Pivonkova et al., 2010). Spinal cord injury of adult rats revealed a dramatic increase in the NG2 expression, which reached a maximum level at 4 weeks following the injury (Wu et al., 2005). NG2 expression was shown to be upregulated following hippocampal excitotoxic injury produced by kainic

acid, in which NG2 (+) cells underwent reactive changes (Bu et al., 2001). A similar result was observed in reactive NG2 (+) cells following a punctured adult rat cerebellum (Levine, 1994). These findings demonstrate the active role of these cells in CNS injuries, and a possible contribution to the inhibition of CNS damage.

NG2 (+) cells are found in association with multiple CNS diseases. Some evidence has shown that NG2 (+) cells play a role in remyelination, which is important in the case of multiple sclerosis (MS), an inflammatory demyelinating disease (Wilson et al., 2006, Jennings and Carroll, 2010). In the case of hippocampal chronic temporal lobe epilepsy (TLE), it has been reported that around 79% of newborns' cells give rise to astrocytes or NG2 (+) cells, which is around three times higher than the 25% produced in the intact hippocampus (Hattiangady and Shetty, 2010). Moreover, it has been found that electroconvulsive therapy (ECT), an effective treatment for severe depression, mediated reactivation changes in glial cells, especially in the microglia, astrocytes and NG2 (+) cells tested in different brain regions (Jansson et al., 2009). It is suggested that electroconvulsive shock (ECS), the rat counterpart of ECT, interferes with the glial proliferation inhibition effect of glucocorticoid, which is associated with depression (Wennstrom et al., 2006). These studies suggest the significant contributions of NG2 (+) cells to CNS diseases.

2.3.4 Progeny:

NG2 (+) Cells and OPCs:

In 1983, Raff et al. were the first to observe the ability of A2B5 (+) glial progenitor cells (of perinatal rat optic nerve cultures) to differentiate into process-bearing GFAP (+) type 2 astrocytes in the presence of serum, or into GC (+)

oligodendrocytes in serum-free solution (Raff et al., 1983). Several years later, it was shown that NG2 is expressed on the surface of A2B5 (+) O2A cells in perinatal optic nerve cultures (Stallcup and Beasley, 1987) and perinatal cerebellar culture (Levine and Stallcup, 1987). NG2 (+) A2B5 (+) cells are shown to differentiate to GFAP (+) astrocytes or GC (+) oligodendrocytes in perinatal RON and cerebellar culture, depending on the culture conditions (Levine and Stallcup, 1987, Stallcup and Beasley, 1987). Since then, NG2 expressing glia have been known as OPCs or O2A cells. However, to test these results *in vivo*, it is difficult to rely on A2B5 for the identification of OPCs, since some neurons also express this antigen. Instead, PDGF α receptor is used as a reliable marker for OPCs with high cell specificity (Eisenbarth et al., 1979, Richardson et al., 1988). An immunofluorescence study revealed a similar distribution of NG2 and PDGF α R staining throughout the rat CNS in both the grey and white matter (Nishiyama et al., 1996a). Moreover, a close colocalisation of PDGF α R and NG2 (+) cells is found in the rat CNS at embryonic day 15 (E15) and reaches a peak in the first postnatal week (Nishiyama et al., 1996a). Double immunostaining studies showed a clear co-expression of PDGF α R and NG2 in O2A cells cultured from the neonatal rat cerebrum (Nishiyama et al., 1996b). It has been reported that NG2 (+) O2A cells can differentiate into O4 (+) oligodendrocytes and GFAP (+) astrocytes, while the level of NG2 staining is down-regulated with the cell differentiation (Nishiyama et al., 1996b). AN2, a mouse type of rat NG2 antibody, is found to be distributed in the mouse brain, optic nerves and spinal cord during development and even after maturation (Diers-Fenger et al., 2001). Immunohistochemistry showed an almost complete colocalisation between AN2 and PDGF α R staining during development, while only around 50% of the AN2 (+) cells were stained with PDGF α R antibodies (Diers-Fenger et al., 2001). AN2 (+) cells of P2 mouse forebrain cultures can

be differentiated into both O4 (+) oligodendrocytes and GFAP (+) astrocytes (Diers-Fenger et al., 2001). Double immunofluorescence labelling of adult rat cerebral cortex for NG2 with CNP, O4 or PDGF α R showed a high colocalisation of NG2 and PDGF α R in OPCs and confirmed the expression of NG2 in O4 (+) immature oligodendrocytes but never in CNP (+) mature oligodendrocytes (Dawson et al., 2003). All of these *in vivo* and *in vitro* studies suggest that NG2 (+) glial cells are OPCs but are not mature oligodendrocytes. This raises several questions regarding the identity of these cells in the mature CNS, such as the following: Do NG (+) cells represent a homogenous population? And do adult NG2 glial cells represent a distinct population?

NG2 (+) Cells and Astrocytes:

Several studies have reported bipotential NG2 (+) cells which can generate both astrocytes and oligodendrocytes *in vitro* (Levine and Stallcup, 1987, Raff et al., 1983, Stallcup and Beasley, 1987). On the other hand, immunofluorescence studies have reported no overlap between NG2 and GFAP staining of adult and developing rat cerebellum (Levine and Card, 1987, Levine et al., 1993). Similar findings were established in developing rat corpus callosum and showed that NG2 (+) cells did not express GFAP astrocyte markers (Nishiyama et al., 1996a). It is also reported that NG2 staining did not overlap with GFAP staining in the white matter of the adult rat spinal cord, optic nerves or cerebral cortex (Redwine et al., 1997, Hamilton et al., 2009, Dawson et al., 2003). However, double immunostaining with GFAP and NG2 revealed a significant expression of NG2 antigen in GFAP (+) adult reactive astrocytes and a lower uniform expression of NG2 antigen in GFAP (+) neonatal reactive astrocytes of cultured rat cortex (Hirsch and Bahr, 1999). Double immunolabelling with NG2 antigen and protoplasmic astrocyte markers, such as the calcium-binding protein S100 β , vimentin and glutamine synthetase (GS), revealed that there are distinct populations in

the P3 corpus callosum, adult cortex and cerebellum (Nishiyama et al., 1996a, Reynolds and Hardy, 1997, Levine and Card, 1987). In contrast, Dawson et al. found low levels of glutamine synthetase, a marker for some astrocyte populations, expressed by NG2 (+) cells in the adult rat cerebral cortex (2003). These studies have raised some confusion, and since then, the astrocyte progeny have become the centre of debate.

NG2 (+) Cells and Macrophages:

In the early 1990s, it was reported that embryonic but not neonatal NG2 (+) cells can be labelled with GD3 antibody, in which the latter is used as a marker for oligodendrocyte precursor cells (Levine et al., 1993). Later, it was clearly demonstrated that GD3 (+) cells in mature RON and cerebellum were microglia rather than oligodendrocyte precursors (Wolswijk, 1994, Wolswijk, 1995). Since then, there have been many studies which support this finding. Double immunolabelling with NG2 and different microglial or macrophage markers such as 4H1, GSA I-B4 (Griffonia simplicifolia isolectin B4), ED2 and F4/80 have revealed no overlap between these two populations in the adult and neonatal corpus callosum, cortex, cerebellum or spinal cord in normal or EME (experimental autoimmune encephalitis) conditions (Nishiyama et al., 1997). However, double immunolabelling with NG2 and ED1 or OX42 microglial markers showed NG2 expression in a subpopulation of macrophages in the case of kainic acid-induced excitotoxic lesions of the hippocampus, but not in normal conditions (Bu et al., 2001). Consistently, double immunolabelling has reported NG2 expression in IBA1 (+) macrophages following spinal cord injury (Jones et al., 2002). Moreover, an overlap between NG2 (+) cells and OX42 (+) macrophages has been noticed following trimethyltin (TMT)-induced neurodegeneration in the dentate gyrus at the site of injury, but not in areas away from the degeneration area (Fiedorowicz et al., 2008). Similar results have been reported following facial nerve injuries in which

OX42 (+) and lectin (+) microglial cells were labelled with NG2 (Zhu et al., 2010). In conclusion, these data suggest that NG2 could be expressed in reactive macrophages rather than resting microglia, and it has been suggested that this represents phagocytosis of NG2 (+) cells by macrophages. However, it has been suggested that NG2 expression in macrophages could diversely affect their functions (Zhu et al., 2012).

NG2 (+) Cells and Neurons:

The neuronal fate of NG2 (+) has been a matter of debate for several years. It has been shown that NF (+) neurons can be generated from A2B5 (+) OPC cultures of neonatal RON, only in the case of prolonged exposure to basic fibroblast growth factor (bFGF) (Kondo and Raff, 2000). Using early postnatal CNP-GFP transgenic mouse brain showed that NG2 (+) cells represent a majority of purely purified GFP (+) cells, which can differentiate into NeuN (+) or type 2a,b microtubule-associated protein (MAP2_{a,b}) (+) neurons *in vitro* (Belachew et al., 2003). Moreover, it has been reported that SVZ purified NG2 (+) cells can generate Er81 (+) neurons in the olfactory bulb, hippocampus and striatum after transplantation (Aguirre and Gallo, 2004). In contrast, purified DsRed (+) / NG2 (+) cells from NG2:DsRedBAC transgenic mice were examined and revealed that NG2 (+) cells did not differentiate into NeuN (+) neurons *in vitro* (Zhu et al., 2008a). Fate mapping study of Plp-Cre-ER transgenic mice in which Plp promoter was expressed by NG2 (+) cells showed that NG2 (+) cells can generate immature neurons in the hippocampus, forebrain and cerebral cortex (Guo et al., 2009). Fate mapping *in vivo* studies have reported some neuronal generation from oligodendrocyte precursor cells in the piriform cortex of PDGF α R-CreER transgenic mice (Rivers et al., 2008). However, using a similar line of PDGF α R-CreER transgenic mice revealed the absence of neuronal generation from oligodendrocyte precursor cells in the piriform cortex (Clarke et al., 2012). Similar results were achieved using a

different line of PDGF α R-CreER transgenic mice, which revealed no neuronal differentiation from NG2 (+) cells in the postnatal brain (Kang et al., 2010).

2.3.5 Morphology:

Stallcup was the first to describe NG2 (+) cells in IHC as multiple process-bearing cells in rat CNS cultures (Stallcup et al., 1983). The morphological development of NG2 (+) cells has been studied in the cerebellum *in vivo* (Levine and Card, 1987), and immunocytochemistry of the cerebellum shows embryonic (E16) NG2 (+) cells as simple in shape, with only one or two main processes emanating from the cell body, and having a lack of branches. Multiple branches begin to appear and increase in number during the postnatal period (Levine and Card, 1987). However, adult NG2 (+) cells are far more complex than the neonatal cells. Adult NG2 (+) cells are stellate in shape and are distributed throughout the neocortex, hippocampus and cerebellum and are relatively lower in density in areas of compact neuronal somas such as stratum pyramidale and stratum granulosum (Ong and Levine, 1999, Levine and Card, 1987). Adult NG2 (+) cells usually have irregular cell bodies which give rise to straight main processes (thick or thin) (Ong and Levine, 1999). Thin secondary and tertiary processes are usually highly branched from the main processes and have the ability to expand distally (Ong and Levine, 1999). It is reported that around 8–30 processes can branch from a single NG2 (+) cell (Dawson et al., 2003). However, white matter NG2 (+) cells have a more elongated shape than the spherical stellate NG (+) cells in grey matter (Dawson et al., 2003, Butt et al., 2002a). This reflects the high packing density of myelinated axons in the white matter, in which NG2 (+) cells extend their processes parallel to the axons. However, radial stellate NG2 (+) can be detected in white matter when axons are not longitudinally oriented with the cells (Butt et al., 2002a). It is established that NG2 (+) cells send their processes to contact nodes of

Ranvier, pial surface and synapses (Butt et al., 1999, Bergles et al., 2000, Levine and Card, 1987). In grey matter, NG2 (+) cell processes extend between neurons, forming a network which facilitates their contact with neurons, as seen in the cerebellum, cerebral cortex and hippocampus (Butt et al., 2002a). For this reason, Butt and his colleagues have given NG2 (+) cells the name “synantocytes”, since *synanto* means “contact” in Greek (2002).

At the ultrastructural level, several articles have studied NG2 (+) cells but lacked the use of I-EM. First, these cells were identified as beta astrocytes in the adult cerebral cortex, in which they are characterised by stellate morphology, an irregular nucleus containing clumped chromatin accumulated under the nuclear membrane, scanty cytoplasm containing small mitochondria and short rough ERs, and thick processes and a lack of glial filaments (Reyners et al., 1982). Later, it was reported that beta astrocytes are mitotically active and work as multipotent glial precursors in the adult cerebral cortex (Reyners et al., 1986). A good fixed tissue sample of an adult monkey cortex was examined for NG2 (+) cells by excluding other known cell types, in which NG2 cells were called “ β neuroglial” cells (Peters, 2004). This study revealed pale cells characterised by a pale and irregularly contoured nucleus containing a thin layer of chromatin under the nuclear envelope, pale cytoplasm consisting of thin mitochondria, centrioles, and a lack of intermediate filaments (Peters, 2004). Several studies have reported the close localisation of NG2 (+) cells to the blood vessels (Peters, 2004, Ong and Levine, 1999, Reyners et al., 1982).

On the other hand, there are only a couple of studies that have used I-EM to identify NG2 (+) cells and study their morphology. NG2 (+) cells were identified as “smooth protoplasmic astrocytes” in immunoperoxidase adult cerebellum sections, and these cells were characterised by a prominent, oval nucleus with a thin rim of chromatin

accumulated at the inner surface of the nuclear membrane, which was surrounded by scanty cytoplasm and few organelles (Levine and Card, 1987). The cell processes of these cells were extended distally between the axons and dendrites and lack of glial filaments (Levine and Card, 1987). In 1999, NG2 (+) cells were known as OPCs and were distinguished by their regular soma, heterochromatin accumulated under the nuclear membrane, scanty cytoplasm containing mitochondria with hardly any other organelles, and their irregular processes with no glial filaments or glycogen stores (Ong and Levine, 1999). It was found that NG2 (+) cells send their processes into the neuropil facing axonal terminal and dendrites, recognised by their synaptic vesicles and post-synaptic densities, respectively (Ong and Levine, 1999). This feature of multiple processes emanating from NG2 (+) cell bodies allowed Nishiyama and his colleagues to give NG2 (+) cells the name, “polydendrocytes” (Nishiyama et al., 2002). Depending on the nuclear shape, NG2 (+) cells of P21 rat hippocampus and cortex have been classified into two groups, in which the first is characterised by a regular shaped nucleus with clustered chromatin, while the other nuclear type has an irregular shape and scattered chromatin forming a thin rim under the nuclear envelope (Nishiyama et al., 2002). However, these studies have used pre-embedding I-EM and peroxidase, which greatly affected the contrast of the micrographs and limited the amount of detail in the figures.

2.4 Objectives:

NG2 (+) cells are a distinct type of glial cell which, over time, have come to be known as the fourth type of glial cell. These cells are distributed throughout the developing and adult CNS and are known to be mitotically active even in the adult CNS. Some studies have shown some similarities between NG2 (+) cells and astrocytes. The astrocyte fate of NG2 (+) cells is a matter of debate. In the current study, the focus was on studying the morphological features of NG2 (+) cells at the ultrastructural level and under confocal microscope, and more precisely, their relation with astrocytes in nRON following post-embedding immunolabelling technique. However, few studies have examined the morphology of the NG2 (+) glial cells at the ultrastructural level. Previous studies have used light fixation and immunoperoxidase techniques, which adversely affect the tissue contrast. It is important to reach an adequate level of fixation in order to clearly recognise the ultrastructural features while refacing the tissue antigenicity.

CHAPTER 3 : MATERIALS AND METHODS

3.1 Animals:

Wister rats aged P8—P14 and adults were provided by the animal house of Leicester University. They were culled by cervical dislocation following schedule 1, in accordance with the regulations of the British Home Office. The brain and the two optic nerves were immediately washed in Sorensen's buffer, and under a microscope, both optic nerves were dissected and submerged in the fixatives.

3.2 Immuno-Electron Microscope:

3.2.1 Buffer and Fixatives:

Phosphate buffer solution (PBS) (discussed in Section 2.12.1) and sodium cacodylate buffer were used in this technique. Sodium cacodylate buffer is found to be a good alternative to Sorensen's buffer, as it prevents the phosphate overexposure produced by PBS (Bozzola and Russell, 1999). It is made by mixing cacodylic acid with DDW and adjusting the pH to 7.2 with concentrated hydrochloric acid (HCl). Two types of primary fixatives were used. The first was 0.1 M Sorensen's with 3% glutaraldehyde, prepared by mixing 2.5 ml of 0.2 M Sorensen's with 0.6 ml of 25% glutaraldehyde and 1.9 ml of distilled deionised water. The second one was 0.1 M Sorensen's with 3% glutaraldehyde and 2% paraformaldehyde, made by mixing 2.5 ml of 0.2 M Sorensen's with 0.6 ml of 25% glutaraldehyde, 0.95 ml of 8% paraformaldehyde and 0.95 ml of distilled deionised water. A secondary fixation, 1% osmium tetroxide in 0.05 M buffer, was used in addition to the primary fixation.

3.2.2 Embedding Protocols:

There were several issues with the embedding protocols. The important issue is to reach an adequate level of fixation while refacing the tissue antigenicity. For this reason, non-osmicated P10 RON samples were examined, and they revealed a poorly fixed tissue in which only some cell remnants appeared (Figure 3-1). As a result, osmium was used in embedding the samples for all subsequent studies. Moreover, a wide variety of resins was available, and three types were tested in the following protocols (see Table 3-1). The samples went through several steps during the embedding, some of which can affect tissue antigenicity, such as fixation, dehydration, resin embedding and polymerisation (Spehner et al., 2002, Bozzola and Russell, 1999).

3.2.2.1 Protocol 1:

This protocol is used to embed P10 RON based on a common previously used protocol for RON (Constantinou and Fern, 2009, Salter and Fern, 2008a, Thomas et al., 2004, Wilke et al., 2004). Table 3-2 shows the details of protocol 1, in which the sample is passed through four main steps: fixation, dehydration, embedding and polymerisation. Samples were primarily fixed by 3% glutaraldehyde and then secondarily fixed by 1% osmium tetroxide. A gradual dehydration was introduced by using 70%, 90% and 100% ethanol. Samples were then embedded in Spurr's resin, a low viscosity epoxy resin, and polymerised in an oven. As a result, the tissue was fixed well and looked good under the electron microscope (Figure 3-2). However, due to poor availability of Spurr's resin due to health and safety concerns, I was unable to continue with this protocol, and alternative protocols were tried.

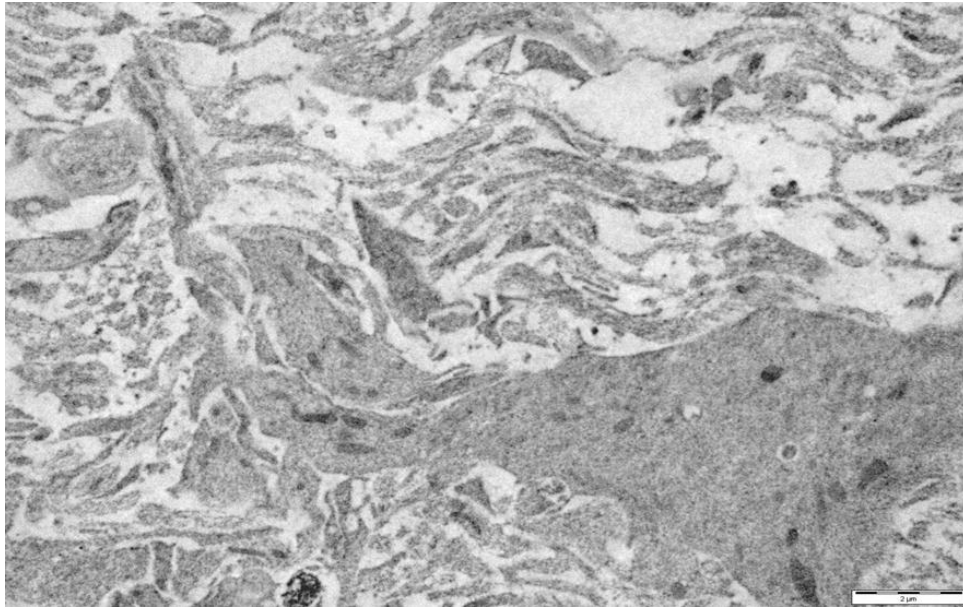


Figure 3-1: A non-osmicated P10 nRON.

Note the poor degree of fixation and contrast. All the tissue was destroyed, and only some cell remnants appeared.

Scale bar = 2 μ m.

Table 3-1: Types of resins utilised in this study.

Type of resin	Brief Background
Spurr's Resin & Modified Spurr's Resin	A widely used type of resin with a low viscosity that helps in penetrating different types of tissue. However, VCD resin is known to be highly toxic and carcinogenic, and for that reason, production of this type of resin is now prohibited. On the other hand, ERL 4221D resin, used in the modified Spurr's resin, is non-toxic and may be a good choice for embedding instead of the original Spurr's (Spurr, 1969, Ellis, 2006, Bozzola and Russell, 1999).
Agar Low Viscosity Resin	This type of resin is a low viscosity resin that is known to be a good replacement for Spurr's resin, due to its ability to penetrate the sample and provide a good contrast for the sections under the scope (Bozzola and Russell, 1999).
LR White Resin	This type of resin is recommended for immunolabelling. First, it is hydrophilic (i.e., it helps the solution to penetrate the section), and therefore, the antibodies attach to the receptor easily. LR White resin has a lower ability to dissolve lipids, and it preserves the structure of cell membrane and cytosol organelles. In addition, this type of resin does not interfere with the antigen (i.e., there is no need to etch the sections) (Bozzola and Russell, 1999).

Table 3-2: Embedding protocol 1 (original Spurr's resin).

Steps	Description	Duration
Primary fixation	3% glutaraldehyde in 0.1 M Sorensen's buffer at pH 7.4	90 minutes
Wash	Three washes in 0.1 M buffer	10 minutes each
Secondary fixation	1% osmium tetroxide in 0.1 M buffer	2 hours
Wash	Two washes in buffer	20 minutes each
	One wash with DDW	20 minutes
Dehydration	70% ethanol	40 minutes
	70% ethanol	Overnight
	90% ethanol	30 minutes
	100% ethanol twice	30 minutes each
Embedding	Propylene oxide twice	10 minutes each
	50:50 propylene oxide/Spurr's resin	4 hours
	Lid was pierced to allow propylene oxide evaporation	Overnight
	Resin was replaced with fresh Spurr's resin and left in mixer	5 hours
Polymerisation	Samples were embedded and polymerised in a 60 °C oven	16 hours

Table 3-3: Original Spurr's resin formulation. (Spurr, 1969)

	40 ml	30 ml	20 ml
1 VCD	10.0 gm	7.5 gm	5.0 gm
2 DER 736	4.0 gm	3.0 gm	2.0 gm
3 NSA	26.0 gm	19.5 gm	13.0 gm
4 Mix 5 minutes			
5 DMAE	0.4 gm	0.3 gm	0.2 gm
6 Mix 5 minutes			

VCD: vinyl cyclohexene dioxide; DER 736: diglycidyl ether of polypropylene glycol flexibiliser; NSA: nonenyl succinic anhydride; DMAE: accelerator dimethylaminoethanol.

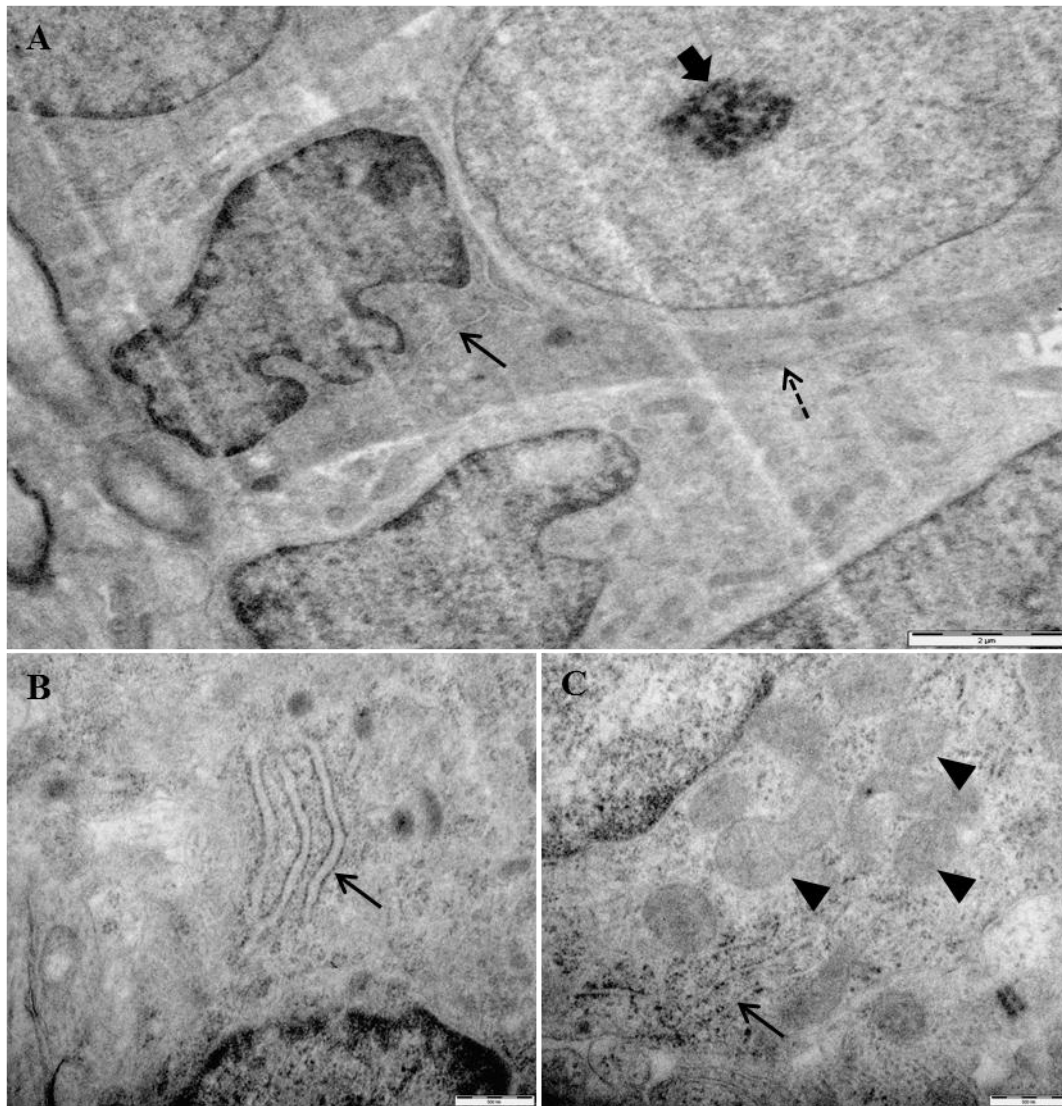


Figure 3-2: P10 nRON following protocol 1 and embedded in original Spurr's resin.

The sections appear well-fixed. (A) Cells, glial processes (dashed arrow) and intracellular organelles are shown as follows: ER (arrow) and nucleolus (bold arrow). Note the good contrast between the cell process (dashed arrow) and the adjacent cells. High magnification micrographs show rough, wide-bore ER studded with ribosomes (B, arrow), narrow pore ER (C, arrow) and mitochondria (C, arrowheads).

Scale bar = 2 μ m except in (B) and (C) = 500 nm.

3.2.2.2 Protocol 2:

This protocol was used to embed P14 neonatal RON. Table 3-4 shows protocol 2 steps, in which two types of primary fixation were tried: 3% glutaraldehyde and a combination of 3% glutaraldehyde / 2% paraformaldehyde. In trying to preserve some antigenicity, osmium tetroxide was applied for 60 minutes instead of 120 minutes as in protocol 1. Agar low viscosity resin was used in this protocol (see Table 3-1 and Table 3-5 for more details). After remounting the nerves, the glue used in the remounting was soft and did not provide enough support for the blocks during the cutting stage; as a result, gold ultra-thin sections could not be taken. The blocks were left for one week to let the glue dry, and they were polymerised again in a 60 °C oven for 16 hours; unfortunately, however, the glue was still soft and sticky. The next step was to trim out all the soft glue and replace it with a new one. The new glue was hard enough to support the block, but nice gold ultra-thin sections still could not be taken, and we started to think that the problem was with the resin itself. The blocks were again placed in the 65 °C oven for 16 hours, but unfortunately, there was no improvement in the resin condition. Before repeating the whole process, I thought it would be a good idea to know, at least, if the type of fixative had any effect on immunolabelling. As such, ultra-thin sections were taken by the technician in the EM lab using a diamond knife, and immunolabelling was conducted on these sections. The sections were poorly fixed, with a high background staining under the scope (Figure 3-3); therefore, this protocol was abandoned.

Table 3-4: Protocol 2 (Agar low viscosity resin).

Steps	Description	Duration
Primary fixation	3% glutaraldehyde or 3% glutaraldehyde/ 2% paraformaldehyde in 0.1 M Sorensen's buffer at PH 7.4	90 minutes
Wash	Three washes in 0.05 M buffer	10 minutes each
Secondary fixation	1% osmium tetroxide in 0.05 M buffer	60 minutes
Wash	Two washes in buffer	20 minutes each
	Two washes with DDW	20 minutes each
Dehydration	70% ethanol	20 minutes
	70% ethanol	Overnight
	90% ethanol	20 minutes
	100% ethanol three times	20 minutes each
Embedding	Propylene oxide twice	10 minutes each
	3 propylene oxide : 1 Agar low viscosity resin	60 minutes
	1 propylene oxide : 1 Agar low viscosity resin	90 minutes
	1 propylene oxide : 3 Agar low viscosity resin	60 minutes
	100% fresh agar low viscosity resin twice	30 minutes and overnight at 4 °C
	fresh 100% agar low viscosity resin twice	3 hours each
Polymerisation	Samples were embedded and polymerised in a 60 °C oven	16 hours

Table 3-5: Agar low viscosity resin formulation.

		60.0 g	50.0 g	40.0 g	30.0 g	%
1	LV resin (R1372)	28.0 g	23.25 g	18.5 g	14.0 g	46.5
2	VH1 Hardener (R1375)	6.0 g	5.0 g	4.0 g	3.0 g	10.0
3	VH2 Hardener (R1378)	24.5 g	20.5 g	16.5 g	12.2 g	41.0
4	Mix 5 minutes					
5	LV Accelerator (R1381)	1.5 ml	1.25 ml	1.0 ml	0.75 ml	2.5
6	Mix 5 minutes					

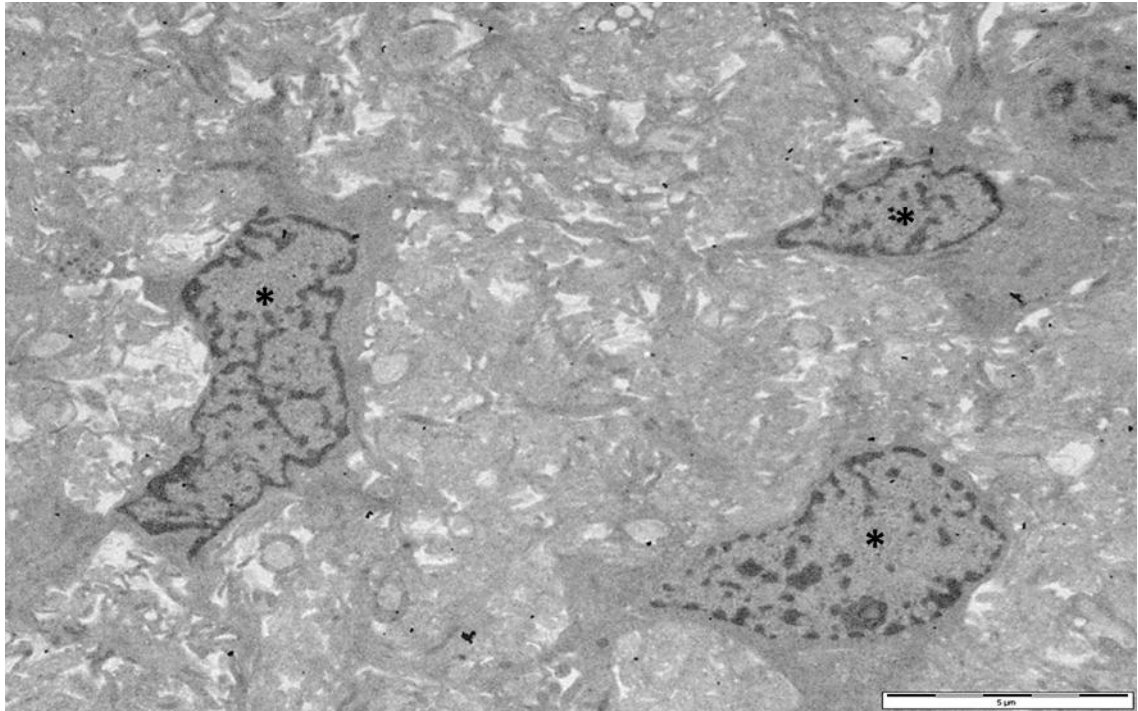


Figure 3-3: P14 nRON followed protocol 2 and was embedded in agar low viscosity resin.

Note the poor level of fixation, in which only the nucleus (asterisk) can be identified.
Scale bar = 5 μm .

3.2.2.3 Protocol 3:

This protocol was used to embed P8 neonatal RON; some nerves were fixed with 3% glutaraldehyde and the others with 3% glutaraldehyde / 2% paraformaldehyde (see Table 3-6). Secondary fixation was applied for 90 minutes instead of the 60 minutes of protocol 2. The samples were immersed and embedded in LR White resin (see Table 3-1).

As a result, the blocks were sectioned well. Semi-thick sections looked normal under the light microscope. After trimming down around 2 ml of the nerve, ultra-thin sections looked well fixed under the electron microscope. However, there was expanded extracellular space, as well as some holes at the edge of the nerve, and a lot of peppering (see Figure 3-4). Otherwise, the cells, cytoplasmic organelles, cell membrane, glial processes and axons could be identified. After immunolabelling, the sections had many large folds in each square of the grid, and holes at the edge of the nerve interfered with the identification of the cells and the semi-quantitative method needed for this study. Some steps were skipped in the protocol of immunolabelling in order to decrease the chance of these folds appearing (e.g., etching the sections and dichloromethane the sections). Unfortunately, the folds were still present. Chloroform vapour was tried instead of dichloromethane, because it is known to have a stronger effect than dichloromethane, but it made the problem worse. Blocks were again polymerised in a 50 °C oven for 16 hours. After that, similar immunolabelling steps were taken with these sections, without using antibodies, and the result was the same, without any change in the appearance of the folds (see Figure 3-4 B and C). The reason was thought to be the permeability characteristic of this type of resin, which became expanded by the multiple washes in the immunolabelling; therefore, this protocol was abandoned.

Table 3-6: Protocol 3 (LR White resin).

Steps	Description	Duration
Primary fixation	3% glutaraldehyde or 3% glutaraldehyde/ 2% paraformaldehyde in 0.1 M Sorensen's buffer at pH 7.4	90 minutes
Wash	Three washes in 0.05 M buffer	15 minutes each
Secondary fixation	1% osmium tetroxide in 0.05 M buffer	90 minutes
Wash	One wash in buffer	20 minutes
	Two wash with DDW	20 minutes each
Dehydration	70% ethanol	20 minutes
	70% ethanol	Overnight
	90% ethanol	30 minutes
	100% ethanol three times	20 minutes each
Embedding	3 100% analytic grade ethanol : 1 LR White resin	90 minutes
	1 100% analytic grade ethanol : 1 LR White resin	120 minutes
	1 100% analytic grade ethanol : 3 LR White resin	90 minutes
	100% fresh LR White resin twice	30 minutes and overnight at 4 °C
	100% fresh LR White resin twice	3 hours each
Polymerisation	Samples were embedded and polymerised in a 55 °C oven	24 hours

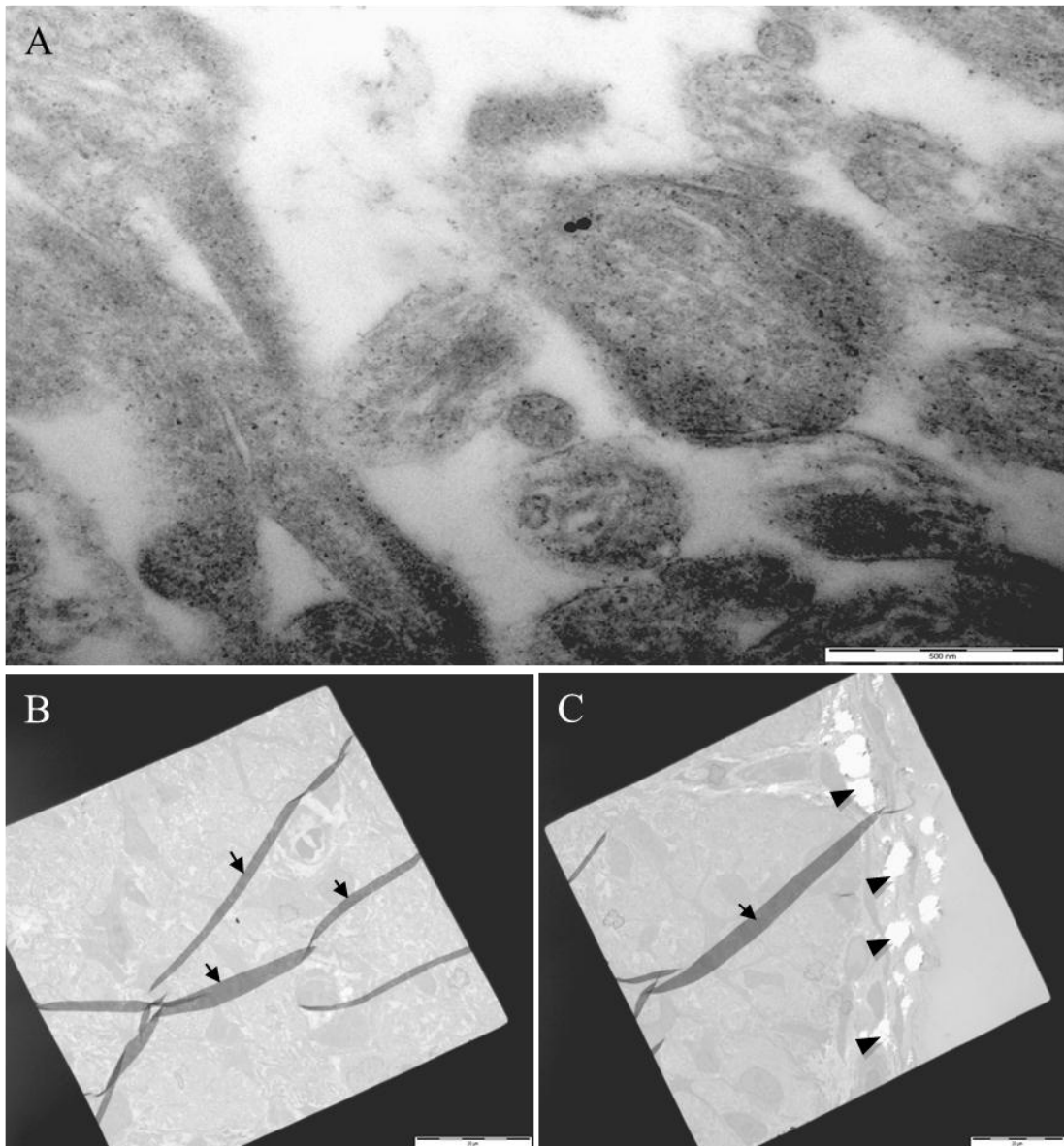


Figure 3-4: P8 RON following protocol 3 and embedded in LR White resin.

Micrographs show background peppering (A), large folds (arrows in B and C), and holes at the nerve edge (arrowheads in C).

Scale bar = 500 nm in A and 20 μm in B and C.

3.2.2.4 Protocol 4:

This protocol was used to embed a P10 neonatal RON using two types of resin: the original Spurr's resin, with only a small quantity available, and modified Spurr's resin (see Table 3-1). Some of the nerves were fixed with 3% glutaraldehyde, and the others with 3% glutaraldehyde / 2% paraformaldehyde. The fixation duration was increased, and the gradual dehydration was expanded from 30% to 100% (see Table 3-7). Blocks of original Spurr's resin sectioned well. Semi-thick sections looked normal under the light microscope, and ultra-thin sections looked well fixed under the electron microscope. However, there was a little peppering, and the resin was non-homogeneous in colour, as shown in Figure 3-5A. Moreover, all the cells, intracellular organisms, axons and even the fine glial processes are hardly identifiable, as shown in Figure 3-5B-D. Blocks of modified Spurr's resin sectioned well. The ultra-thin sections were well fixed, but they showed a high level of peppering, which might be due to the paraformaldehyde (see Figure 3-6). For this reason, this protocol was abandoned.

Table 3-7: Protocol 4 (original Spurr's resin and modified Spurr's resin).

Steps	Description	Duration
Primary fixation	3% glutaraldehyde or 3% glutaraldehyde / 2% paraformaldehyde in 0.1 M Sorensen's buffer at pH 7.4	120 minutes
Wash	Three washes in 0.1 M buffer	15 minutes each
Secondary fixation	1% osmium tetroxide in 0.05 M buffer	120 minutes
Wash	One wash in buffer	20 minutes
	Two washes with DDW	20 minutes and overnight
Dehydration	30% ethanol	120 minutes
	50% ethanol	120 minutes
	70% ethanol	90 minutes
	70% ethanol	Overnight
	90% ethanol	35 minutes
	95% ethanol	30 minutes
	100% ethanol twice	30 minutes each
Embedding	Propylene oxide twice	10 minutes each
	50:50 propylene oxide/Spurr's resin or modified Spurr's resin.	5 hours
	Lid was pierced to allow propylene oxide evaporation	Overnight
	Resin was replaced with fresh Spurr's (or modified Spurr's) resin twice.	3 hours each
Polymerisation	Samples were embedded and polymerised in a 60 °C oven	16 hours

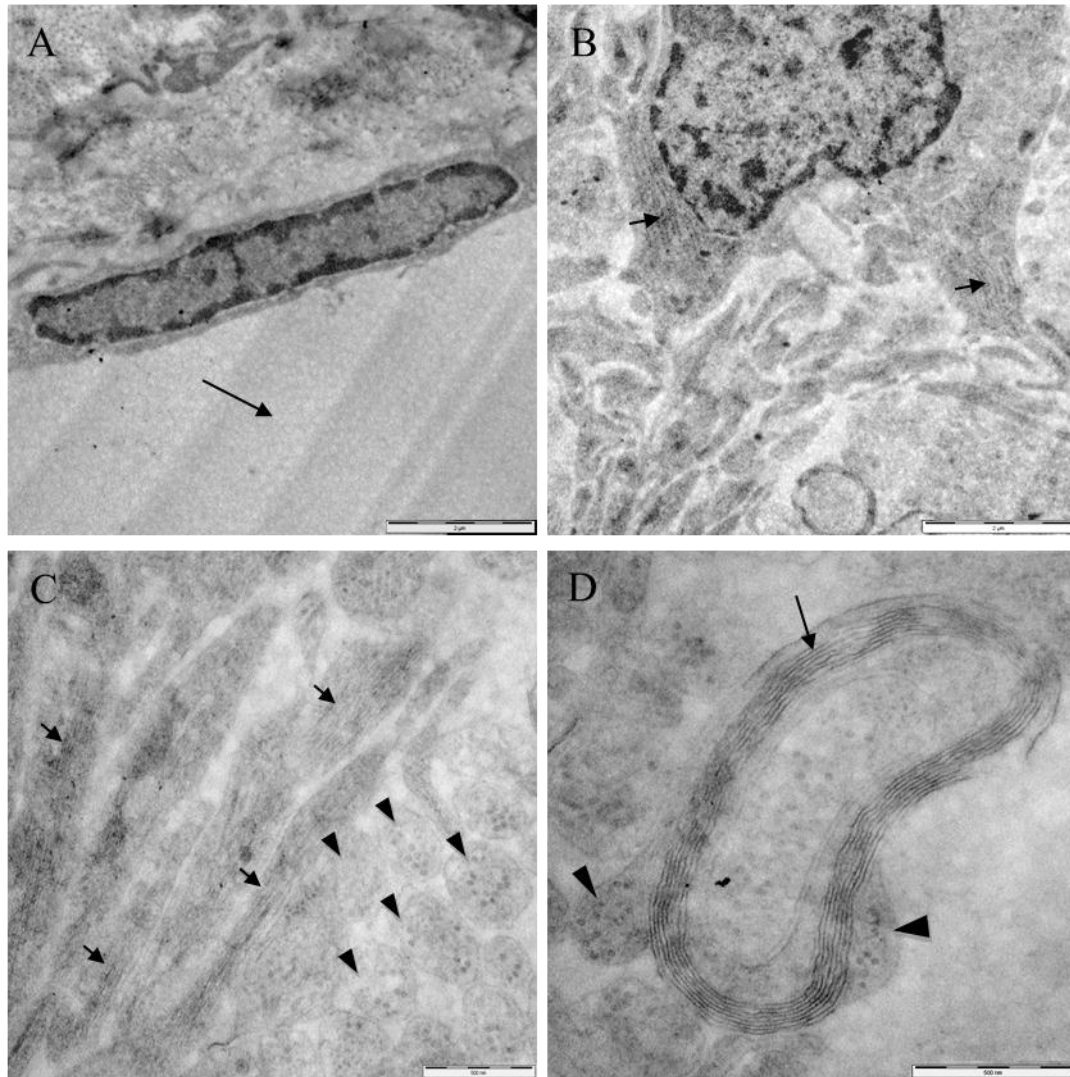


Figure 3-5: P10 RON following protocol 4 and embedded in original Spurr's resin.

Note the non-homogenous resin (arrow, A); cellular and intracellular components are hardly recognisable, such as wide-bore ER (arrows, B), glial filaments (arrows, C), axons (arrowheads, C), layers of myelin sheath (arrow, D) and glial tongue (arrowhead, D).

Scale bar = 2 μ m in A and B and 500 nm in C and D.

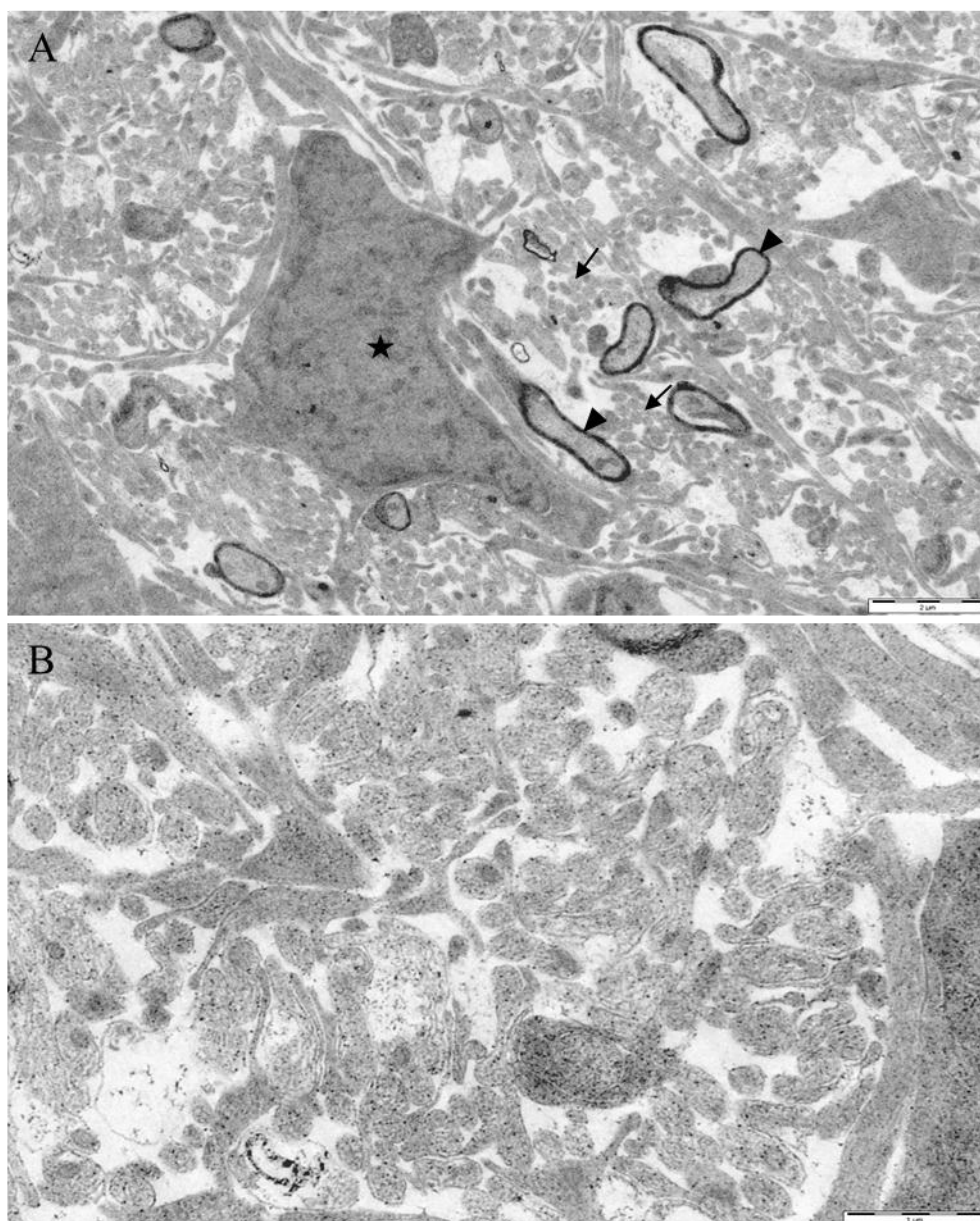


Figure 3-6: P10 RON following protocol 4 and embedded in modified Spurr's resin.

The tissue appeared well fixed, and the cell (star, A), axons (arrows, A) and myelin sheath (arrowhead, A) could be identified. However, the tissue shows a high level of peppering (B).

Scale bar = 2 μm in A and 1 μm in B.

3.2.2.5 Protocol 5:

This protocol was used to try to decrease the duration of the fixative to 30 minutes for the primary fixation and 45 minutes for the secondary fixation. P11 neonatal RON was embedded in original Spurr's resin (see Table 3-8 for more details). Blocks sectioned well but looked poorly fixed under the electron microscope. There was no cell membrane apparent separating cells, and some cells showed a disrupted nuclear membrane (Figure 3-7A). Vacuoles and peri-nuclear gap were obvious in almost all the cells, and the intracellular organelles were unhealthy (e.g. swollen mitochondria and dilated ER) (see Figure 3-7B and C).

Table 3-8: Protocol 5 (Modified Spurr's resin).

Steps	Description	Duration
Primary fixation	3% glutaraldehyde in 0.05 M Sorensen's buffer at pH 7.4	30 minutes
Wash	Three washes in 0.1 M buffer	15 minutes each
Secondary fixation	1% osmium tetroxide in 0.05 M buffer	45 minutes
Wash	One wash in 0.05 M buffer	20 minutes
	Two washes with DDW	20 minutes and overnight at 4°C
Dehydration	30% ethanol	60 minutes
	50% ethanol	90 minutes
	70% ethanol	30 minutes
	70% ethanol	Overnight
	90% ethanol	30 minutes
	100% ethanol twice	30 minutes each
Embedding	Propylene oxide twice	10 minutes each
	50:50 propylene oxide/Spurr's resin	4:30 hours
	Lid was pierced to allow propylene oxide evaporation	Overnight
	Resin was replaced with fresh Spurr's resin twice and left in the mixer	3 hours each
Polymerisation	Samples were embedded and polymerised in a 60 °C oven	16 hours

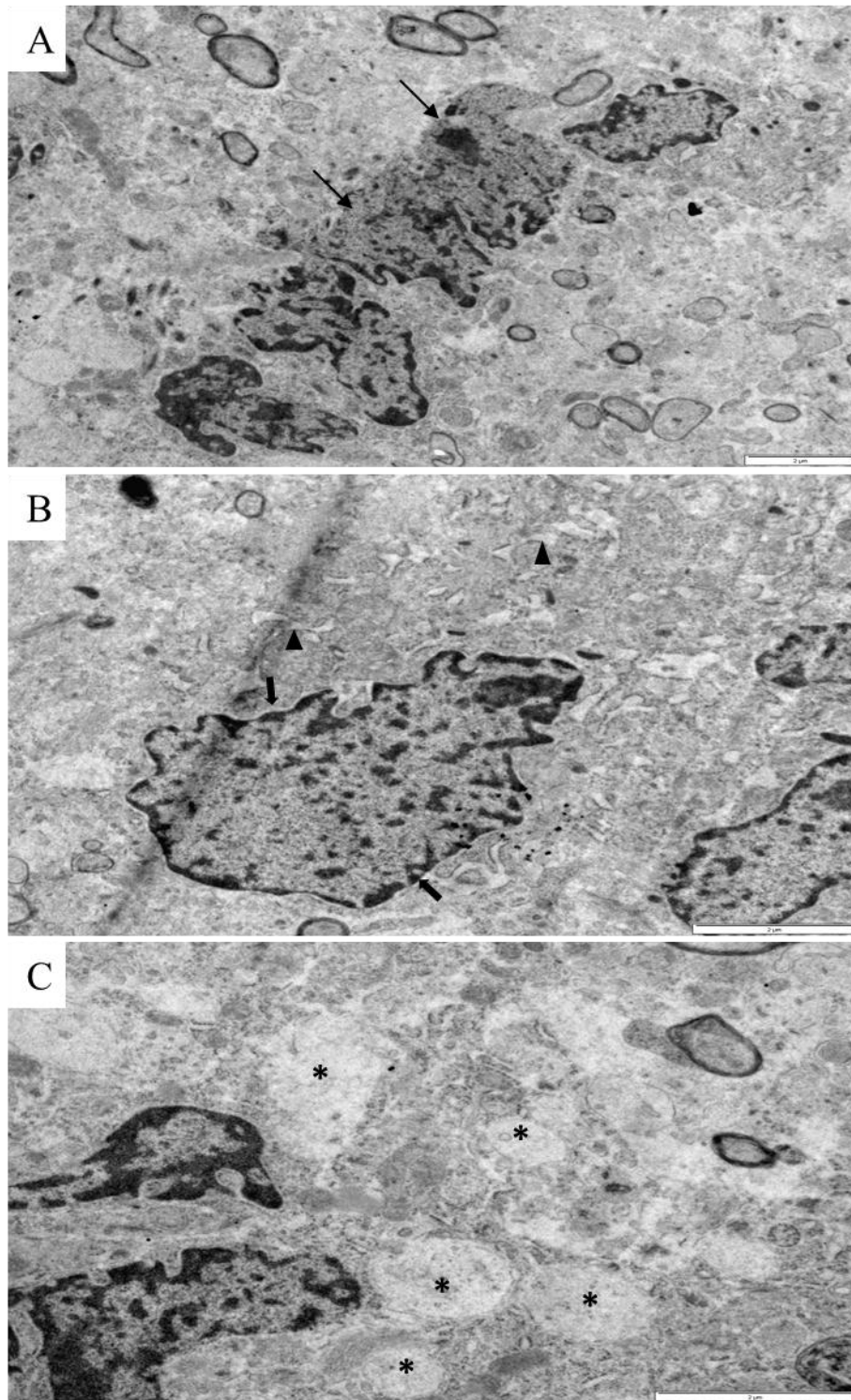


Figure 3-7: P11 RON following protocol 5 and embedded in original Spurr's resin.

The micrographs show a poorly fixed tissue with no obvious cell membranes; damaged nuclear membrane (arrows, A), peri-nuclear gap (bold arrows, B), dilated organelles (arrowheads, B) and large vacuoles (asterisks, C) are apparent.

Scale bar = 2 μm.

3.2.2.6 Protocol 6:

Some studies have shown a great level of fixation and contrast achieved when the tissue is treated with sodium cacodylate buffer and fixed with glutaraldehyde, osmium with potassium ferricyanide, and finally, urenyl acetate (Murphy et al., 2011, Thong et al., 2003). In this protocol, sodium cacodylate buffer was used, and three fixation steps were performed, as shown in Table 3-9. As a result, the ultra-thin sections looked well fixed under the electron microscope, and the cell processes and intracellular organelles were clearly identified, as shown in Figure 3-8. This protocol was used to generate RON which contributed to the data analysis.

Table 3-9: Protocol 6 (modified Spurr's resin).

Steps	Description	Duration
Primary fixation	2% formaldehyde/ 3% glutaraldehyde in 0.1 sodium cacodylate buffer/ 2 mM calcium chloride at pH 7.4	Overnight
Wash	Three washes in 0.1 M buffer	20 minutes each
Secondary fixation	1% osmium tetroxide in 1.5% potassium ferricyanide in DDW buffer	2.5 hours minutes
Wash	Three washes with DDW	20 minutes each
Tertiary fixation	2% aqueous urenyl acetate	1 hour at 4 °C
Wash	2 washes with DDW	10 minutes each
Dehydration	30% ethanol	15 minutes
	50% ethanol	15 minutes
	70% ethanol	15 minutes
	70% ethanol	Overnight
	90% ethanol	30 minutes
	100% ethanol three times	30 minutes each
Embedding	Propylene oxide twice	10 minutes each
	2 propylene oxide : 1 modified Spurr's low viscosity resin (hard formula)	90 minutes
	1 propylene oxide : 1 modified Spurr's low viscosity resin	60 minutes
	1 propylene oxide : 2 modified Spurr's low viscosity resin	60 minutes
	100% modified Spurr's low viscosity resin	30 minutes
	100% modified Spurr's low viscosity resin	overnight
	fresh modified Spurr's low viscosity resin	3 hours each
Polymerisation	Samples were polymerised in a 60 °C oven	16 hours

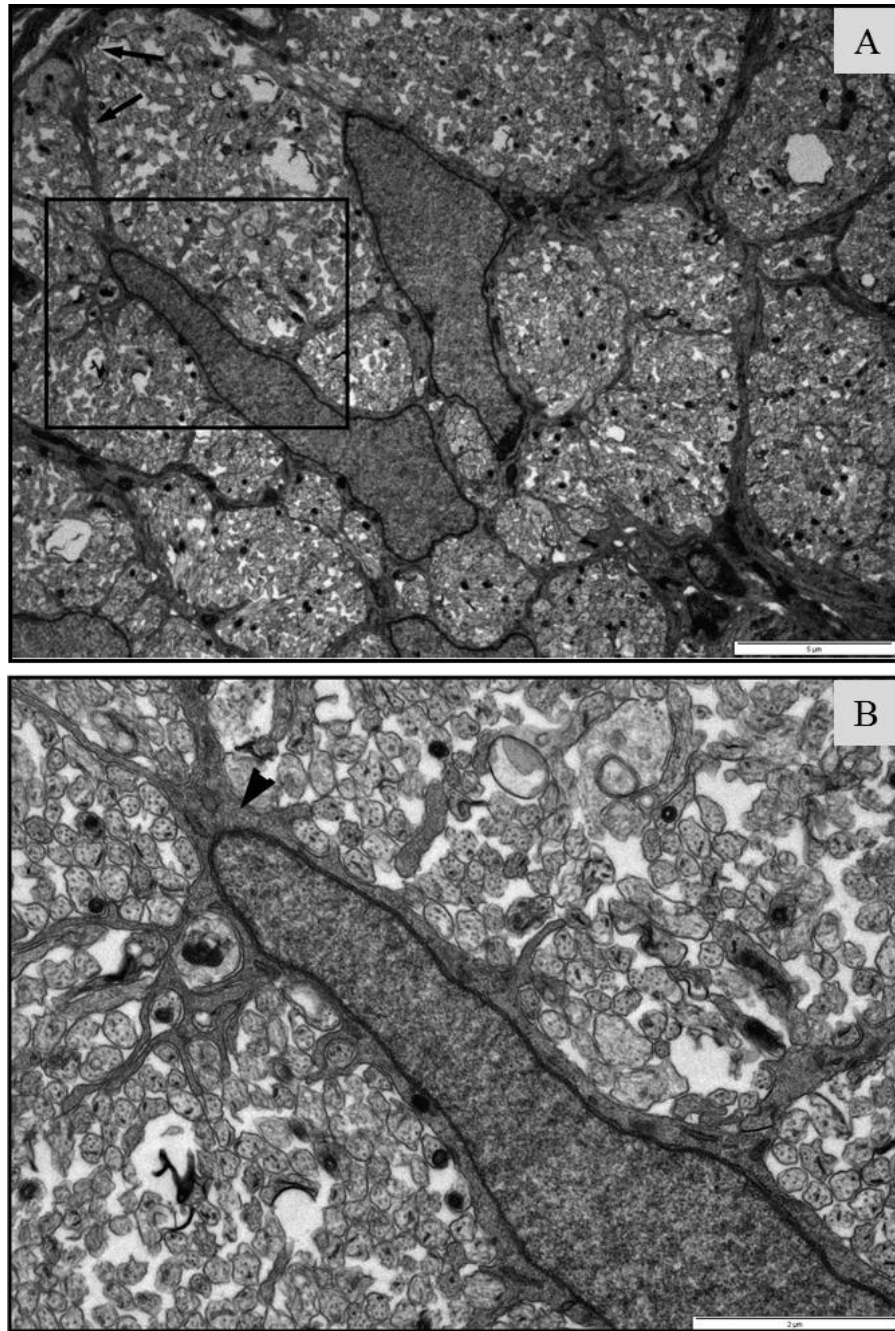


Figure 3-8: P10 RON following protocol 6 and embedded in modified Spurr's resin.

(A) The electron micrograph revealed a well fixed tissue and showed two glial cells and glial processes that extended from glial soma to reach glial limitans (bold arrows). The box in (A) is shown in higher magnification in (B), revealing the fine processes of astrocyte containing glial filament (arrowheads).

3.2.3 Immunolabelling:

3.2.3.1 Solution:

PBST buffer solution was used in immune-electron microscopy (I-EM). Sodium metaperiodate (Sigma-Aldrich), made by dissolving sodium metaperiodate in DDW until saturated, was used for etching the sections.

3.2.3.2 Primary Antibodies:

Four types of NG2 antibodies were tested, and only one of them seemed to be specific for I-EM. The first antibody was a mouse anti-NG2 cocktail obtained from Zymed Laboratories. This antibody cocktail consists of a mixture of four mouse monoclonal antibodies known to react with the N-terminal domain, the central collagen binding domain and a membrane proximal epitope of the NG2 proteoglycan; to the best of my knowledge, it has not been used in EM before. The second antibody was a mouse anti-NG2 monoclonal antibody obtained from the Millipore Corporation. This antibody is known to react with native, recombinant, and protein from cells expressing NG2 proteoglycan. The data suggest that this antibody was more specific than the other types used. The third antibody was a mouse monoclonal mixture, which was a kind gift from Dr William Stallcup (Burnham Institute, La Jolla, CA) (Stallcup et al., 1990). The fourth antibody was a rabbit polyclonal antibody obtained from Abcam Company. Another rabbit polyclonal antibody, prepared by using three rat cell lines —B49, B111, and B28— and two human cell lines —IMR-90 and M21— was also a kind gift from Dr Stallcup (Stallcup et al., 1983) (see Table 3-10).

All the antibodies were tested in a range of concentrations (1:200, 1:100, 1:50 and 1:20). Only one antibody, the rabbit anti-NG2 polyclonal antibody which was gift from Dr Stallcup, showed specific labelling, and the best concentration was 1:20.

Sections were left in a primary antibody solution for different durations, and no differences were found between leaving the sections for 4 hours or for 20 hours.

Table 3-10: Primary antibodies used in I-EM.

Antibody against:	Species	Monoclonal/ Polyclonal	Dilution	Company	Product code
NG2	Rabbit	Polyclonal	1:20	Abcam	Ab62341
NG2	Rabbit	Polyclonal	1:20	Generous gift from Dr Stallcup	
NG2	Mouse	Monoclonal	1:20	Millipore Corporation	MAB5384
NG2	Mouse	Monoclonal Mixture	1:20	Generous gift from Dr Stallcup	
NG2	Mouse	Monoclonal cocktail	1:20	Zymed Laboratories	37-2700

3.2.3.3 Secondary Antibodies:

The secondary antibody used for mouse monoclonal anti-NG2 primary antibodies was goat anti-mouse IgG, 30 nm gold conjugate (Sigma-Aldrich). Goat anti-rabbit IgG, 30 nm gold conjugate (Sigma-Aldrich) was used with the rabbit anti-NG2 polyclonal antibody. Both were used in a 1:50 concentration.

3.2.3.4 General Protocol:

The general protocol used in immunolabelling started with submerging grids in DDW for 10 minutes. Then the grids were etched in filtered sodium metaperiodate for 30 minutes, followed by four washes with DDW. After that, the grids were transferred to PBST/BSA for another 30 minutes. Grids were then submerged in the primary antibody in PBST/BSA solution for ~4 hours, followed by five washes with PBST/BSA solution. The grids were transferred to the secondary antibodies in PBST/BSA for ~1

hour, followed by two washes in PBST/BSA solution and then four washes with DDW. This protocol was based on a commonly used protocol in the lab for nRON I-EM (Wilke et al., 2004, Alix and Fern, 2009).

Several problems were encountered in immunolabelling. First, staining was almost non-specific until 10% goat serum (GS) was added to the PBST/BSA solution, and the grids were submerged in PBST/BSA/GS for 30 minutes before transferring them to the primary antibody. This step was important to block any non-specific binding sites. Second, the gold particles were seen in clusters with high background staining, as shown in Figure 3-9. This problem was solved by filtering the solution, especially the goat serum, before using it, and by keeping the PBST/BSA solution in the freezer. However, using two types of PBST/BSA solution in terms of pH (7.26 and 8.16) resulted in no difference in their effect on immunolabelling.

3.2.4 Data Analysis:

To estimate the density of gold particle staining, electron micrographs were taken, and a semi-quantitative method was applied. All the cells within one section of the grid were identified, and the area was calculated using J Image software. Then, the site of each gold particle in the cell-free area (CFA) of the entire grid was identified to study the distribution of these gold particles in various cell types, axons and undifferentiated glial processes. As NG2 should not be present in axons or collagen, this indicates background staining.

3.2.5 Difficulties in the Study:

Some difficulties were encountered during the data collection process. Sections sometimes carried dirt, bacteria or precipitation of staining, all of which could affect the contrast of the micrograph and interfere with identification of the ultrastructural features of the cell, as shown in Figure 3-10 A-C. Moreover, some sections showed knife marks, which became expanded by the effect of the electron beam, as shown in Figure 3-10D. In this case, identification of the cell type could be very difficult, especially if the tear appeared inside a cell.

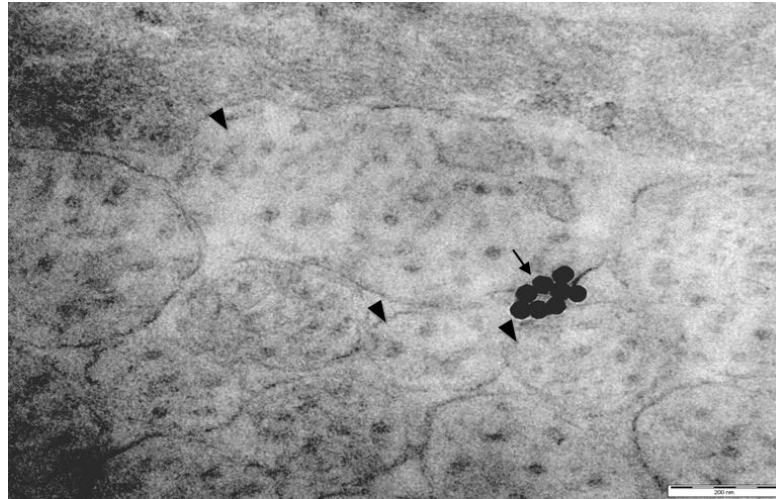


Figure 3-9: The micrograph shows a cluster of gold particles (arrow) attached to axons (arrowheads).

Scale bar = 200 nm.

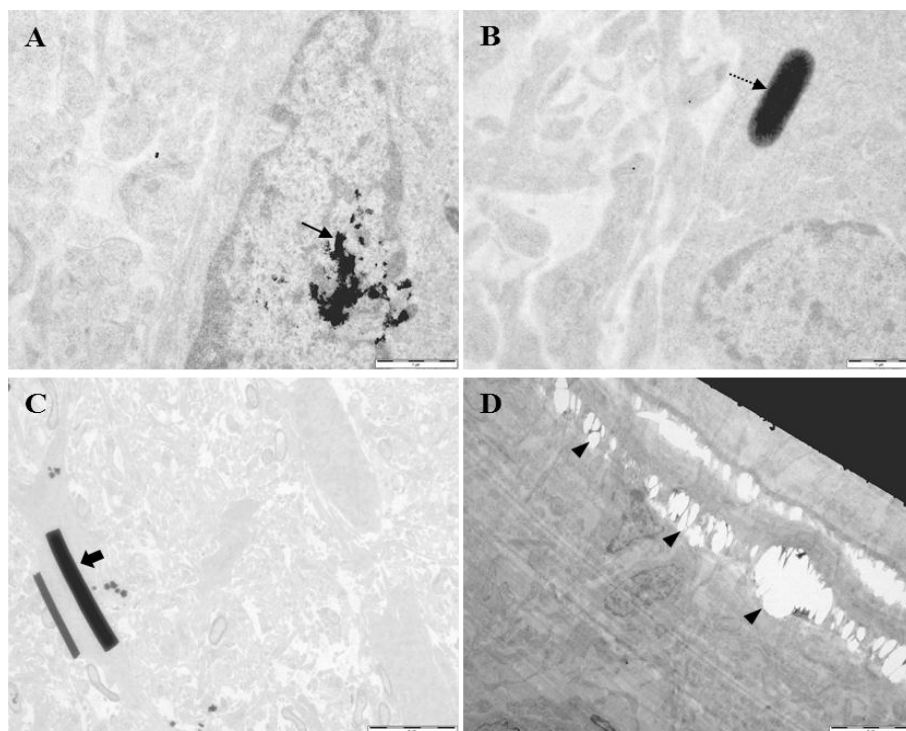


Figure 3-10: Limitation of study

The micrographs show some dirt (arrow, A), a bacterium (dashed arrow, B) and a remnant of urenyle acetate staining (bold arrow, C), all of which adversely affect the contrast. Moreover, knife marks can produce tears (arrowheads, D), which affect the analysis.

3.3 Immunohistochemistry:

The protocol used in this section is similar to the previous protocol discussed in Section 2.11.1. However, the fixation was reduced to 2% paraformaldehyde for 30 minutes in P10 and 60 minutes in adult RON. Table 3-11 shows the antibodies used in IHC and their concentration. Anti-NG2 antibody is recommended for immunocytochemistry and evaluated by Western blot on rat brain lysates (www.millipore.com). This type of antibody has been widely used in previous studies (Fernandez et al., 2004, Svenningsen et al., 2004, Barberi et al., 2003, Dugas et al., 2007, Ponti et al., 2008, Norsted et al., 2008, Orre et al., 2009, Labombarda et al., 2010, Katayama et al., 2010, Verreault et al., 2011, Steiner et al., 2011, Holopainen et al., 2012).

Table 3-11: Primary and secondary antibodies used in IHC.

Primary antibodies:

Antibody against:	Species	Monoclonal/ Polyclonal	Dilution	Company	Product code
Anti-glial fibrillary acidic protein (GFAP)	Rabbit	Polyclonal	1:200	Sigma	G4546
NG2	Mouse	Monoclonal	1:100	Millipore Corporation	MAB5384

Secondary antibodies:

Fluorophore	species	Dilution	Company	Product code
Alexa-488	Goat anti-mouse IgG (highly cross-adsorbed)	1:1000	Molecular Probes	A11029
Alexa-568	Goat anti-rabbit IgG (highly cross-adsorbed)	1:1000	Molecular Probes	A11036

CHAPTER 4 : RESULTS AND DISCUSSION

4.1 Results:

4.2 Immunogold Labelling:

NG2 immunogold staining was performed on three different nerves, all of which followed embedding protocol 6. Negative controls were done by applying secondary antibodies without primaries and showed no gold staining, see Figure 4-2. However, the gold staining level in the processed RON was low, representing the remaining antigenicity after long steps of fixation, embedding, and etching. Polyclonal antibodies have been shown to produce more cell-specific staining than monoclonal antibodies. Blinded counting of gold particles in 10 sections revealed that around 87.3% of staining is found in glia, while 13.5% is background staining, determined as staining of nuclei and axons where the protein is thought to be absent. At this stage, about 37% of the nerve volume is made up of glia, which will include their nuclei (Black et al., 1986), and, therefore, this represents specific glial staining. Glial-specific staining is represented by staining in glial processes (27.9%) and in the glial somata cytoplasm or membrane (59.4%), while the background staining is found in axons (7.4%) and glial nuclei (6.1%), as shown in Figure 4-1. This level of background staining is not dissimilar from that achieved by several studies that used the I-EM technique in this tissue (Arranz et al., 2008, Wilke et al., 2004). NG2 staining was found in the astrocytes' somata cytoplasm of P10 RON, which could be identified by its wide pore ER and glial filaments; see Figure 4-3A–D. Moreover, NG2 reactivity is seen in astrocyte processes which are characterised by the expression of glial filaments (see Figure 4-3E–G). NG2 staining is also seen in oligodendrocyte processes ensheathing axons and contributing to myelin formation (see Figure 4-3H).

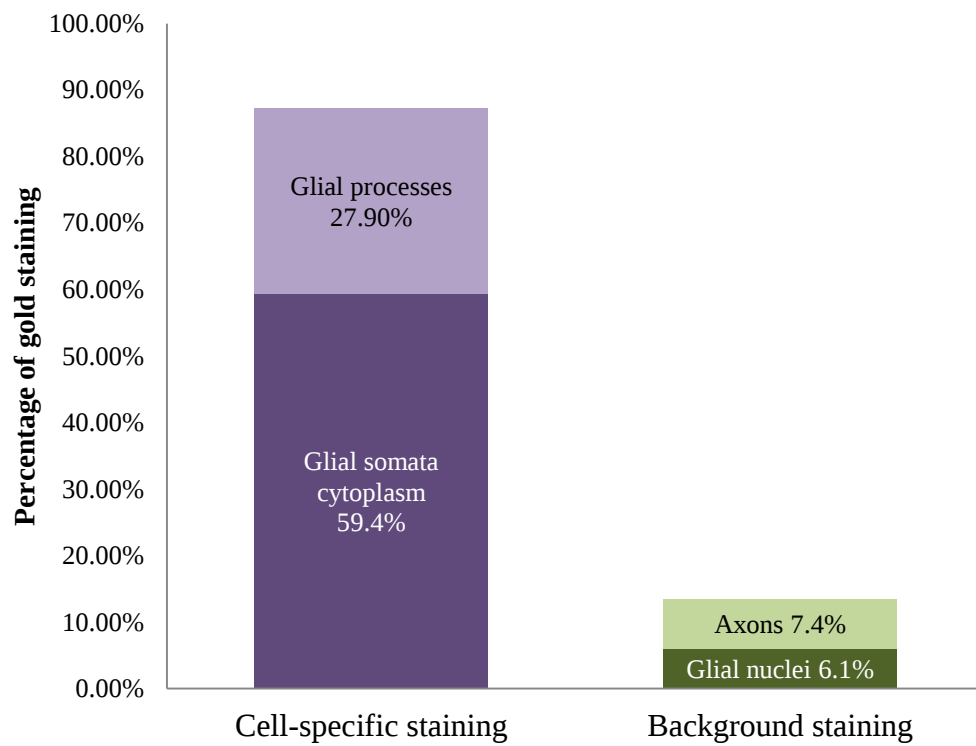


Figure 4-1: Gold staining distribution in RON.

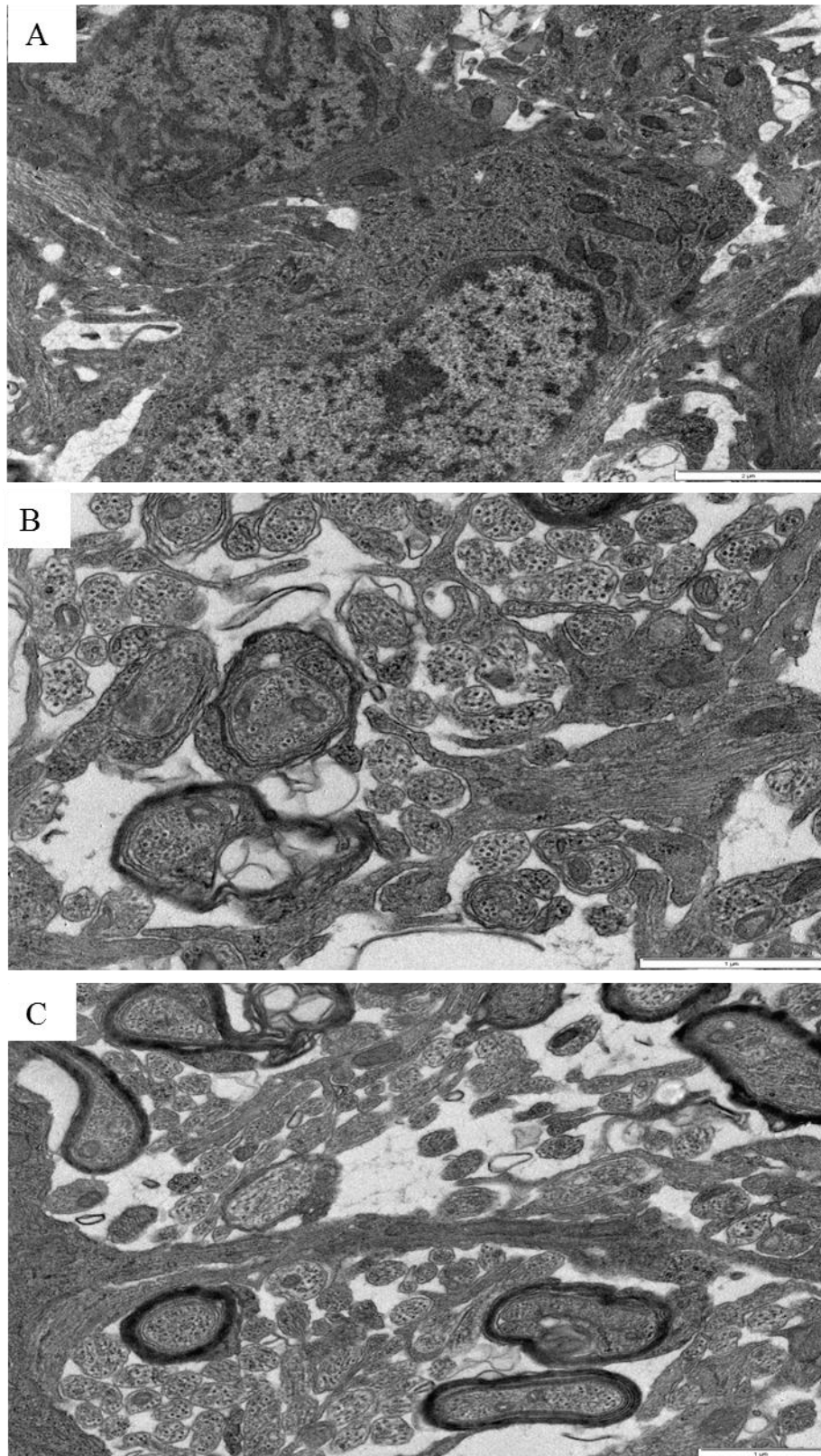


Figure 4-2: Negative control of P10 RON following protocol 6.

Micrographs show no gold staining after application of secondary antibody without primary antibody.

Scale bar= 2μm in (A), 1μm in (B) and (C).

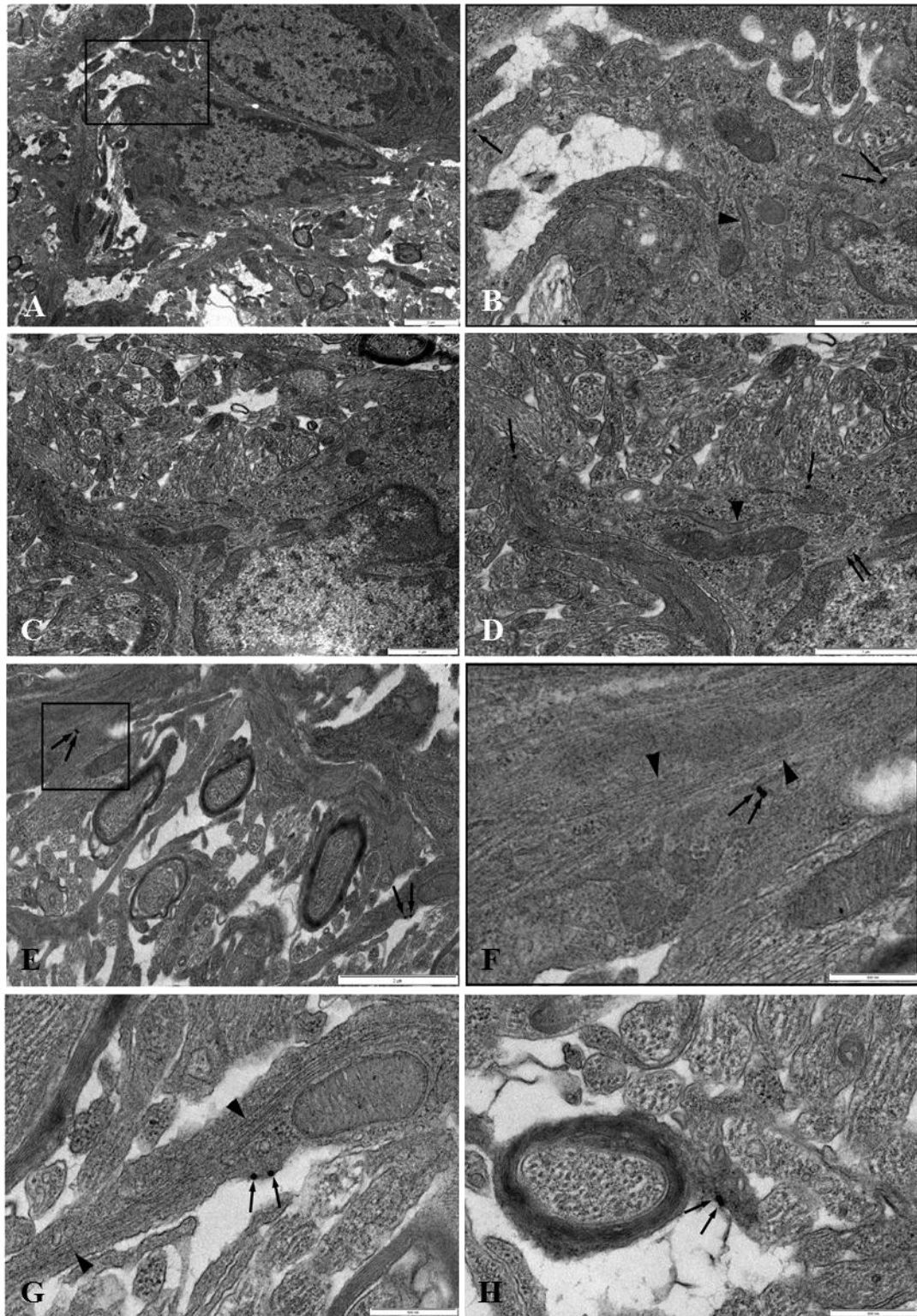


Figure 4-3: NG2 immunogold labelling of P10 RON.

(A–B) Two closely apposed glial cells with similar features, the lower of which is NG2 (+). Boxed area shown at higher gain in (B). Note the gold particles (arrows), wide-bore ER (arrowheads) and microtubules (e.g., double arrows). Glial filaments cannot be positively identified in this cell.

(C–D) Another NG2 (+) cell with similar features, which does express GFAP filaments (double arrows). Boxed area shown at higher gain in (D).

(E–F): NG2 staining in glial processes (arrows), some of which contain GFAP filaments (arrowhead). Boxed area shown at higher gain in (F).

(G) A further example of a GFAP expressing (arrowheads), NG2 (+) processes (arrows).

(H) An example of NG2 (+) (arrows) oligodendrocyte processes ensheathing an axon.

Scale bar = 2 μm in (A), and (E); 1 μm in (B), (C), and (D); 500 nm in (F), (G), and (H).

4.3 NG2 Immunohistochemistry in RON:

Double immunostaining with NG2 and GFAP was performed in lightly fixed P10 and adult RON and cortex. Taking advantage of the confocal microscope to collect stack images, the depth of images can be controlled, which improves the image contrast and facilitates a three-dimensional image formation. NG2 immunoreactivity was seen throughout the neonatal and adult RON in three-dimensional images. Double immunostaining revealed a co-localisation between NG2 and GFAP in neonatal and adult RON and adult cortical grey matter, as shown in Figure 4-4, Figure 4-5 and Figure 4-6. In cortical grey matter, NG2 staining was shown in astrocyte somata rather than processes. However, there is still an NG2 population which does not co-express GFAP in RON and cortical grey matter.

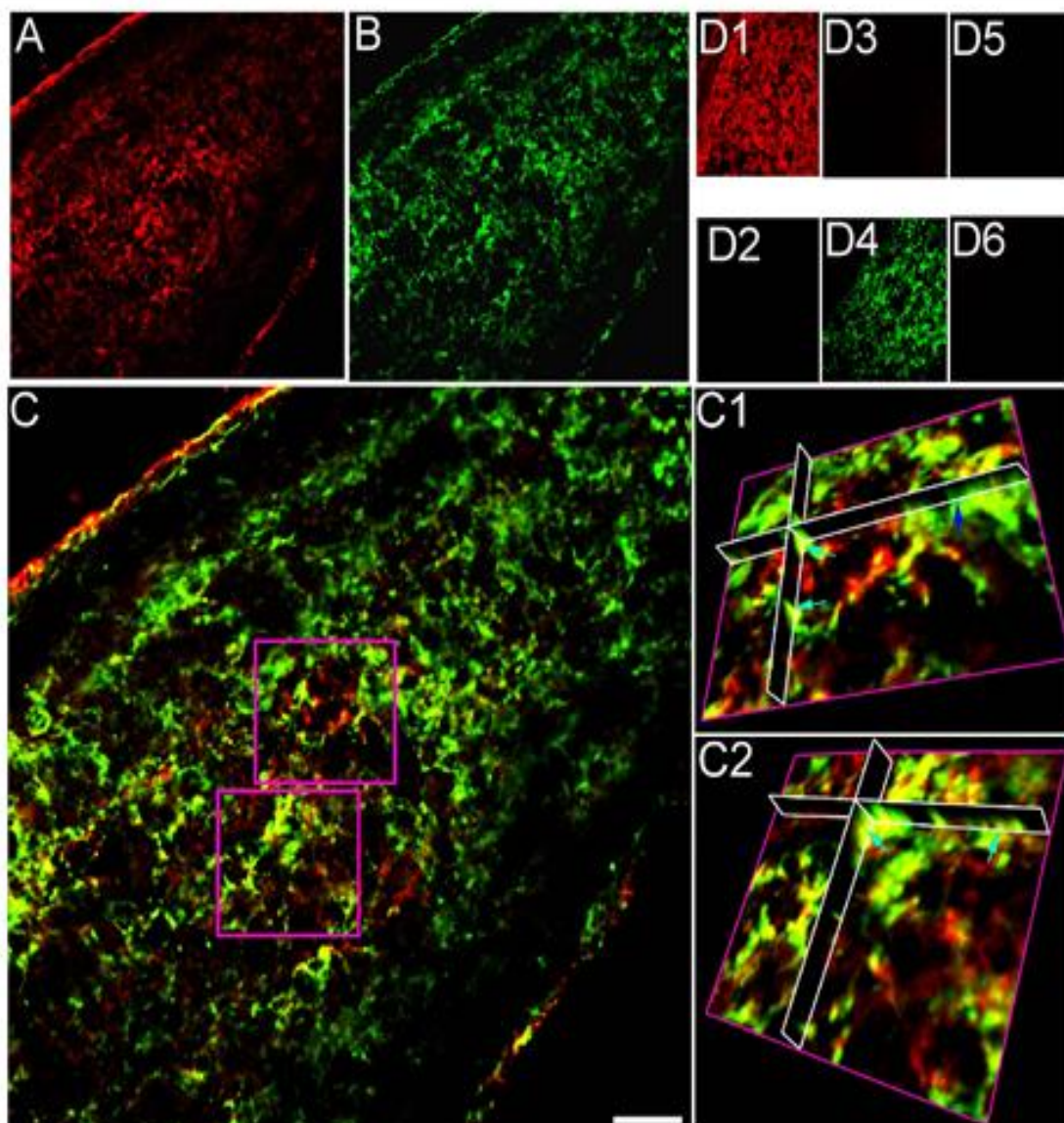


Figure 4-4: GFAP and NG2 co-localisation in P10 RON.

(A) GFAP immunoreactivity (red).

(B) NG2 immunoreactivity (green).

(C) Overlay with boxed areas shown at higher gain and as 3-D projections in (C1) and (C2). Note the NG2 co-localisation (e.g., light blue arrows) in parts of cells that may also have regions that are only GFAP (+) (e.g., dark blue arrow).

(D) Controls showing GFAP staining (D1) and absence of NG2 staining (D2) when the NG2 antibody was omitted from the otherwise identical protocol; NG2 staining (D3) and no GFAP staining (D4) when the GFAP antibody was omitted, and the absence of any staining when both primary antibodies were omitted (D5–6).

All images were collected and displayed using identical settings. Scale bar = 10 μm .

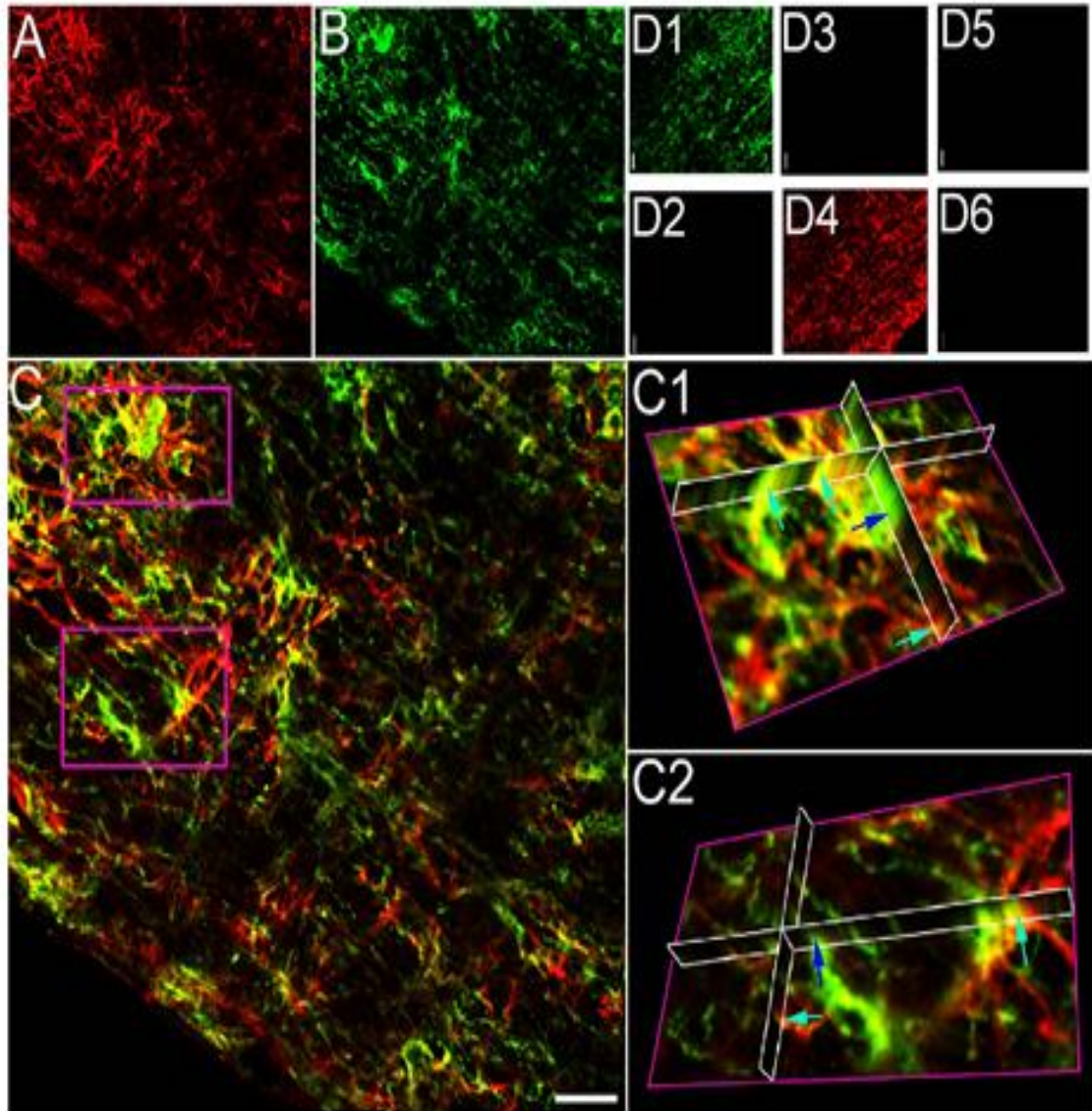


Figure 4-5: GFAP and NG2 co-localisation in adult RON.

(A) GFAP immunoreactivity (red).

(B) NG2 immunoreactivity (green).

(C) Overlay with boxed areas shown at higher gain and as 3-D projections in (C1) and (C2). Note the NG2 co-localisation (e.g., light blue arrows) in parts of cells that express GFAP alone in other regions, and cells that are only GFAP (+) (e.g., dark blue arrow).

(D): Controls showing NG2 staining (D1) and absence of GFAP staining (D2) when the GFAP antibody was omitted from the otherwise identical protocol; GFAP staining (D4) and no NG2 staining (D3) when the NG2 antibody was omitted, and the absence of any staining when both primary antibodies were omitted (D5–6).

All images were collected and displayed using identical settings. Scale bar = 10 μ m.

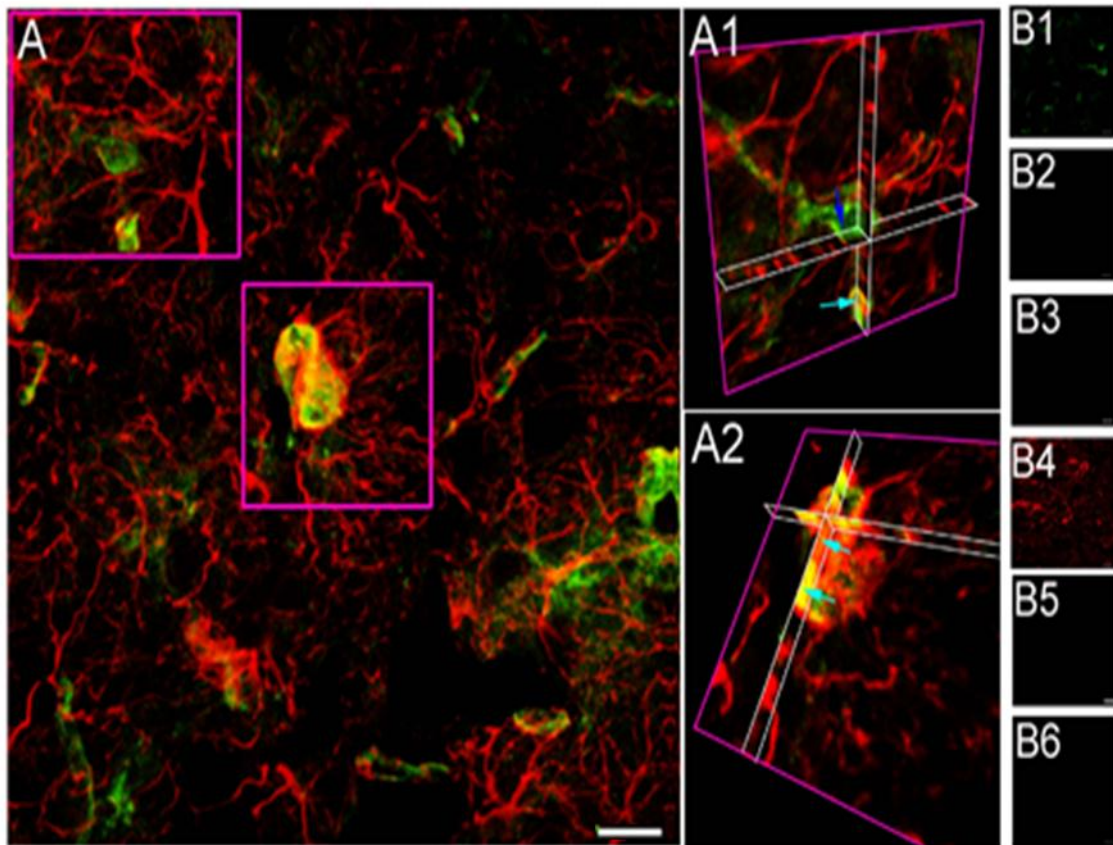


Figure 4-6: GFAP and NG2 co-localisation in adult cortical grey matter.

(A) GFAP immunoreactivity (red) and NG2 immunoreactivity (green), with boxed areas shown at higher gain and as 3-D projections in (A1) and (A2). Note the NG2 co-localisation (e.g., light blue arrows) in parts of cells that may also have regions that are only GFAP (+) while other NG2 (+) cells are GFAP (-) (e.g., dark blue arrow).

(B) Controls showing GFAP staining (B1) and absence of NG2 staining (B2) when the NG2 antibody was omitted from the otherwise identical protocol; NG2 staining (B4) and no GFAP staining (B3) when the GFAP antibody was omitted, and the absence of any staining when both primary antibodies were omitted (B5–6).

All images were collected and displayed using identical settings. Scale bar = 10 μm .

4.4 Discussion:

Several studies have reported that NG2 (+) cells are distinct populations from GFAP (+) astrocytes in different CNS regions such as cerebellum, corpus callosum, spinal cord, optic nerves and cerebral cortex (Dawson et al., 2003, Hamilton et al., 2009, Levine and Card, 1987, Levine et al., 1993, Nishiyama et al., 1996a, Redwine and Armstrong, 1998). Similar results were established in corpus callosum, cortex and cerebellum using other astrocyte markers such as S100 β , vimentin and GS (Levine and Card, 1987, Nishiyama et al., 1996a, Reynolds and Hardy, 1997). However, a close inspection of these studies reveals partial co-expression between GFAP and NG2, although these results were not commented on by the authors (Hamilton et al., 2009, Nishiyama et al., 1996a, Redwine et al., 1997). Moreover, a low level of GS was found to be expressed by NG2 (+) cells in the cerebral cortex (Dawson et al., 2003). NG2 expression was reported in reactive astrocytes of the adult and neonatal cultured rat cortex (Hirsch and Bahr, 1999). In addition, GFAP-EGFP transgenic mice showed GFAP (+) staining in astrocytes of EGFP bright cells, with lower NG2 staining in astrocytes of low EGFP expression in the P2 mouse brainstem (Grass et al., 2004). P10 CA1 stratum radiatum immunostaining with GFAP, S100 β and AN2 in GFAP-EGFP mice revealed incomplete overlap between GFAP and S100 β staining, in which S100 β was found in astrocytes of low EGFP expression and showed a co-localisation with AN2 (Matthias et al., 2003). NG2 staining was reported on GFAP (+) astrocytes of P29 pons while it was not seen on GFAP (+) astrocytes in the cerebellum, hippocampus, and cortex of GFAP-EGFP transgenic mice (Leoni et al., 2009). However, this low level of NG2 staining on GFAP could have been due to insufficiency in Cre reporter lines of transgenic mice, which might have limited GFAP expression only to a small proportion of astrocytes (Casper and McCarthy, 2006, Malatesta et al., 2003).

Retroviral (β -actin_p-GFP retrovirus) marking of GFAP (+) cells in the adult subventricular zone (SVZ) and corpus callosum revealed NG2 cell generation from GFAP (+) astrocytes, in normal and demyelinated conditions generation (Menn et al., 2006, Gonzalez-Perez et al., 2009).

Transgenic mice have been used to help in resolving this confusion. NG2CreBAC:Z/EG double-transgenic mice were used to study the astrocyte fate of NG2 (+) cells in which Cre recombinase is expressed in NG2 (+) cells (Zhu et al., 2008a, Zhu et al., 2008b). These studies revealed a detectable EGFP in S100 β (+) and some GFAP (+) astrocytes only at the ventral brain and grey matter of the spinal cord but not in the neocortex, white matter or cerebellum, while EGFP was seen in adenomatous polyposis coli (APC+) oligodendrocytes at the spinal cord, dorsal and ventral cortex, and in white matter (Zhu et al., 2008a, Zhu et al., 2008b). Similar NG2CreBAC:ZEG double-transgenic mice suggested S100 β (+) astrocyte generation from EGFP NG2 (+) cells in the mice olfactory bulb (Komitova et al., 2009). Another type of double-transgenic mice of PLP-CreER: Rosa26-EYFP (PCE/R) was used, in which the proteolipid promoter (PLP) was restricted to the myelin proteolipid protein found in oligodendrocytes (Guo et al., 2009). Interestingly, this study revealed that plp-promoter-expressing NG2 progenitors (PEEP) can generate immature astrocytes in the ventral forebrain and grey matter of the spinal cord but not in white matter (Guo et al., 2009). Immunocytochemistry was performed in plp-CreER transgenic mice in which Cre recombinase was expressed by oligodendrocytes, and this study revealed Cre (+) cells co-labelled with NG2 and GFAP in mice spinal cord, optic nerve and cerebellum during the postnatal period (Michalski et al., 2011). Oligo2::CreERTM;Z/EG (GFP reporter) mice showed reporter expression in GFAP+/S100 β + astrocytes in brain grey matter after adult recombination (Dimou et al., 2008). This was also seen in MBPlacZ

transgenic mice, which reported GFAP expression on MBP (+) cells in cerebral white and grey matter (Dyer et al., 2000). These studies gave some evidence that astrocytes shared NG2/oligodendrocyte lineage. Moreover, some studies suggested that NG2 (+) could be immature astrocytes, as they express S100 β (Karram et al., 2008, Lytle et al., 2009). CNP-EGFP transgenic mice revealed a colocalisation of NG2 and S100 β in the EGFP (-) areas of the developing spinal cord (Lytle et al., 2009). However, this particular line is known to be limited to the cells of oligodendrocyte lineage (Yuan et al., 2002). Similar CNP-EGFP studies showed NG2 (+) EGFP (-) areas which represent around 1–2% of the NG2 population in P8 cortex (Chittajallu et al., 2004) and in the adult brain (Mangin and Gallo, 2011). Moreover, NG2 knock-in mice revealed co-expression of enhanced yellow fluorescence protein (EYFP) with only markers of immature cells such as O4, PDGF α R, S100 β and GC in developing and adult hippocampus and cortex (Karram et al., 2008). However, the use of two different lines of PDGF CreER transgenic mice has reported no astrocyte production from PDGF expressing cells in the developing or adult white and grey matters of the mice brain (Kang et al., 2010, Rivers et al., 2008). These studies provide some evidence that PDGF α R (+) NG2 (+) cells do not produce astrocytes. However, there is still some population of PDGF α R (-) NG2 (+) cells reported in some studies (Diers-Fenger et al., 2001, Liu et al., 2002, Wilson et al., 2006, Nishiyama et al., 1996a). Using PDGF α R GFP transgenic mice has clarified the GFP/NG2 co-expression range, which appeared to be between 93% and 30%, depending on the brain region and postnatal age (Clarke et al., 2012).

A conclusion which can be drawn from these studies is the possibility that the NG2 (+) population consists of two distinct groups of different lineage potential, in

which the NG2/PLP/MBP/Olig2 lineage can generate astrocytes while the NG2/PDGFR lineage produces oligodendrocytes, see Figure 4-7.

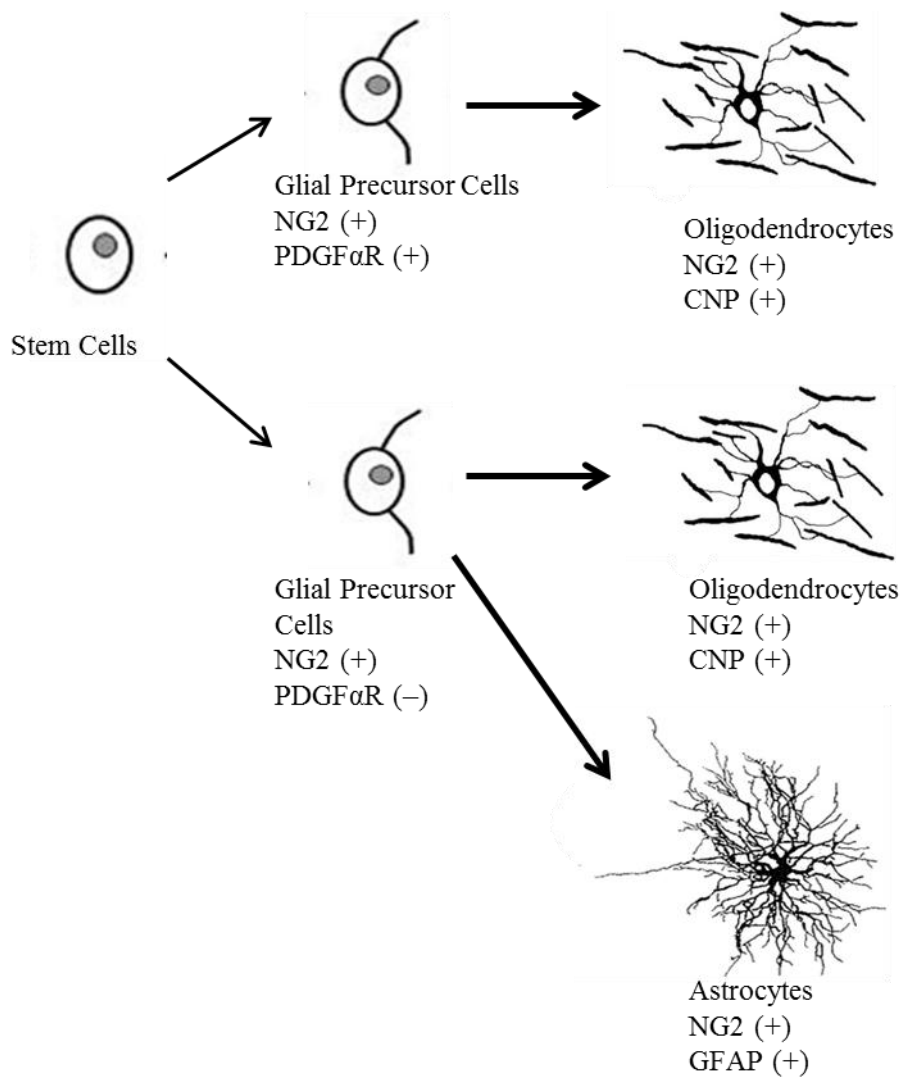


Figure 4-7: Astrocyte fate of NG2 (+) cells.

Note that NG(+)/GFAP(+) mature astrocytes and NG2(+)/CNP(+) myelinating oligodendrocytes can be generated from NG2(+)/PDGFR(-) precursor cells. On the other hand, NG2(+)/PDGFR(+) precursor cells can generate myelinating oligodendrocytes but not mature astrocytes.

4.5 **Conclusion:**

NG2 (+) cells occupy around 5–10% of glial cells in mature and immature CNS (Trotter et al., 2010). In the last 30 years, extensive studies focusing on NG2 (+) cells have produced controversy regarding the definite character of these cells. In the current study, we have shown that a subpopulation of NG2 (+) cells is GFAP (+) astrocytes in neonatal and adult RON and in adult cortical grey matter. This is consistent with a large body of evidence showing some similarities between these two populations, although it is not the consensus view in the field. Both types of cell are known by their stellate morphology and multiple processes emanating from cell somas (Montgomery, 1994, Butt et al., 2002a, Dawson et al., 2003, Levine and Card, 1987, Nishiyama et al., 2002, Ong and Levine, 1999, Butt et al., 1994a, Butt et al., 1994b). However, it is noticed that astrocyte processes are branching in a more extensive way than the NG2 (+) cells (Nishiyama et al., 2005). Both NG2 (+) cell and astrocyte processes contact nodes of Ranvier, pial surface, blood vessels and synapses in grey matter (Butt et al., 1999, Butt et al., 1994b, Levine and Card, 1987, Ong and Levine, 1999, Bergles et al., 2000). However, it has been estimated that astrocytes can contact around 140,000 synapses, while NG2 (+) cells can contact fewer than 20 (Bushong et al., 2002, Ge et al., 2009). It has been suggested that NG2 (+) cells lack glial filaments, relying on studies which ignored NG2 I-EM or on others with poor electron-micrographs contrast due to peroxidase (Levine and Card, 1987, Peters, 2004). Interestingly, both NG2 (+) cells and astrocytes are shown to undergo reactive changes during injury and to contribute to glial scar formation (Honsa et al., 2012, Busch et al., 2010, Buss et al., 2009, Levine, 1994, Tan et al., 2005, Hirsch and Bahr, 1999).

Focusing on the physiological aspects, both types of cells were shown to express similar neurotransmitter receptors. Astrocytes are known to express different

types of glutamate receptors (ionotropic such as NMDA, AMPA, and kainiate; metabotropic such as mGluR1, mGluR5, mGluR3) and glutamate transporters such as EAAT-1 and EAAT-2 (Parpura and Verkhratsky, 2013, Parpura and Verkhratsky, 2012, Lee and Pow, 2010). Astrocytes have an important role in extracellular glutamate homeostasis by picking up extracellular glutamate and converting it to a non-transmitter form (glutamine) by glutamine synthetase (Coulter and Eid, 2012, Lee and Pow, 2010). On the other hand, NG2 (+) cells express only two types of ionotropic glutamate receptors (AMPA and kainiate) and lack glutamate transporter and glutamate synthetase (Bergles et al., 2000, Hamilton et al., 2009, Lin et al., 2005, Nishiyama et al., 2005). The use of GFAP/EGFP transgenic mice and patch clamp techniques reveals two types of EGFP (+) astrocytes, in which one type expresses glutamate receptors (ionotropic) while the other has glutamate transporters (Matthias et al., 2003). It has been found that the AN2 also expresses the first type of EGFP (+) astrocytes, which only express glutamate receptors (Matthias et al., 2003). These results support our suggestion that some NG2 (+) cells are astrocytes. Moreover, astrocytes are known to express ionotropic GABA_A and metabotropic GABA_B receptors (Parpura and Verkhratsky, 2013). On the other hand, NG2 (+) cells are found to express GABA_A receptors, but the expression of GABA_B receptors is still unknown (Lin and Bergles, 2004, Williamson et al., 1998).

Both NG2 (+) cells and astrocytes are shown to contribute in controlling the extracellular K⁺ level. Following neuronal stimulation, the level of [K⁺]_e increases from the resting level of 3 mM to around 4 mM (Walz, 2000). Astrocytes are responsible for uptaking the excess [K⁺]_e through a passive inward K⁺ current which is transported into other adjacent areas by gap junction (Walz, 2000). On the other hand, NG2 (+) cells are reported to control the [K⁺]_e to less of an extent than astrocytes. One of the NG2 (+)

cells' cell membrane features is weak permeability to ions due to its high input resistance (between 200 and 400M) and its low negative resting membrane potential between -70 mV and -90 mV (Bergles et al., 2000, Chittajallu et al., 2004, Lin and Bergles, 2002, Lin and Bergles, 2004, Lin et al., 2005). For this reason, NG2 (+) cells have a smaller inward K^+ current as compared to astrocytes stimulated by ATP-sensitive K^+ channels (Butt et al., 2005, Lin and Bergles, 2002). However, unlike astrocytes, NG2 (+) cells lack gap junctions (Bergles et al., 2000, Chittajallu et al., 2004, Lin and Bergles, 2002, Lin and Bergles, 2004, Lin et al., 2005).

In conclusion, these studies produced evidence that at least some NG2 (+) cells share features of astrocytes in some aspects such as morphology, antigen expression and physiological properties. This is consistent with our finding of GFAP and NG2 colocalisation in neonatal and adult RON and cortical grey matter.

Part II:

The role of GABA, glycine, nicotine, adrenergic receptors in developing central white matter Ischaemic Injury

CHAPTER 5 : INTRODUCTION

5.1 Glial Synapses:

The ability of glial cells to form functional synapses is a significant discovery over recent years. Bergles et al. (2000) were the first to suggest the presence of NG2 glial functional synapse in hippocampus. By using patch clamp recording, they recorded the inward current in oligodendrocytes precursor cells (OPCs) after the activation of excitatory axons of pyramidal neurons, which was mediated by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) – Glutamate receptors (GlutR) (Bergles et al., 2000). Electron micrographs showed that NG2 glial processes opposed the axonal presynaptic transmitter-releasing sites, which are recognised by the clustering appearance of synaptic vesicles (Bergles et al., 2000). Since then, “glial synapses” have become a major field of research.

In white matter, axons and glial cells communicate with each other through neurotransmitter signals, in which glutamate is one of the most extensively studied neurotransmitters. Several studies have demonstrated white matter axons and glial cells (oligodendrocytes, astrocytes, and microglia) expression of a wide range of neurotransmitter receptors, such as AMPA, kainate, N-methyl-D-aspartate (NMDA), and purinergic receptors such as P2X, and P2Y receptors (Matute, 2010). Moreover, glial cells are distributed in a way that makes for easy communication with axons; for example, astrocytes and NG2 glial cell processes have been found to contact nodes of Ranvier (Butt et al., 2004), and oligodendrocytes made clear contact with axons. Electron microscopy has shown the existence of vesicle-like structures that are loaded with glutamate in the axons, oligodendrocytes and astrocytes of optic nerves (Back et al., 2007). Voltage-gated Ca^{2+} channels (VGCCs) are expressed by optic nerve axons and mediate activity-dependent $[\text{Ca}^{2+}]_i$ increase, which is needed for exocytosis (Kukley et al., 2007, Alix et al., 2008). Such data are consistent with the presence of

functional synapses in white matter. Moreover, it is suggested that synapses are present with the glial cells after their division in mice brains (Kukley et al., 2008). However, glial synapses have generated a number of controversies; for example, some argue about the pattern of glial synaptic signalling as to whether it is synaptic or extrasynaptic (Maldonado et al., 2011).

Synaptic signal transmission in white matter is quite different from that in grey matter. Membrane depolarisation in grey matter triggers Ca^{2+} -dependent vesicular release of neurotransmitters from presynaptic membrane to the synaptic cleft, in which neurotransmitters are subjected to unidirectional targeting (Alix and Domingues, 2011). On the other hand, neurotransmitters released from axons of white matter diffuse in the extracellular space with no clearly subjected targets (Alix and Domingues, 2011).

5.2 Astrocyte-Axon Communication in White Matter:

Astrocytes are well equipped with multiple ion channels and membrane receptors, which help them to detect signals and respond in milliseconds (Barres et al., 1988, Barres et al., 1990a, Scemes and Giaume, 2006). Astrocytes respond to the stimuli by increase $[\text{Ca}^{2+}]_i$ level and spread these Ca^{2+} signals to the adjacent non-stimulated astrocytes via intracellular Ca^{2+} waves (Cornellbell and Finkbeiner, 1991, Charles et al., 1991a). Ca^{2+} waves propagate in astrocytes either through direct intracellular diffusion via gap junctions or through extracellular gliotransmitters such as ATP (Scemes and Giaume, 2006). It has been shown that astroglial communication is dependent on the Ca^{2+} signalling, which is mediated by glutamate and ATP release (Anderson et al., 2004, Guthrie et al., 1999, Newman, 2001, Hamilton et al., 2008). Moreover, multiple astrocyte processes can contact the same node of Ranvier and

axonal stimulation mediated by neurotransmitters can generate Ca^{2+} signalling in astrocytes (Butt et al., 2004, James and Butt, 2001). All of that gives astrocytes an important role in synaptic signalling and propagation (Haydon, 2001, Jourdain et al., 2007, Auld and Robitaille, 2003).

Glutamate and ATP are released from both axons (Kukley et al., 2007, Ziskin et al., 2007, Stevens et al., 2002) and astrocytes (Scemes and Giaume, 2006, Hamilton et al., 2008). Luciferin-luciferase assays have been used to measure the level of extracellular ATP released from retinal astrocytes following mechanical stimulation, which was found to be 78 μM at the site of injury and 6.8 μM 100 μm away from the injury (Newman, 2001). 10 nM of ATP was enough to mediate a detectable Ca^{2+} signal in Fluo-4 loaded astrocytes of optic nerves (Hamilton et al., 2008), suggesting that a low level of ATP release can initiate Ca^{2+} signals in optic nerve astrocytes. Astrocytes express P2Y_1 metapotropic ATP receptor, as well as a wide range of P2X_{1-6} (except P2X_5) ionotropic ATP receptors (Moran-Jimenez and Matute, 2000, James and Butt, 2001, James and Butt, 2002, Kukley et al., 2001). Applying broad-spectrum $\text{P2X}/\text{P2Y}$ receptor blockers (suramin and PPADS), broad-spectrum P2X receptor blocker (NF023), SERCA pump blocker (thapsigargin) and Ca^{2+} -free solutions significantly inhibits ATP response in optic nerve astrocyte. This means that ATP triggers a rise in astrocyte $[\text{Ca}^{2+}]_i$ through the activation of both P2Y and P2X purinoceptors, and the latter mediate Ca^{2+} release from intracellular sources (mediated by P2Y) and Ca^{2+} influx (mediated by P2X) (Hamilton et al., 2008).

Immunohistochemistry, PCR, and Western blotting in the hippocampus, cortex, and cerebellum have revealed astrocyte expression of some members of the ectonucleoside triphosphate diphosphohydrolase (ecto-NTDase) family, which are the ectoenzymes responsible for ATP hydrolysis (Wink et al., 2006). It has been suggested that

NTPDase2 plays an important role in ATP hydrolysis in astrocytes, as the ATPase activity is inhibited by suramin and Evans Blue but not sodium azide, a NTPDase1inhibitor (Wink et al., 2006). However, this study suggested that extracellular ATP is rapidly broken down to ADP and AMP by the action of ectonucleotidases (Wink et al., 2006). Intracellular ATP hydrolysis in astrocytes has been ruled out, as the ATPase and ADPase activities were not affected by intracellular ATPase inhibitors such as Na^+ - K^+ -ATPase inhibitors (ouabain and orthovanadate), Ca^{2+} - Mg^{2+} -ATPase inhibitors (N-ethylmaleimide, NEM, and lanthanum), or mitochondrial ATPase inhibitors (oligomycin and sodium azide) (Plesner, 1995, Wink et al., 2006). It has been argued about the potency of different agonists for purinoceptors and demonstrated that ADP is more potent than ATP in P2Y_1 receptors, while ATP is more potent than ADP P2X receptors (Burnstock and Knight, 2004). However, it has been shown that ADP is more potent than ATP in optic nerve astrocytes, which has been proven by the significantly reduced ATP response that occurs after applying multiple enzymes that inhibit ADP, such as $\text{ATP}\gamma\text{S}$, a nonhydrolysable type of ATPases, ARL67156, an inhibitor for ATP to ADP conversion, and apyrase, an enzyme which catalyses ADP hydrolysis (Hamilton et al., 2008).

Immunohistochemical studies of AMPA and NMDA receptors in optic nerve and corpus callosum have revealed their expression in oligodendrocytes, but not in astrocytes (Salter and Fern, 2005, Karadottir et al., 2005). Both NMDA and AMPA/KA receptor activation has been found to mediate membrane current and Ca^{2+} influx in oligodendrocytes (Bakiri et al., 2009). Moreover, the application of AMPA, NMDA, and metabotropic GlutR receptor agonists generates small Ca^{2+} signals in optic nerve astrocytes (Hamilton et al., 2008). It has thus been suggested that glutamate activates

astrocytes indirectly through the activation of oligodendrocytes, which release ATP and therefore activate astrocytes (Hamilton et al., 2008). This is consistent with the expression of P2X₇ receptors in oligodendrocytes (Matute et al., 2007). However, due to a lack of information regarding NMDA and AMPA receptor expression in optic nerve astrocytes, the possibility of glutamate binding directly to astrocyte cannot be ruled out, particularly as the NMDARs have been demonstrated in spinal cord astrocytes (Ziak et al., 1998, Bradesi et al., 2011, Lee et al., 2010).

Mechanical or electrical stimulation triggers a Ca²⁺ wave in the optic nerve and retinal astrocytes at an estimated speed of ~20–23 µm/s and decreases gradually over several hundred micrometres (Hamilton et al., 2008, Newman and Zahs, 1997). However, it has been shown that Ca²⁺ signalling produced by mechanical or electrical stimulation can be inhibited by suramin but not by NBQX, which indicates the important role of ATP, but not glutamate, as a gliotransmitter in optic nerve (Hamilton et al., 2008). This is consistent with the greater astrocyte response to ATP than to glutamate in other regions of the brain, such as the cortex (Guthrie et al., 1999), retina (Newman and Zahs, 1997) and spinal cord (Scemes et al., 2000). However, it has been found that glutamate in optic nerve mainly triggers ATP release via P2X₇, and that ATP therefore amplifies the glutamate-mediated Ca²⁺ signals by propagating Ca²⁺ signals via P2Y₁ (Hamilton et al., 2008).

To sum up, ATP and glutamate are important neurotransmitters in white matter. In the optic nerve, ATP has the predominant role in astrocyte Ca²⁺ signalling. Axon depolarisation mediates ATP and glutamate release from axons, and both of them mediate a Ca²⁺ influx either directly by binding to astrocyte receptors or indirectly via other cells. ATP is then released from astrocytes via P2X₇ -dependent release which exaggerates the initial Ca²⁺ signal via binding to P2Y₁ of the same astrocyte (autocrine

properties) and propagates the Ca^{2+} wave via binding to P2Y receptors of adjacent cells (paracrine properties).

In the grey matter, astrocytes participate in a tripartite synapse which facilitates an easy communication between neurons and astrocytes (Perea et al., 2009). It has been shown that astrocytes respond to signals through an increase in $[\text{Ca}^{2+}]_i$ (Charles et al., 1991b, Cornell-Bell et al., 1990). Astrocytes can modulate the synaptic activity through secreting some neuroactive substances such as glutamate, ATP, GABA, tumour necrosis factor α ($\text{TNF}\alpha$) and prostaglandins (Perea et al., 2009). Moreover, astrocytes have an important role in re-uptaking the excitotoxic neurotransmitters in which glutamate is the most extensive one studied (discussed later in 5.7.3).

5.3 Membrane potential and electrical signals:

The membrane potential is controlled by ion permeabilities in which ions have specific intracellular and extracellular concentrations, see Table 5-1. Ions pass through the cell membrane via ion channels which allow passive ions flow with their gradients (Hammond, 2008). The exact intracellular concentration of ions is controlled by active transporters such as $\text{Na}^+\text{-K}^+$ ATPase (Hammond, 2008). However, both the concentration gradient and membrane potential are responsible to determine the direction and quantity of ion diffusion and that can be calculated by Nernst equation, see Equation 1 (Brady S. T., 2012, Hammond, 2008, Squire L.R., 2008). Nernst equation calculates the equilibrium potential for one specific ion in which K^+ , Na^+ , Ca^{2+} , and Cl^- are important ions known to contribute in equilibrium, see Table 5-1. With respect to the relative permeability of different ions, Alan Hodgkin and Bernard Katz wrote Goldman-Hodgkin-Katz (GHK) equation which can calculate the membrane potential from the contribution of different ions, see Equation 2 (Hammond, 2008, Squire L.R., 2008).

Table 5-1: Nernst potential for different ions.

Ion	$[\text{ion}]_e$ (mmol/l)	$[\text{ion}]_i$ (mmol/l)	Nernst potential (mV)
Na^+	140	7	+58
K^+	3	140	-84
Ca^{2+}	1.5	10^{-7}	+116
Cl^-	140	7	-58

Equation 1: **Nernst Equation:**

$$E_{ion} = \left(\frac{RT}{zF} \right) \ln \left(\frac{[ion]_e}{[ion]_I} \right)$$

E_{ion} is the equilibrium constant for a specific ion; R is the ideal gas constant (8.314 VCK⁻¹ mol⁻¹); T is the absolute temperature in kelvin; F is the Faraday constant (96500 C mol⁻¹); z is the valence of the ion (-1 for Cl⁻ and +1 for K⁺); [ion]_e is the extracellular concentration for the ion; and [ion]_I is the intracellular concentration for the ion.

Equation 2: **Goldman-Hodgkin-Katz (GHK) equation:**

$$E_m = \frac{RT}{F} \cdot \ln \frac{(P_K[K^+]_e + P_{Na}[Na^+]_e + P_{Cl}[Cl^-]_I)}{(P_K[K^+]_I + P_{Na}[Na^+]_I + P_{Cl}[Cl^-]_e)}$$

E_m is the membrane potential; R is the ideal gas constant (8.314 VCK⁻¹ mol⁻¹); T is the absolute temperature in kelvin; F is the Faraday constant (96500 C mol⁻¹); P is the permeability of the ion.

5.4 Ischaemia:

Ischaemia develops as a consequence of inadequate blood supply. The brain is a very active organ that consumes high levels of glucose and oxygen to meet its demands (Brady S. T., 2012). For this reason, it is very sensitive to any disturbance in energy supply and is highly vulnerable to injury (Brady S. T., 2012).

The extent of ischaemic damage in the brain varies according to the person's age and the site of the injury. For example, white matter can tolerate ischaemia longer than grey matter and has a better recovery outcome (Fern et al., 1998). This is because grey and white matter have different mechanism of action towards injury. For example, reversal of Na^+ - Ca^{2+} exchange is important in white matter, while VDCC plays a significant role in grey matter (Stys, 1998). Moreover, although an immature brain is typically more resistant to ischaemic injury than a mature brain, this is not always the case, as it depends on the developmental stage at the time of injury (Fern et al., 1998, Vannucci, 2007). Fern et al. (1998) correlate this fact to a specific developmental stage and show that P0–P4 RON is highly resistant to ischaemia (anoxia + aglycemia), while P5–P20 RON is partially resistant to either anoxia or aglycemia, but not both; P21–P40 has very poor resistance to anoxia, aglycemia or ischaemia, and >P50 RON has poor resistance to anoxia, aglycemia or ischaemia. This reflects the fact that the immature brain has heightened periods of vulnerability to ischaemia, which, as this study reveals, is between P21–P40 in RON; this is related to the myelination period (Fern et al., 1998).

5.5 Periventricular Leukomalacia (PVL):

Hypoxic-ischaemic injury to white matter during the perinatal period is the underlying cause of multiple types of CNS insults (Rezaie and Dean, 2002, Johnston et

al., 2001). Disorders will vary according to the developmental stage of the brain at the time of the injury; i.e., preterm ischaemic injury is commonly located in periventricular white matter, causing PVL, while an ischaemic injury of a full-term infant is commonly located near the cerebral cortex and leads to the development of hypoxic ischaemic encephalopathy (Anca, 2011). The period of high vulnerability for developing PVL is in the mid-gestational period (approximately between gestational weeks 23 and 32) (Back, 2006, Rezaie and Dean, 2002, Obenaus and Ashwal, 2008).

PVL is the most common white matter pathology associated with CP: around 1.5 to 2.5 per 1,000 live births per year are associated with infants who suffer from CP (Back, 2006, Fern et al., 1998, Haynes et al., 2005, Rezaie and Dean, 2002). It is estimated that around 68% of PVL patients may have CP; the mortality rate in these situations reaches 59% (Anca, 2011, Guzzetta et al., 1986). PVL, known as Little's disease in the 1840s, was first recognised by W.J. Little, who attributed the cause of PVL to "abnormal factors" due to prematurity and asphyxia (Folkerth, 2005, Rezaie and Dean, 2002). Little's formulation was followed in 1867 by Virchow, who gave this disorder the term, "congenital encephalomyelitis", and described the lesion macroscopically, linking it to acute infection and inflammation (Folkerth, 2005, Rezaie and Dean, 2002). Later on, Parrot identified additional pathological features for PVL as prematurity, infarction and haemorrhage (Folkerth, 2005, Rezaie and Dean, 2002). In 1962, Banker and Larroche defined this pathology as "periventricular leukomalacia", and they considered anoxia as a main factor predisposing to PVL (Rezaie and Dean, 2002).

The disorder is characterised by symmetrical multifocal necrotic regions surrounded by diffuse, non-necrotic injury areas of white matter (Folkerth, 2005, Rezaie and Dean, 2002, Volpe et al., 2011). Focal necrosis is more severe than the

diffuse injury in which focal necrosis is associated with cell death of all cellular elements, while diffuse injury is accompanied by pre-myelinating oligodendrocytes (pre-OLs) death, astrogliosis, and microglial infiltration (Folkerth, 2005, Rezaie and Dean, 2002). Later on, these necrotic areas either convert to cysts or collapse to form a white matter scar (Folkerth, 2005). Classification of PVL is established depending on the lesion extension (Anca, 2011).

Perinatal brain damage usually starts with non-specific signs such as irritability, apnoea, seizures, hypotonia, and poor feeding; progression of the disorder is usually accompanied by permanent neurophysiological handicaps such as motor and mental retardation (88%), epilepsy (50%), spastic hemiparesis (88%), cognitive defects (88%) and visual impairment (28%) (Vannucci, 2007, Obenaus and Ashwal, 2008, Mercuri et al., 2003, Nelson, 2007, Koelfen et al., 1993). However, there are some cases of infants who die after birth or within the new born period, with numbers reaching as high as 20–50% (Vannucci, 2007).

Several perinatal factors, which could be maternal or foetal in origin, contribute to the development of PVL. Some risk factors are found to be related to this disorder, such as birth trauma, asphyxia, respiratory failure, cardiac defects (bradycardia, congenital heart disease), prematurity, intrauterine infection (chorioamnionitis), pre-eclampsia and sepsis (Back, 2006, Khwaja and Volpe, 2008, Rezaie and Dean, 2002). Infections are recognised to be predisposing factors for preterm delivery (Back, 2006). Premature infants suffer from impaired cerebrovascular auto-regulation due to changes in cerebral blood pressure, which leads to pressure passive circulation and, therefore, ischaemia (Back, 2006, Khwaja and Volpe, 2008, Rezaie and Dean, 2002, Volpe et al., 2011). In general, hypoxia-ischaemia looks to be an important characteristic underlying these risk factors.

5.6 Mechanism of Ischaemic Injury in White Matter:

The demands of high energy requirements and limited energy stores in the CNS make the brain highly sensitive to energy deficiency and susceptible to injury (Stys, 2005). Absence of cytoplasmic ATP leads to failure of all ATP-dependent pumps, such as the $\text{Na}^+ - \text{K}^+$ ATPase and Ca^{2+} -ATPase; therefore, ions move down their concentration gradients (Stys, 2005).

Failure of $\text{Na}^+ - \text{K}^+$ -ATPase mediates K^+ efflux and Na^+ influx into the axoplasm, and elevated level of $[\text{K}^+]_e$ produces anoxic depolarisation (Stys, 1998, Stys, 2004, Stys, 2005, Ransom et al., 1992). A high concentration of intracellular Na^+ ultimately reverses the $\text{Na}^+ - \text{Ca}^{2+}$ exchanger and evokes Ca^{2+} influx through the reversed $\text{Na}^+ - \text{Ca}^{2+}$ exchanger (Stys, 1998). Intracellular Ca^{2+} accumulation is also mediated through voltage-gated Ca^{2+} channel, and Ca^{2+} is released from intracellular stores in some axon populations (Muller and Ballanyi, 2003, Stys, 2005). Membrane depolarisation triggers excitotoxic neurotransmitter releases, such as glutamate. A high level of glutamate in the extracellular space may result from vesicular release, failure of reuptake, and reversal of glutamate uptake (Stys, 1998, Stys, 2005). Glutamate can subsequently activate metabotropic receptors, as well as three types of ionotropic receptors: NMDA, AMPA and Kainate (Harukuni and Bhardwaj, 2006). Ionotropic glutamate receptor activation can mediate toxic Ca^{2+} influx. An elevated level of intracellular Ca^{2+} triggers a series of subcellular destructive events, as it causes mitochondrial Ca^{2+} overload and dysfunction and activates a number of Ca^{2+} -dependent enzymes, such as phospholipase C, phospholipase A2, protein kinase C, and nitric oxide synthetase, which produces nitric oxide (NO). NO causes DNA damage and energy failure due to mitochondrial dysfunction and phospholipase activation enhances cell membrane breakdown and death (Harukuni and Bhardwaj, 2006). While glutamate-

mediated excitotoxicity has largely been described in neurons of the brain's grey matter, recent evidence has suggested an important role during ischaemia in white matter (Kukley et al., 2007, Ziskin et al., 2007).

5.6.1 Mechanism of ischemic injury in axons:

Identification of the mechanism of axon injury is of paramount importance for the rational design of therapeutic intervention against PVL and other forms of white matter injury. The response to energy failure is quite different in white matter compared to grey matter and depends on developmental stage. It has been found that the ischaemic injury in myelinated axons is Na^+ and Ca^{2+} -dependent while it is Ca^{2+} -dependent and Na^+ -independent in pre-myelinated axons (Stys, 1998, Alix and Fern, 2009). Oligodendrocyte GluRs play a main role in mediating Ca^{2+} influx, and injury of these cells is closely associated with axons in which GluRs are implicated in axon injury, as shown in myelinated spinal cord axons (Ouardouz et al., 2009a, Ouardouz et al., 2009b). Alex and Fern provide a number of important mechanistic insights into ischaemic injury in P10 pre-myelinated axons (2009) and show that 60 minutes of OGD causes extensive injury of P10 RON pre-myelinated axons. Administration of NMDA and non-NMDA receptor blockers, MK-801 and NBQX respectively, also prevented pre-myelinating axon ischaemic injury. Immuno-labelling combined with confocal and electron microscopy localized the GluR-NMDA on oligodendrocyte processes at the site of contact with pre-myelinating axons, while the GluR-AMPA was located on the axolemma and oligodendrocyte soma. GluR-NMDA activation in P10 mouse optic nerve (MON) is also a main cause of damage of oligodendrocyte processes during ischaemia (Salter and Fern, 2005). GluRs-NMDA also mediate pre-myelinated axon damage in P10 RON (Alix and Fern, 2009). This is consistent with the NMDA receptor

expression in P10 RON via PCR detecting mRNAs of each NR1, NR2A-B, NR3A-B subunit (Domingues et al., 2011).

It has been suggested that the distribution of NMDA receptors on glia processes facing pre-myelinated axons at this stage may result in the propagation of damage from oligodendrocytes to the neighbouring axon via some unknown intermediate. However, the contribution of AMPA receptors cannot be ruled out, as oligodendrocyte NMDA receptors depend on AMPA receptors to overcome Mg^{2+} block (Karadottir et al., 2005). It has been found that axon-oligodendrocyte precursor cell synapse in the P6 hippocampus is deeply injured during ischaemia through AMPA receptor-mediated Ca^{2+} influx and excitotoxicity (Shen et al., 2012).

5.6.2 Mechanism of ischemic injury in astrocytes:

Ischaemia evokes different mechanisms of injury in astrocytes according to their maturation. Ischaemic injury in P10 RON astrocytes is mediated by a $Na^+-K^+-Cl^-$ cotransporter, while injury is Ca^{2+} -dependent in P0–P2 RON astrocytes where VGCCs are key (Thomas et al., 2004, Fern, 1998). It has been found that ischaemic accumulation of $[Ca^{2+}]_i$ in P0 RON astrocytes is mediated by VGCC but not by Ca^{2+} released from intracellular stores or glutamate-mediated Ca^{2+} influx (Fern, 1998). This is consistent with a tissue print that was used to examine the presence of VGCC in P2 and P10 RON astrocytes and suggested the presence of type T and type L VGCC in P2 RON, while type T VGCC was clearly reduced in P10 RON (Barres et al., 1990a). It has been noticed that astrocytes lose their processes during ischaemia (Thomas et al., 2004, Hulse et al., 2001). The mechanism of P10 astrocyte injury to both somata and processes is promoted by both $Na^+-K^+-Cl^-$ cotransporters and Na^+-K^+ dependent HCO_3^- transporters (Salter and Fern, 2008a).

5.7 Astrocytes and Ischaemia:

5.7.1 Reactive Astrocytes:

Until recently it was believed that astrocytes are more resistant to ischaemia than neurons (Chen and Swanson, 2003, Liu et al., 1999). In fact, several studies have shown that astrocytes are highly sensitive to ischaemic injury and document a significant percentage of cell death over acute periods of modelled ischaemia, which could reach 50% (Fern, 1998, Thomas et al., 2004, Petito et al., 1998). Several *in vivo* studies, including four-vessel occlusion, middle cerebral artery occlusion (MCAO), and global ischaemia report astrocyte death within 2 to 60 minutes of ischaemia; this is not dissimilar to the injury sensitivity of neurons (Petito et al., 1998, Davies et al., 1998, Fern, 1998, Thomas et al., 2004).

Human neonatal ischaemic cerebral specimens revealed 15–40% white matter astrogliosis (Rezaie and Dean, 2002), while gliosis is an important component in PVL as well (Hirayama et al., 2001). A large number of CNS injuries trigger the appearance of reactive astrocytes including ischaemia, mechanical trauma, tumours and neurodegenerative diseases (Li et al., 2008). Reactive astrocytes are characterised by an increase in cell proliferation, hypertrophy, and the upregulation of intermediate filaments, of which the latter is composed of GFAP, nestine and vimentin expression (Li et al., 2008, Ridet et al., 1997, Buffo et al., 2010). However, there is some debate about the effect of acute ischaemia on astrocytes and whether the appearance of reactive astrocytes will mask the real picture, i.e., whether astrocyte death would be hidden by astrocyte proliferation. To avoid any queries raised about inaccuracy of some methods such as IHC and GFAP expression (Fern, 2001), live cell imaging of GFP expressed astrocytes was used to allow a calculation of the astrocyte number in a time

series, as well as cell morphology. This study suggested the absence of reactive astrocytes within 180 minutes of perfusion of modelled ischaemia in P7–14 MON (Shannon et al., 2007). Despite the absence of their roles in the acute events of ischaemia, they still play important roles in the post-ischaemic period. These cells can limit the toxic spreading from dead cells, regulating excessive glutamate and K^+ , and control the second stage of damage (Rolls et al., 2009). In addition, they can support the living cells after ischaemia by providing glucose, nutrients, and growth factors (Rolls et al., 2009).

5.7.2 Glutamate Homeostasis:

Astrocytes play a central role in glutamate homeostasis. Glutamate is an important neurotransmitter that mediates excitatory signalling in the nervous system. There is an abundance of glutamate in the brain, but most is intracellular, and the extracellular level is tightly controlled. Excessive uncontrolled levels of extracellular glutamate can overactivate GluR and evoke excitotoxicity and cell death. The level of extracellular glutamate increases during brain ischaemia (Benveniste et al., 1984). Glutamate receptors are expressed in both grey and white matter (Belachew and Gallo, 2004, Verkhratsky and Steinhauser, 2000, Matute et al., 2006). Astrocytes are well supplied with both ionotropic and metabotropic glutamate receptors (Gallo and Ghiani, 2000, Matute et al., 2006, Belachew and Gallo, 2004).

Glutamate is synthesised in neurons and astrocytes from glutamine via glutaminase or from α -ketoglutarate (α -KG) via aspartate aminotransferase (AAT) (Schousboe and Waagepetersen, 2005). It is important to know that glutamine synthetase and pyruvate carboxylase (the latter is needed by tricarboxylic acid (TCA) cycles) are astrocytes specific enzymes; this reveals the importance of astrocytes in glutamate synthesis (Schousboe and Waagepetersen, 2005). Glutamate reuptake from

the extracellular space depends on a number of neuronal and glial glutamate transporters. Five types of glutamate transporters have been cloned and are known as GLT (EAAT2), GLAST (EAAT1), EAAC (EAAT3), EAAT4 and EAAT5 (Danbolt, 2001). Adult astrocytes express GLT-1, GLAST and EAAC1, while P14–P17 astrocytes express only GLT-1 in the RON (Arranz et al., 2008). Astrocytes play an essential role in glutamate uptake and maintaining a non-toxic level of extracellular glutamate. This fact is based on data from several studies using GLAST or GLT-1, but not neuronal subtype (EAAC1), for knockdown in rats, and the result shows elevated levels of extracellular glutamate and excitotoxic neurodegeneration (Rothstein et al., 1996, Watase et al., 1998, Tanaka et al., 1997). Other studies show that neuronal cortical cultures that lack astrocytes are 100-fold more susceptible to glutamate injury than the culture with abundant astrocytes (Rosenberg and Aizenman, 1989). Astrocytes will have a high energetic cost to mediate this glutamate uptake, as the process expends energy, estimated as a large percentage of total brain ATP turnover (Sibson et al., 1998). Depolarisation triggers glutamate release by ATP-dependent vesicle secretion or by reverse glutamate transporters (Schousboe and Waagepetersen, 2005). Because both glutamate reuptake by glutamate transporters and glutamate secretion by vesicles are energy consuming, astrocytes are sensitive to any energy decline. During ischaemia, astrocytes fail to control the glutamate level and tend to secrete glutamate through reversal of glutamate transporters (Rossi et al., 2000). However, during ischaemia, a high level of extracellular glutamate is not only due to glutamate efflux from astrocytes, but it has been shown that a greater quantity of glutamate is secreted from neurons (Rossi et al., 2000). At the same time, it has been shown that astrocytes try, at least for some time, to tolerate a high level of extracellular glutamate during ischaemia by

increasing the level of intracellular glutamate, as demonstrated by increasing glutamate to glutamine ratio *in vivo* (Ottersen et al., 1996).

5.7.3 Astrocyte Metabolism:

Astrocytes are able to produce energy via both oxidative phosphorylation and glycolysis (Dienel and Hertz, 2005). Glycolysis produces 2 net ATP per glucose molecules while oxidative phosphorylation produces 36 net ATP per glucose molecules (Dienel and Hertz, 2005). However, in normal conditions, it is suggested that 25–32% of astrocyte metabolism is mediated by glycolysis, while the majority is covered by oxidative phosphorylation (Silver and Erecinska, 1997). By comparing astrocytes to neurons, it is not surprising that around 75% of glucose phosphorylation in parenchyma is undergone in astrocytes (Hyder et al., 2006). In addition to that, most glycogen reserves in the adult brain are shown only in astrocytes (Brown, 2004).

Astrocyte metabolism has a crucial role in maintaining ionic homeostasis, as many ionic transporters are ATP-dependent (e.g., Na^+/K^+ ATPase) (Dienel and Hertz, 2005). In physiological conditions, astrocyte metabolism is stimulated by high levels of neurotransmitters such as glutamate, and this is needed to meet the high energy cost processes of glutamate uptake and Na^+ extrusion by Na^+/K^+ -ATPase (Dienel and Hertz, 2005). Glycolysis, as well, plays an important role in astrocyte metabolism and is usually localised in areas with a lack of mitochondria, such as filopodia (Dienel and Cruz, 2003). However, in the case of hypoglycaemia, glycogen can be a good energy source when the tissue energy demand increases (Wang and Bordey, 2008). Not only glycogen can be used as an alternative to glucose; astrocytes have other sources for energy, such as lactate and fatty acids (Rossi et al., 2007). On the other hand, ischaemia is a more severe case than hypoglycaemia, as oxygen is needed for mitochondrial respiration and production of ATP (Rossi et al., 2007). Some evidence shows that only

cells in the penumbra of focal ischaemia, where the blood supply drops to around 20 to 40% of its normal level, have the chance to survive following anaerobic glycolysis (Dienel and Hertz, 2005, Back, 1998, Kato and Kogure, 2000).

5.8 Neurotransmitter Receptors

Neurotransmitters mediate signal transmission and cell communication through binding to specific receptors. These receptors are generally classified into two main groups: ionotropics and metabotropics (see Table 5-2 for some examples). They are structurally and functionally different. Activation of ionotropic receptors mediates the direct opening of an ion channel, which allows ion movement down its gradients and therefore generates a current rapidly. On the other hand, metabotropic receptor action is mediated by a G protein coupled to its intracellular surface, in which the latter activates a series of intracellular responses and, therefore, spends more time to generate a response, estimated from tenths of seconds to hours (Squire L.R., 2008).

5.8.1 Ionotropic Receptors Structure:

Ionotropic receptors are relatively larger than metabotropic receptors and comprise four or more large protein subunits (except VGKC, which can have one or more). Each subunit has a large extracellular hydrophilic N-terminal domain, and four hydrophobic membranes spanning segments (M1 to M4) (Hammond, 2008). The ionotropic receptor is lined by an M2 segment which may play a role in ion selectivity and transport (Hammond, 2008). There is a phosphorylation site in the long intracellular hydrophilic domain connecting M3 and M4 segments. The large N-terminal domain is exposed to the synaptic cleft and contains neurotransmitter and glycosylated binding sites (Hammond, 2008).

5.8.2 Metabotropic Receptors Structure:

Metabotropic receptors are made up of seven hydrophobic α helices transmembrane segments that wrap back and forth within the cell membrane (TM1-TM7), an intracellular hydrophilic carboxyl terminus (C-terminus) that carries the cysteine residue, and an extracellular hydrophilic amino terminus (N-terminus). These parts are connected by three extracellular (loops 1–3) and four intracellular (loops 1–4) polypeptide chains (Shenker, 1995).

Table 5-2: Some examples of neurotransmitters receptors.

	<i>Ionotropic</i>	<i>Metabotropic</i>
Glutamate receptors	Glut-AMPA Glut-NMDA Glut-Kainate	Metabotropic GlutRs
GABA receptors	GABA _A , GABA _C	GABA _B
Glycine receptors	Glycine receptor	
Acetylcholine receptors	Nicotinic AChRs	Muscarinic AChRs
Catecholamine receptors	Adrenergic receptor	Dopamine receptors
Serotonin receptors	5-HT ₃ receptor	5-HT ₁₋₇ (except 5-HT ₃)

5.9 GABA_A Receptor:

GABA_A receptor is a member of the pentameric ligand-gated ion channel receptor superfamily (LGIC), to which glycine, nicotine (nAChR), serotonin (5-HT₃R), GABA_C, glutamate and histidine receptors also belong (Betz and Laube, 2006, Lynch, 2004, Gisselmann et al., 2002). GABA (γ-aminobutyric acid) is the most widely distributed inhibitory neurotransmitter in the brain. It is released by presynaptic terminals and activates two types of receptors: ionotropic (GABA_A and GABA_C), and metabotropic (GABA_B) receptors. GABA_A mediates the majority of GABA functions.

5.9.1 Structure and Classification:

GABA_A is an ionotropic receptor in which nineteen subunits of GABA_AR have been identified in the mammalian nervous system (α_1 , α_2 , α_3 , α_4 , α_5 , α_6 , β_1 , β_2 , β_3 , γ_1 , γ_2 , γ_3 , ρ_1 , ρ_2 , ρ_3 , δ , ϵ , π and, θ), giving rise to the multiplicity of different GABA receptor subtypes, characterised by different functions and pharmacology (Olsen and Sieghart, 2008, Sieghart, 1995, Hammond, 2008, Bormann, 2000). The majority of GABA receptors have two α , two β , and one γ subunit ($\beta \alpha \beta \alpha \gamma$); usually, these are of α_1 , β_2 , and γ_2 subtypes (Shrivastava et al., 2011).

The International Union of Pharmacology (IUPHAR Committee) has classified GABA_A receptors, according to the presence of α subunit, into GABA_{A1} to GABA_{A6} (Bormann, 2000). GABA_A receptors of $\alpha \beta \delta$, or $\alpha \beta \epsilon$ subunits, which are insensitive to benzodiazepine, are categorised as GABA_{A0} (Bormann, 2000). GABA_{A0r} classification was created for any α containing GABA receptors if they are bicuculline insensitive (Bormann, 2000).

5.9.2 Binding Sites:

It has been found that α and β subunits are enough to form homomeric functional GABA_A receptors that induce a current in the presence of GABA (Hammond, 2008). However, these types of receptors are not blocked with benzodiazepines (Hammond, 2008). Expression of γ subunit together with α and β subunits is needed for GABA_A receptor formation that is affected by benzodiazepine (Zorumski and Isenberg, 1991, Bormann, 2000, Hammond, 2008). That clarifies the presence of GABA binding site in α and β subunits and the contribution of γ subunit in benzodiazepine sensitivity. To be more specific, glutamate (E) 155, tyrosine (Y) 157, and threonine (T) 160 are amino acids identified in the β subunits that affect GABA receptor activity, while phenylalanine (F) 64 to leucine (L) are in α subunits. β subunit and the nearby α subunit share the formation of the GABA binding site (Hammond, 2008). That means there are two GABA binding sites and one benzodiazepine binding site in the GABA receptor of $\beta \alpha \beta \alpha \gamma$ subunits.

5.9.3 Function:

GABA_A receptors are selectively permeable to Cl^- . However, these receptors behave differently in the mature and developing nervous systems. Activation of GABA_A receptors in the adult nervous system generates influx and membrane hyperpolarisation through Cl^- reversal potential following Nernst equation discussed previously in section 5.3. Therefore, GABA is an important inhibitory neurotransmitter in the adult nervous system, and its absence initiates epileptiform activities. On the other hand, activation of GABA_A receptors in neonates initiates membrane depolarisation and excitation via generation of sodium action potential (Ben-Ari, 1997, Debray et al., 1997, Hammond, 2008, Cherubini and Ben-Ari, 2011). This issue was

observed in all regions of the CNS, including the spinal cord, hypothalamus, neocortex, cerebellum, cortex, hippocampus and olfactory bulb (Ben-Ari, 1997, Debray et al., 1997, Wu et al., 1992, Serafini et al., 1995, Wang et al., 1994, Obrietan and Vandenpol, 1995, Luhmann and Prince, 1991, Owens et al., 1996, Fiszman et al., 1990, Mueller et al., 1984, Connor et al., 1987, Ben-Ari et al., 1989, Rheims et al., 2008). This could be related to the high level of intracellular Cl^- ions in neonates as a result of developmental changes in the intracellular homeostasis system, which reverses the Cl^- driving force (Serafini et al., 1995, Hara et al., 1992, Staley et al., 1996, Hammond, 2008). A Ca^{2+} influx has been reported after activation of the GABA_A receptor (Wang et al., 1994, Connor et al., 1987, LoTurco et al., 1995, Hales et al., 1994, Chen et al., 1996, Yuste and Katz, 1991). It is known that the GABA_A receptor is impermeable to Ca^{2+} , and it has been found that both NMDA receptors and voltage-gated Ca^{2+} channels play a role in this intracellular Ca^{2+} increase (Ben-Ari, 1997, Hammond, 2008, Wang et al., 1994, Connor et al., 1987, LoTurco et al., 1995, Hales et al., 1994, Chen et al., 1996, Yuste and Katz, 1991, Ben-Ari et al., 1997). A study done by Ben-Ari and his team showed that activation of the GABA_A receptor in the hippocampus during the early postnatal period (P0–P2) triggers membrane depolarisation and thus the opening of both voltage gated Ca^{2+} and Na^+ channels (Ben-Ari et al., 1997). Between P2 and P5, GABA_A depolarisation activates NMDA receptors in addition to voltage-gated Ca^{2+} and Na^+ channels, and that mediates more Ca^{2+} influx (Ben-Ari et al., 1997). Following maturation, GABA_A receptor activation generates membrane hyperpolarisation via Cl^- influx and AMPA receptors play the excitatory role through permitting cation (K^+/Na^+) crossing the cell membrane (Ben-Ari et al., 1997).

5.9.4 Pharmacology:

A couple of nervous system medications act on GABA_A receptors allosteric sites and modulate its activity (Zorumski and Isenberg, 1991). There are multiple GABA_A receptor agonists, such as benzodiazepine (which is used as an anxiolytic agent, muscle relaxant, and anticonvulsant), barbiturates (which is a hypnotic), and anti-epileptic agents and neurosteroids (Delorey and Brown, 1992). These GABA_A agonists bind to the GABA_A receptor at specific receptor sites. On the other hand, there are some well-known GABA_A non-competitive antagonists, such as picrotoxin, and competitive antagonists, such as bicuculline. Bicuculline binds to the GABA binding site in the receptor, while picrotoxin is a channel blocker and binds to the M2 segment (Zorumski and Isenberg, 1991). The exact binding site of picrotoxin is unclear, but it is suggested that it binds to residue 6, a highly conservative residue of the M2 segment (Hammond, 2008).

5.9.5 Synthesis and Distribution:

GABA is synthesised from glutamate by GAD (glutamic acid decarboxylase) and is stored inside vesicles at the presynaptic terminal (Uusi-Oukari and Korpi, 2010). During depolarisation, Ca²⁺ channels are opened and mediate Ca²⁺ influx. This results in neurotransmitter release into the synaptic cleft following vesicular docking. GABA in the synaptic cleft can bind either to GABA_A or GABA_B receptors. This response is ended by receptor desensitisation and reuptake of excess GABA by GABA transporters (GAT) into either presynaptic terminals or surrounding glia (Uusi-Oukari and Korpi, 2010).

GABA is a major neurotransmitter with widespread distribution in the central nervous system. Studies have examined the distribution of GABA subunits in the brain

(Uusi-Oukari and Korpi, 2010), which have indicated the presence of the GABA system in non-synaptic white matter regions such as the optic nerve. A significant amount of data indicates the presence of GABA in the optic nerve, starting with the immune-electron microscopy which showed GABA immunostaining in pre-myelinated axons and glial cells of neonatal rat optic nerves, with lower levels in adult rats (Sakatani et al., 1992). The mRNA of GABA transporters, GAT-1, GAT-2 and GAT-3, are expressed in neonatal optic nerve detected via PCR (Howd et al., 1997). Electrophysiological studies of neonatal optic nerves showed a depolarising effect of GABA after GABA_A activation by applying agonists or GABA uptake inhibitors (nipecotate: NPA) (Sakatani et al., 1992, Butt and Jennings, 1994b, Sakatani et al., 1991b). It is believed that GABA_A, but not GABA_B, is responsible for GABA actions in the optic nerve. In the neonatal optic nerve, GABA_A action is blocked by applying the GABA_A antagonist, bicuculline (Sakatani et al., 1992), while neonatal optic nerve depolarisation of GABA looks similar to that induced by the GABA_A agonist, muscimol (5.5 ± 1.1 mv) (Sakatani et al., 1992). There is no effect on the membrane potential after applying the GABA_B agonist baclofen (Sakatani et al., 1992). All of the evidence suggests that GABA action in the optic nerve is mediated by the GABA_A receptor subtype.

5.10 Glycine Receptor:

5.10.1 Structure:

The glycine receptor is a pentameric receptor which could be homomeric and contains five α subunits or heteromeric receptors and has α and β subunits (Kuhse et al., 1993, Grudzinska et al., 2005). Cross linking experiments suggest a stoichiometry of 3 α and 2 β subunits (Betz and Laube, 2006, Laube et al., 2002). However, another study using wild type α and β subunits co-expression with α and β tandem construct indicate a stoichiometry of 2 α and 3 β subunits (Grudzinska et al., 2005). Four subtypes of α subunits (α_1 , α_2 , α_3 and α_4) and only one type of β subunit have been distinguished. It is believed that α subunit of α_2 subtype is an embryonic type that gradually disappears after birth with an increase in α_1 and α_3 subtypes (Becker et al., 1992, Singer et al., 1998).

5.10.2 Pharmacology:

Glycine is one of the major inhibitory neurotransmitters in the adult CNS. Activation of glycine receptors mediates Cl^- influx and produces inhibitory post-synaptic potentials. There are a variety of agents that act on glycine receptors and contribute in some therapeutic treatments. Some of these antagonise glycine: opioids, which are used for narcotic and antitussive purposes (Rajendra et al., 1997), tropeines, which are used as antispastics and anaesthetics (Ren et al., 1999), benzodiazepine, which is used as a sedative and anxiolytic (Rajendra et al., 1997), and steroids (Wu et al., 1990, Rajendra et al., 1997). Others potentiate glycine effect, such as penicillin G (Breitinger and Becker, 1998), alkylbenzene sulphonate (Machu et al., 1998), and chlormethiazole (Hales and Lambert, 1992). Unfortunately, most of these agents lack glycine receptor specificity (Laube et al., 2002). However, it has been found that

strychnine is a potent competitive antagonist of glycine receptors (Palma et al., 1999). It has been shown that glycine and strychnine bind to the α subunits only (Becker et al., 1986, Graham et al., 1983). However, Grudzinska and colleagues suggest some differences in the number of agonist and antagonist binding sites in homomeric and heteromeric glycine receptors (Grudzinska et al., 2005).

5.10.3 Function and Regulation:

It is accepted that both GABA and glycine act as excitatory neurotransmitters in the developing nervous system, i.e., they mediate depolarisation (Cherubini et al., 1991, Flint et al., 1998, Reichling et al., 1994, Singer and Berger, 2000, Singer et al., 1998). Depolarisation via GABA and glycine provoke Ca^{2+} influx through voltage-gated Ca^{2+} channels (Flint et al., 1998, Owens et al., 1996, Reichling et al., 1994, Wang et al., 1994). It is known that glycine is also a co-agonist for NMDAR, distinct from its action at glycine receptors. It mediates the excitatory effect of a glutamate receptor via direct activation of its ion channel (Chatterton et al., 2002, Lynch, 2004).

Generation of action potential mediates ATP hydrolysis through Na^+/K^+ ATPase, which leads to increased $[\text{K}^+]_i$ and decreased $[\text{Na}^+]_i$ with changes in the intracellular pH (Rudnick, 1998). These changes in intracellular pH and membrane potential mediate glycine accumulation inside the vesicular lumen (Rudnick, 1998). Extracellular glycine is regulated by reuptake by sodium/chloride-dependent glycine transporters (GLYT_s) (Aragon and Lopez-Corcuera, 2005, Eulenburg et al., 2005). There are two types of GLYT_s: GLYT₁, which is distributed through CNS and located mainly in glial cells (Adams et al., 1995, Zafra et al., 1995), and GLYT₂, which is identified in axons and presynaptic terminals of glycinergic neurons of the lower brain regions and spinal cord (Liu et al., 1993, Zafra et al., 1995). VIAAT (vesicular inhibitory amino acid transporter) is a glycine transporter that localises on the synaptic

vesicle in glycinergic terminal (Dumoulin et al., 1999, Legendre, 2001); this intracellular glycine transporter does not depend on Na^+/Cl^- ions but is mediated by membrane potential and antiport of H^+ (Rudnick, 1998).

5.11 Nicotinic Cholinergic Receptors:

Cholinergic receptors have an important role in nervous system pathways and are classified as muscarinic, localised at the neuromuscular junction, or neuronal nicotinic (nAChR), which is found throughout the central and peripheral nervous system (Hogg et al., 2003).

5.11.1 Structure:

NACHRs are pentameric receptors and have a similar structure to the other ionotropic receptors, as discussed previously in section 1.8.1. Different subunits of AChRs with specific stoichiometry have been identified, e.g., AChRs in neuromuscular junctions consist of $\alpha 1$, $\beta 1$, γ , δ , and ϵ subunits, which are commonly in 2:1:1:1:1 stoichiometry ($2\alpha 1: 1\beta 1: 1\gamma: 1\delta: \text{or } 1\epsilon$), while neuronal nAChRs are made up of α ($\alpha 2$ – $\alpha 9$) and β ($\beta 2$ and $\beta 4$) subunits in $2\alpha:3\beta$ stoichiometry (Paterson and Nordberg, 2000). In addition, neuronal nAChRs can be homomeric receptors composed of only one subunit type ($\alpha 7$, $\alpha 8$, or $\alpha 9$) (Couturier et al., 1990). Around nine nAChR subunits, six α subunits ($\alpha 2$ – $\alpha 7$), and three β subunits ($\beta 2$ – $\beta 4$) are identified in the human brain (Paterson and Nordberg, 2000). The presence of $\alpha 3$, $\alpha 4$, $\alpha 7$, $\beta 3$, and $\beta 4$ subunits has been documented in hippocampal astrocytes (Graham et al., 2003, Sharma and Vijayaraghavan, 2002, Gotti and Clementi, 2004). It was elucidated that the glial somas express $\alpha 3\beta 4$, while glial processes express $\alpha 4\beta 2$ receptor (Graham et al., 2003).

5.11.2 Classification:

Based on the pharmacological aspect, nAChRs are classified into two main groups. The first group is α Bgt-nAChRs, which is characterised by selective α Bgt binding (Gotti and Clementi, 2004, Govind et al., 2009). This type of receptor could be homomeric and constituted of $\alpha 7$ – $\alpha 9$ subunits, or heteromeric and composed of $\alpha 7$, $\alpha 8$,

or $\alpha 9$, $\alpha 10$ subunits (Gotti and Clementi, 2004). The second group is heteromeric receptors, composed of $\alpha 2$ - $\alpha 6$ and $\beta 2$ - $\beta 4$ subunits and characterised by high affinity binding to other agonists (nM affinity) (Govind et al., 2009).

5.11.3 Pharmacology and Binding Sites:

Nicotinic receptors have multiple binding sites for different agonists, antagonists and allosteric drugs. Acetylcholine (ACh) is a well-known agonist that binds to both homomeric and heteromeric nAChRs. The heteromeric nAChRs carry two ACh binding sites that are located at the interface between α and β subunits, while homomeric nAChRs carry five ACh binding sites at the interface between α receptors (Paterson and Nordberg, 2000, Gotti and Clementi, 2004). Several allosteric compounds modulate nAChRs, and these compounds can be either activating or inhibitory; for more details. Non-competitive allosteric activators, such as physostigmine, bind to the α subunits away from ACh binding sites and trigger channel activation (Pereira et al., 1993). On the other hand, a non-competitive allosteric inhibitors, such as chlorpromazine or MK 801, act on two different sites: a high affinity M2 binding site, which is present inside the ion channel, and a lower affinity binding site, located between the receptor and the outer layer of cell membrane (Lena and Changeux, 1993). Steroids such as progesterone, corticosterone and dexamethasone are known to desensitise nicotinic receptors by binding to the N-terminal hydrophilic domain (Bertrand et al., 1991). In addition, L type Ca^{2+} blockers, such as nimodipine and nifedipine, can bind to the ion channel of nAChRs and inhibit their effect (Lopez et al., 1993). Several compounds, such as substance P, can bind to the intracellular loop connecting M3 to M4 and enhance phosphorylation and, therefore, nAChR desensitisation (Simmons et al., 1990). A high affinity blocker, α Bgt binds at ACh binding sites in α Bgt-nAChRs and triggers a rapid receptor desensitisation (Gotti and

Clementi, 2004). Mecamylamine has been used as a broad spectrum blocker for nAChRs (Papke et al., 2001b). It has the power to block the physiological, behavioural, and reinforcing features of nicotine (Martin et al., 1990, Martin et al., 1989). Mecamylamine is classified as a nAChRs channel blocker (Paterson and Nordberg, 2000); however, it has been demonstrated to share some features of both competitive and non-competitive antagonists (Martin et al., 1990).

5.11.4 Synthesis and Function:

Acetylcholine is synthesised inside the soma after choline is combined with acetyl CoA and converted to acetylcholine by the enzyme choline acetyltransferase (ChAT) (Abreu-Villaca et al., 2011, Niewiadomska et al., 2009). ACh is then transferred and stored inside vesicles by vesicular acetylcholine transporters (VACHT) (Abreu-Villaca et al., 2011). Activation of presynaptic terminals enhances vesicle fusion to the cell membrane and ACh excretion, which the latter can bind to pre- or post-synaptic nicotinic nAChRs or to muscarinic receptors. Within the synaptic cleft, ACh is cleaved into choline and acetate by acetylcholinesterase (AChE). The choline is then re uptaken to the presynaptic terminals by a high affinity choline transporter (CHT) (Abreu-Villaca et al., 2011).

Activation of nAChRs facilitates influx of both monovalent cations, such as Na^+ , and divalent cations, such as Ca^{2+} (Gotti and Clementi, 2004). However, Ca^{2+} permeability differs according to the receptor subunits and species, e.g., pure $\alpha 7$ nAChRs are more permeable to Ca^{2+} , with a fractional Ca^{2+} current (Pf) that reaches 6–12%, than the heteromeric receptors, which have a Pf of 2–5% (Dajas-Bailador and Wonnacott, 2004). Activation of nAChRs plays a role in Ca^{2+} homeostasis, as it triggers membrane depolarisation and voltage-gated Ca^{2+} channel activation, which

triggers further Ca^{2+} influx (Gotti and Clementi, 2004). It has been demonstrated that activation of heteromeric receptors consisting of $\alpha 3$ and/or $\beta 2$ subunits mediates a Ca^{2+} current with VGCC assistance, while homomeric $\alpha 7$ can also trigger a Ca^{2+} current alone (independently of VGCC) (Dajas-Bailador and Wonnacott, 2004). Activation of nAChRs enhances neurotransmitter release as GABA (Lu et al., 1998), noradrenaline (Clarke and Reuben, 1996), and ACh (Grady et al., 2001).

5.12 Adrenergic Receptors:

5.12.1 Structure and Classification:

Adrenoceptors are metabotropic receptors. Despite the differences between receptors in this classification, they share some general features, as discussed previously in section 1.8.2. Nine types of adrenoceptors are identified and are divided into three main groups: α_1 , α_2 , and β receptors. Each group is classified into three subdivisions: α_1 (α_{1A} , α_{1B} , α_{1D}), α_2 (α_{2A} , α_{2B} , α_{2C}), and β (β_1 , β_2 , β_3). They are classified as α and β receptors according to their agonist selectivity, e.g., α receptors are more sensitive to norepinephrine and epinephrine than to the synthetic isoproterenol (ISO), while β receptors bind more often to ISO than to natural catecholamines (Brady S. T., 2012). These receptors are located pre- and post-synaptically (Rho and Storey, 2001). The α_1 , α_2 , and β receptors are metabotropic receptors that bind to G_q/G_{11} , G_i/G_o , G_s proteins, respectively, and activation of each type triggers some cellular effects (see Table 5-3 for more information). These receptors are not limited to neurons, but it has been found that astrocytes express α_{1A} , α_{2A} , and β adrenergic receptors (Hertz et al., 2010, Muyderman et al., 2001, Gibbs and Bowser, 2010).

5.12.2 Function:

Adrenergic receptors are widely distributed all over the body with a high concentration in the CNS; see Table 5-3. The central effect of norepinephrine on the receptors is the enhancement of G protein activation. Stimulating G_q/G_{11} protein coupled to α_1 activates phospholipase C and phosphatidylinositol second messenger systems, which facilitate Ca^{2+} influx and diacylglycerol formation (Rho and Storey, 2001). On the other hand, activation of G_i/G_o protein coupled to α_2 and of G_s protein coupled to β receptor inhibits and activates adenylyl cyclase (which is an important

enzyme for cyclic AMP synthesis), respectively (Ulloa-Aguirre et al., 1999, Rho and Storey, 2001).

Table 5-3: Distribution and effect of adrenoceptors subtypes. (Ahlquist, 1976b, Ahlquist, 1976a, Ahlquist, 1977, Brady S. T., 2012)

	<i>Effect</i>	<i>Location</i>
α_{1A}	$\uparrow$ Ca^{2+} , protein kinase C	Olfactory system, hypothalamic nuclei, motor areas in brain-stem and spinal cord, cerebellum, cerebral cortex, blood vessels heart, liver
α_{1B}	$\uparrow$ Ca^{2+} , protein kinase C	Spleen, kidney, foetal brain, blood vessels, cerebral cortex, thalamus, raphe nuclei, cranial and spinal motor neurons, pineal gland
α_{1D}	$\uparrow$ Ca^{2+} , protein kinase C	Aorta, cerebral cortex, olfactory bulb, hippocampus, dentate gyrus, reticular thalamic nucleus, motor neurons, inferior olivary complex
α_{2A}	$\downarrow$ cAMP	Pancreas, small intestine, locus coeruleus, hippocampus, cerebral cortex, amygdala, hypothalamic paraventricular nucleus, nucleus tractus solitarii, ventrolateral reticular formation, spinal cord, and dorsal root ganglion
α_{2B}	$\downarrow$ cAMP	Liver, thalamus
α_{2C}	$\downarrow$ cAMP	Heart, lung, aorta, hippocampus, olfactory bulb, islands of Calleja, cerebral and cerebellar cortex, dorsal root ganglion
β_1	$\uparrow$ cAMP	Heart, kidney, cerebral cortex, hypothalamus, hippocampus, striatum
β_2	$\uparrow$ cAMP	Lung, liver, hippocampus, cerebral and cerebellar cortex, smooth muscle, olfactory bulb
β_3	$\uparrow$ cAMP	Fat

5.12.3 Catecholamine Synthesis:

In the nervous system, catecholamines are synthesised in neurons near the area of secretion (either neuron terminals or soma) (Brady S. T., 2012). Tyrosine hydroxylase mediates the conversion of tyrosine to 3, 4-dihydroxyphenylalanine (L-DOPA) (Vieira-Coelho et al., 2009). L-DOPA originates from the sympathoneural pathway as well as from non-neuronal cells and could be derived from arterial plasma and carried by L-type amino acid transporters (LAT) (Goldstein et al., 2003, Vieira-Coelho et al., 2009). The concentration of L-DOPA in plasma is found to be 10 times higher than norepinephrine; this is due to the active reuptake of norepinephrine (Goldstein et al., 2003). Amino acid decarboxylase catalyses the conversion of L-DOPA to dopamine; the latter is stored inside vesicles through vesicular monoamine transporters (Vieira-Coelho et al., 2009). Dopamine is converted to norepinephrine by dopamine β -hydroxylase, while norepinephrine is converted to epinephrine by phenylethanolamine N-methyltransferase (Vieira-Coelho et al., 2009, Kanagy, 2005). It has been found that the concentration of tyrosine hydroxylase and dopamine β -hydroxylase at birth equals only 10% to 20% of that of the adult level, which could be reached at the end of the fifth postnatal week (Rho and Storey, 2001). The mature form of noradrenergic innervation is first found on postnatal day 7 (Rho and Storey, 2001).

5.12.4 Pharmacology and Binding Sites:

GPCRs share similar transmembrane structure, while showing some differences in the amino terminal chain, as it is shorter in monoamine and peptide receptors than in glycoprotein hormone receptors (Kobilka, 2007). These differences in structure of the receptors underlie the different binding sites, e.g., monoamine (as catecholamines) binds to the transmembrane segments, while the glycoproteins bind to the long amino

terminus (Kobilka, 2007). The β AR is one of the best structurally characterised receptors from the GPCR family (Kobilka, 2007). Norepinephrine binds to TM3, TM5, and TM6 of β AR and, more precisely, the amine nitrogen of NE binds to Asp 113 in TM3, the catechol hydroxyls of NE bind to serines in TM5, and the aromatic ring and β hydroxyl of NE attach to TM6 (Kobilka, 2007).

It has been shown that norepinephrine binds to $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ adrenergic receptors (Odowd et al., 1989, Nicoll et al., 1990). NE can produce excitatory or inhibitory effects on the nervous system, and this is thought to depend on the type of receptor that binds to NE (Madison and Nicoll, 1986, Nicoll et al., 1990). Both propranolol, a non-selective competitive β ADRs antagonist, and Phentolamine, a non-selective competitive α ADRs antagonist, have been used to block both α and β ADRs (Madison and Nicoll, 1986, Nicoll et al., 1990, Kang et al., 2000).

5.13 Objectives:

Neonatal CNS ischaemia is a major cause for serious injuries that produce a wide range of disabilities and life suffering. It targets both neurons and glial cells. Glial cells and neurons express many of the same neurotransmitter receptors. Research over the last two decades has focused on glutamate receptors. However, it has been shown that there are multiple neurotransmitter receptors expressed in glial cells, including within CNS white matter (WM) during development. This project focused on studying the role of GABA (section 7.2), glycine (section 7.3), nicotine (section 7.4), and noradrenaline (section 7.5) on developing irreversible ischaemic injury in the immature central WM. The mechanism of astrocyte death in P0 neonatal RON was described in 1998 (Fern, 1998). In the current study, baseline controls (aCSF, and OGD controls) are done (section 7.1) to enable comparison for the other sections.

CHAPTER 6 : MATERIALS AND METHODS

6.1 Animal:

Postnatal day 0–2 (P0–2) (referred to as P0), P9–11 (referred to as P10) and adult (> P50) Wistar rats of both sexes were provided by the animal house of Leicester University. They were culled by cervical dislocation following schedule 1, in accordance with the regulations of the British Home Office.

6.2 The Choice of Animal Age and Model:

The optic nerve is a typical CNS white matter model containing central axon of retinal ganglion cells and glial cells, and it is devoid of synapses (Stys, 1998). Rodent optic nerves (RON) have been widely used as a white matter model to study the mechanism of ischaemic injury, since this avoids any complexity arising from synapses (Stys, 1998). In addition, several articles have examined ischaemic related mechanisms in RON of different ages (Fern, 1998, Fern et al., 1998, Fern and Moller, 2000). As mentioned before, the period of high vulnerability for developing PVL is the mid-gestational period in humans, which corresponds to the period around P0 in rats or mice (Domowicz et al., 2011). Note that in P0 rats, the majority of glial cells are astrocytes.

6.3 Optic Nerve Dissection:

After decapitation, the head is separated from the body using a large scissors, and a clean razor blade is used to make an incision on the scalp along the sagittal suture. After freeing the scalp from the skull, a small fine-tipped scissor is used to cut the optic nerves, behind the eye balls, through the orbit. Another incision is gently made on the soft skull along the sagittal suture in case of a neonate. An adult skull is very hard and rigid and is usually crushed by using bone forceps. The upper part of the skull (vault) is removed to expose the brain. By using a curved forceps, the olfactory bulb is separated and the brain is gently pulled out from the frontal lobe. Both optic nerves should be observed and ensured to be freed from the eye-ball. Particular care was taken in this step to avoid any tearing or transection of the optic nerve. Both optic nerves and the brain are transferred to a petri dish filled with aCSF solution. Under a dissecting microscope, optic nerves are separated from the optic chiasm by using micro forceps. After the nerves are washed several times with aCSF, they are loaded with intracellular dye and kept in an oxygenated chamber for Ca^{2+} imaging experiments, are embedded in Tissue Tek for proceeding with immunohistochemistry experiments, or are immediately fixed for EM.

6.4 Solutions:

6.4.1 aCSF:

Living cells need a physiologically balanced solution to maintain a proper pH, ideal osmotic pressure and a good source of energy. These criteria are achieved by using artificial cerebrospinal fluid (aCSF), the most appropriate physiological solution.

Artificial CSF (aCSF) is composed of (in mM) 153 Na^+ , 3 K^+ , 2 Mg^{2+} , 2 Ca^{2+} , 131 Cl^- , 26 HCO_3^- , 2 H_2PO_4^- , 10 glucose, and pH=7.45 and is bubbled with 95% O_2 / 5% CO_2 . OGD solution has the same components of aCSF, with the exception of glucose, and it is bubbled with 95% N_2 / 5% CO_2 . Applying 5% CO_2 to both solutions helps to correct the bicarbonate buffer system by reducing the hydroxyl ion concentration in the solution. The osmolarity was measured and corrected to 310–320 mOsmol by adding NaCl as required. Usually, a stock solution was made of solution A (NaCl 126 mM, and KCl 3 mM), solution B (NaH_2PO_4 2 mM, MgCl_2 2 mM, NaHCO_3 26 mM, Glucose 10 mM), and Solution C for OGD (NaH_2PO_4 2 mM, MgCl_2 2 mM, NaHCO_3 26 mM) and kept at 4 °C. Before each experiment, a fresh aCSF solution was made by mixing equal amounts of solution A with B (or C for OGD experiments). Two mM of CaCl_2 subscript was then added just before bubbling the solution with gases. After preparing the solution, it was placed in a water bath (~ 40 °C) and bubbled with the required gases for at least one hour before starting the experiment.

6.4.2 Phosphate Buffer Solution (PBS):

PBS was made up by mixing 1.44g (10 mM) Na_2HPO_4 with 0.24g (2 mM) KH_2PO_4 , 8g (137 mM) NaCl, 0.2g (2 mM) KCl and 1000 ml of DD H_2O . The pH was adjusted to 7.4. In immunohistochemistry, usually 10% of goat serum and 0.5% Triton X is added to PBS to make PBGST solution.

6.5 The Choice of Ca²⁺ Indicator:

There is a wide range of Ca²⁺ indicators, and it is important to choose the most appropriate one for the required experiment. Criteria such as Ca²⁺ affinity (K_d), ratiometric properties and selectivity are important to consider. Ratiometric indicators measure [Ca²⁺]_i peaks at different wavelengths and a ratio can be used to minimize artifacts not related to [Ca²⁺]_i changes.

Fura-2 is a high affinity Ca²⁺ indicator that binds to the free Ca²⁺ in cytosol. It is a ratiometric indicator, which helps in recording accurate Ca²⁺ concentration in experiments facing some changing factors, such as uneven dye loading, dye leakage, photobleaching and changes in cell volume. Fura-2 has a good selectivity against other divalent cations. This indicator is available in acetoxymethyl (AM) esters form. AM is membrane permeable and allows loading of hydrophilic Fura-2 dye into the cytosol, where it is then cleaved from Fura-2 by intracellular esterase and leaves the dye trapped inside the cell (Paredes et al., 2008). Fura-2 shares some advantages with the lower affinity indicators, as it can detect a large range of intracellular Ca²⁺, starting from ~100 nM to ~100 μM (Gee et al., 2000). A relative resistance to photobleaching is an important factor in long experiments, as in the case of my experiments (100 min) (Wahl et al., 1990).

6.6 Dye Loading:

Fura-2 AM was mixed with DMSO (dimethyl sulfoxide) and 10% pluronic acid, a non-ionic surfactant used to facilitate loading, to prepare a stock solution of 1 mM. Around 10 μl of Fura-2 AM stock solution was added to 1 ml of aCSF to give a final Fura-2 AM concentration of 10 μM. Optic nerves were placed in a petri dish containing 10 μM Fura-2 AM, which is enough to cover the nerves; the nerves were then

incubated in an oxygenated chamber for around 1 hour at room temperature. Nerves usually have a quick wash with aCSF before imaging starts.

6.7 Mounting:

Before the nerves were mounted, they were washed with aCSF. The optic nerve was fixed to a 22x44 mm glass coverslip by applying glue (Loctite 454 instant adhesive gel) at its ends, leaving the majority of the nerve free from glue. A cover slip was attached to a Plexiglas perfusion chamber (atmosphere chamber, Warner Instruments, Hamden, CT, USA) by high vacuum grease (Dow Corning) to prevent any leakage. The aCSF was connected to the perfusion chamber and run at a rate of 2–3 ml/minute, leaving the nerve completely covered by the solution (fluid level ~1mm). A 95% O₂ / 5% CO₂ gas was blown over the nerve at a rate of 1.5 l/min. The tubes used in the rig were oxygen impermeable. The extra aCSF was sucked out by a vacuum pump through a small tube connected at the other end of perfusion chamber. The chamber has two parts: a large lozenge shaped part where it is connected to the solution and the nerve is fixed in its centre, and a second smaller chamber from which it is connected to the vacuum. Enough fluid (~0.5 ml) was left around the nerve in the large section of the chamber, which was then passed to the small part of the chamber for suctioning, leaving turbulence-free flow. Another cover slip was placed over the large chamber to ensure the quality of air reaching the nerve.

The chamber was placed on a platform and fixed tightly by screws. The platform then was mounted on the stage of a Nikon inverted fluorescent microscope *Eclipse TE200* (Tokyo, Japan). The temperature of both the bath and coverslip was adjusted to around 37 °C using two methods; the first was via a flow-through feedback tubing heater (Warner Instruments) that warmed the solution as it entered to the

chamber; the second method used a feedback objective heater (Bioptechs, Butler, PA) that heated the objective lens, which was a 40x fluorescence oil objective lens (Dapo 40 UV; Olympus, Tokyo, Japan).

6.8 Cell Imaging:

Cells loaded with Fura-2 have maximal emission at 520 nm following excitation at 340 nm, 360 nm, and 380 nm. Excitation was achieved by the passage of light from a high-intensity arc lamp through an Optoscan monochromator (both Cairn Research Ltd., Faversham, UK), while emission was achieved at 520 nm through a Fura filter-fitted dichronic mirror (Chorma Technology Corporation, Rockingham VT, USA). These wavelengths were collected by selecting regions of interest (cells) using MetaFlour software (Molecular Devices, Sunnyvale, CA, USA). The ratio of the 340:380 measures the $[Ca^{2+}]_i$ while a 360 wavelength monitors the fluorescent level inside the cell, which corresponds to cell viability and death. Images were collected every 30 seconds and were captured by a coolSNAP HQ camera (Roper Scientific, Ottobrunn, Germany) and stored on a Windows XP desktop computer.

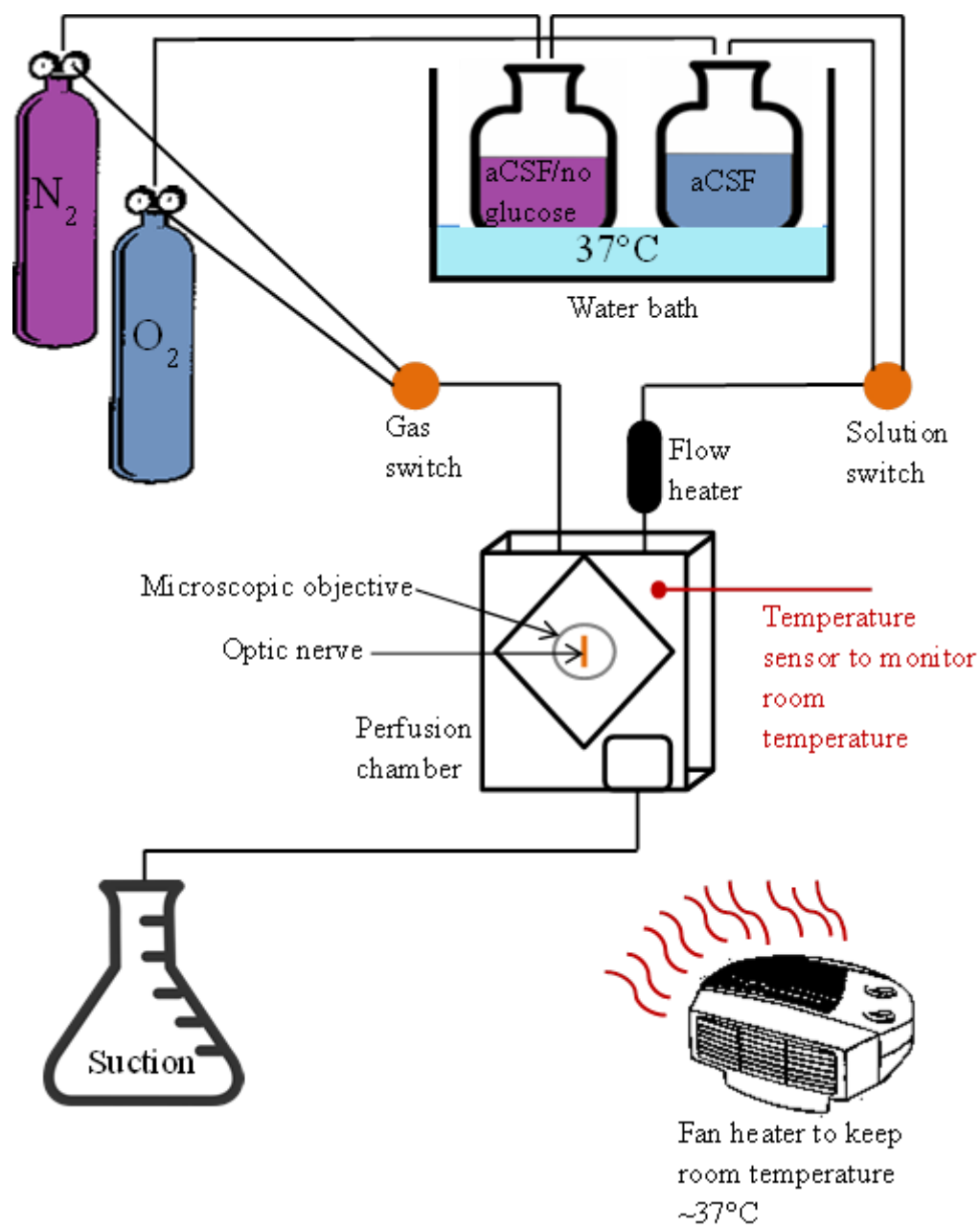


Figure 6-1: Perfusion system setup.

6.9 OGD/aCSF Experiments:

To provide a healthy and physiological environment for control experiments, aCSF solution was kept in a water bath to warm the solution to around 37 °C and was bubbled with 95% O₂ / 5% CO₂ for at least 1 hour; the bottle was carefully sealed to avoid any gas leaking or mixing with atmospheric gas. In an OGD experiment, the solution is replaced by OGD solution (aCSF without glucose) and bubbled with 95% N₂ / 5% CO₂ for at least 1 hour in a water bath. To avoid any protective effect of hypothermia (Bruno et al., 1994), a fan heater was used in the experiment room to ensure that the room temperature was around 37 °C, and that was monitored by an electronic temperature probe placed above a perfusion chamber. During the experiment, switching from one solution/gas to another took 2–3 minutes for a complete solution/gas exchange. The imaging experiment ran for 100 minutes, usually starting within 5 minutes of mounting the nerve, and ratio changes were monitored in regions of interest throughout the experiment.

During aCSF control experiments, the nerve was perfused with aCSF/O₂ throughout the 100 minutes, while in OGD experiments the nerve was perfused with aCSF/O₂ in the first 10 minutes and switched to OGD/N₂ in the 90 remaining minutes. In aCSF experiments, aCSF /O₂ was used in the first 10 minutes and then was switched to the aCSF plus the required drug/O₂ for 90 minutes. In OGD-drug experiments, aCSF plus required drug/O₂ was used for the first 10 minutes, followed by switching the solution to OGD plus the same drug and the gas to N₂.

Table 6-1: Pharmacological reagent.

<i>Name</i>	<i>Concentration</i>	<i>Source</i>	<i>Code</i>
GABA	100 μ M	Sigma	A5835 CAS: 5612-2
Picrotoxin	100 μ M and 300 μ M	TOCRIS	8A/112573
Glycine	100 μ M	Sigma	G7126 CAS: 56-40-6
Strychnine	2 μ M, 5 μ M	Sigma	S8753 CAS: 1421-86-9
Nicotine hydrogen tartrate salt	50 μ M	Sigma	N5260 CAS: 65-31-6
Mecamylamine hydrochloride	10 μ M	TOCRIS	CAS: 826-39-1
α- Bungarotoxin	100 nM	Abcam	Ab120542
Norepinephrine bitartrate salt monohydrate	100 μ M	Sigma	A9512 CAS: 108341-18-0
Propranolol hydrochloride	10 μ M	TOCRIS	A/74250
Phentolamine hydrochloride	10 μ M	Sigma	P7547 CAS: 73-05-2
Ascorbic acid	0.6 μ M	Sigma	CAS: 50-81-7

6.10 Immunohistochemistry:

Immunohistochemistry is a technique used to identify a certain antigen in histological tissue by applying an antibody labelled with a fluorochrome. There are several types of antibody isotypes (IgA, IgD, IgE, IgG and IgM). However, IgG and IgM antibodies represent the majority of research antibodies and can be produced by purifying the serum of an immunised animal. In general, two types of antibodies can be generated: monoclonal and polyclonal. Polyclonal antibodies arise after immunising the host animal (e.g., rabbit, sheep, goat, monkey, mouse, rat and guinea pig) with a target antigen, which produces a large quantity of antibodies with many target epitopes. Monoclonal antibodies are produced from hybridoma cell lines and generate antibodies against one specific epitope.

Sections are usually fixed to preserve the tissue structure, followed by permeabilising the tissue by 0.5% Triton to facilitate antibody access and binding to its epitope. Non-specific binding sites of antibodies are blocked by adding 10% of goat serum. An antigen can be detected directly by the labelled conjugated primary antibody, or indirectly by the labelled secondary antibody that binds to unlabelled primary antibody.

Table-6-2 shows different types of primary and secondary antibodies which were utilised in the current study. Glycine receptor antibodies (GlyR sc-33611) are recommended for detection of glycine receptor $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$ of rat, mouse or human (www.scbt.com) and have been used in several previous studies (Haverkamp et al., 2004, Heinze et al., 2007, Nobles et al., 2012). GABA_A receptor antibodies (GABA_A R $\beta 1/2/3$) are recommended for detection of GABA_A receptor of $\beta 1$, 2, and 3

subunits of rat, mouse and human and have been evaluated by Western blot analysis (www.scbt.com). Anti-neurofilaments- L (NF-L) antibodies are used to detect the low molecular weight (68-70 kDa) subunit of the neurofilaments and were chosen in many previous studies (Alix and Fern, 2009, Arranz et al., 2008). Anti-glial fibrillary acidic protein GFAP is a monoclonal antibody recommended for immunohistochemistry of rat CNS and was used in previous studies (Shannon et al., 2007).

Table-6-2: Antibodies used for immunohistochemistry.

Primary antibodies:

<i>Antibody against:</i>	<i>Species</i>	<i>Monoclonal/ Polyclonal</i>	<i>Dilution</i>	<i>Company</i>	<i>Product code</i>
Anti-neurofilament-L (NF)	Mouse	Monoclonal	1:250	Millipore	MAB1615
Anti-glial fibrillary acidic protein (GFAP)	Mouse	Monoclonal	1:200	Sigma	G3893
Glycine R α	Rabbit	Polyclonal	1:200	Santa Cruz Biotechnology	Sc-33611
GABA A R β 1/2/3	Rabbit	Polyclonal	1:200	Santa Cruz Biotechnology	Sc-28794

Secondary antibodies:

<i>Fluorophore</i>	<i>species</i>	<i>Dilution</i>	<i>Company</i>	<i>Product code</i>
Alexa-488	Goat anti-mouse IgG (highly cross-adsorbed)	1:1000	Molecular Probes	A11029
Alexa-568	Goat anti-rabbit IgG (highly cross-adsorbed)	1:1000	Molecular Probes	A11036

6.10.1 Protocol:

Optic nerves were dissected immediately after killing the animal and were washed with 0.1 M PBS (phosphate buffer saline). The nerves were then carefully submerged in Tissue-Tek (Sakura) and frozen by contact with hexane and dried ice. Cryostat sections were taken at a 10° angle and 20 µm thickness at -20 °C and mounted on SuperFrost Plus slides (Fisher Scientific), followed by fixing the nerve with 4% paraformaldehyde in 0.1 M PBS for 30 minutes in neonatal ON and for 1 hour for the adult specimen, at room temperature. The slides were then washed with 0.1 M PBS before the tissue was permeabilised in 0.1 M PBS containing 10% goat serum (sigma) and 0.5% Triton X (sigma) (PBGST) for 2 hours. Primary antibodies were diluted in PBGST and incubated with the sections overnight at 4 °C. For double labelling, both primary antibodies were mixed together in PBGST, and antibodies from different host species were used, e.g., mouse and rabbit. The secondary antibodies, diluted in PBGST, were applied for around 1 hour after washing the sections with PBGST three times for 5 minutes each. Slides were mounted with PermaFluor mountant medium (Thermo Fisher Scientific) after they were washed twice in PBGST (5 minutes each), twice in 0.1M PBS (5 minutes each), and finally with 0.05 M PBS (5 minutes each).

6.11 Electron Microscope:

6.11.1 Buffer and Fixatives:

The buffer used is 0.2 mol/l Phosphate Sorensen's buffer. It is pre-prepared from a combination of 40.5 ml of Sorensen's A buffer with 9.5 ml of Sorensen's B buffer. The pH is adjusted to 7.4 by adding more Sorensen's B buffer to decrease the pH and

Sorensen's A to increase it. Sorensen's A buffer is composed of 0.2 M of dibasic sodium phosphate (Na_2HPO_4), and Sorensen's B buffer is composed of 0.2 M of monobasic sodium phosphate (NaH_2PO_4).

6.11.2 Embedding Protocol:

Samples were fixed with 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M Sodium Cacodylate buffer / 2 mM calcium chloride of pH 7.4 and kept overnight at 4 °C, followed by several washes (3 x 20 minutes) in 0.1 Sodium Cacodylate buffer / 2 mM calcium chloride of pH 7.4 and stored for 2 days at 4 °C. After that, samples were washed several times (around 3 x 20 minutes) in distilled deionised water (DDW). Then stained with 1% osmium tetroxide / 1.5% potassium ferricyanide in DDW for three hours, followed with three washes in DDW for 20 minutes each. That was followed by fixation in 2% aqueous uranyl acetate for 1 hour at 4 °C. Next the RON samples were washed 2 x 10 minutes in DDW. Gradually dehydration followed using 30%, 50%, and 70% ethanol stepwise for 15 minutes each. Samples were then stored in 70% ethanol overnight at 4 °C. Next day 70% ethanol was replaced by 90% ethanol for 30 minutes and finally by 100% analytic grade ethanol for 30 min, repeated 3 times. Then the samples were twice submerged in propylene oxide for 10 minutes. Samples were immersed in a 2 propylene oxide: 1 modified Spurr's low viscosity resin (hard formula) for 90 minutes on the mixer (see Table 6-3). Media was then replaced by 1 propylene oxide: 1 modified Spurr's low viscosity resin for 60 minutes, 1 propylene oxide: 2 modified Spurr's low viscosity resin for 60 minutes, 100% modified Spurr's low viscosity resin for 30 minutes, and finally modified Spurr's low viscosity resin overnight. After that the resin was replaced two times with a fresh 100% modified Spurr's low viscosity resin and was mixed for 3 hours each time. Samples were

embedded and polymerised in a 60 °C oven for 16 hours (Murphy et al., 2011, Thong et al., 2003).

Table 6-3: Modified Spurr's resin formulation. (Ellis, 2006)

		40.0 ml	30.0 ml	20.0 ml
1	ERL 4221D	16.4 g	12.3 g	8.2 g
2	DER 736	3.8 g	2.7 g	1.9 g
3	NSA	23.6 g	17.7 g	13.0 g
4	Mix 5 minutes			
5	DMAE	0.4 g	0.3 g	0.2 g
6	Mix 5 minutes			

ERL 4221D: vinyl cyclohexene dioxide; DER 736: diglycidyl ether of polypropylene glycol flexibiliser; NSA: nonenyl succinic anhydride; DMAE: accelerator dimethylaminoethanol.

6.11.3 Remounting and Trimming:

After the samples were polymerised, a BEEM capsule removing machine was used to remove the block from the capsule. Embedded samples were remounted using Araldite glue and left overnight at room temperature. Once mounted in the ultramicrotome (Reichert Ultracut S ultramicrotome), the excess resin was roughly trimmed by hand using sharp razor blades. The aim of trimming down the blocks was to remove the Araldite glue near the cutting end to prevent it blocking the knife edge, to expose the sample, and to make the block face small enough to fit in the grid.

6.11.4 Sectioning:

6.11.4.1 *Glass Knives:*

It was important to use sharp knives in order to have good and clear sections. Full protection was used, e.g., gloves, lab coat and eye protection. A glass strip was cleaned with 50% methanol and broken into 25 mm squares and then into triangles

using a Bromma LKB 7800 glass knife maker. Plastic boats were attached to the glass knife using dental wax. The sharpest type of glass knife edge is one bowed up, and this type could be used for longer durations (Bozzola & Russell 1998). A straight-edged knife is reasonably good, but it has a shorter usable edge (Bozzola & Russell 1998). Some types of glass knife edge have horns at their edges, and this type of knife is known to contain many imperfections, so it is not the best choice for cutting ultra-thin sections. However, the Z part of the knife edge, the upper left edge of the knife, is usually better than the E part, the upper right edge of the knife, which could contain some imperfections (Bozzola & Russell, 1998).

6.11.4.2 *Type of Grid:*

There are many types of specimen grids which have different mesh designs. I used 300 square gold grids handled by NOC fine-pointed forceps.

6.11.4.3 *Ultramicrotome:*

Both thick and thin sections were cut using a Reichert Ultracut E microtome and a glass knife filled with DDW in its boat to let sections float on the water surface. Usually the glass knife was adjusted at a 5° angle. Semi-thick sections were taken at around 0.6 µm thickness while ultra-thin sections were cut with around 70 nm. Dichloromethane vapour was spread over the thin sections to remove any wrinkles. After that, thin sections were picked up by the forceps and the grid left to dry under a lamp. However, sometimes it is difficult to pick up sections due to the presence of air bubbles in the grid mesh. In this case, submerging the grids in acetone immediately before cutting the sections can be helpful.

6.11.5 Staining:

Staining the semi-thick sections involved submersion in toluidine blue for 10 seconds followed by a wash with DDW. Staining ultra-thin sections could be done in two ways, either using 2% uranyl acetate for 10 minutes or lead citrate for around three minutes, and both are then followed by washing the sections via several immersions in three beakers filled with DDW. Sections could be stained with both to increase the contrast under an electron microscope. With lead citrate staining, the process of staining took place inside a nitrogen box to avoid CO₂ in air, which can cause lead citrate to precipitate, and the DDW was freshly boiled with the addition of a few drops of sodium hydroxide in the first DDW filled beaker to scavenge any CO₂.

6.11.6 Microscopes:

All the sections were observed under the JOEL 1220 transmission electron microscope, which has a high resolution of 0.4 nm and a high magnification of 1:600,000. The basic design of the transmission electron microscope consists of an illuminating system which contains the electron gun and condenser lenses that control the radiation, a specimen manipulation system that has the specimen stage and holder, an imaging system which has some objective lenses to focus and magnify the image in addition to the SIS Megaview III digital camera, and a vacuum system which is important to remove the air molecules (Bozzola & Russell, 1998).

6.12 Data Analysis:

Raw data collected from regions of interest (ROI) were exported into an Excel spread sheet, where all other data manipulations were undertaken. Two graphs (340/380 ratio and 360 intensity) were performed for each cell, and the background was subtracted to reduce the amount of noise during recording. Experiments of certain drugs

were performed on different days and repeated at least five times to ensure accurate results. In each condition/experiment, the percentage of cell death and the onset of cell death were calculated. All values calculated for a specific condition/experiment were averaged and the standard deviation (SDEV; an indicator of variance within the group) was obtained. The SDEV was then used to calculate the standard error of the mean (SEM; an indicator of how well the mean of a sample estimates the mean of a population). These data were then entered into GraphPad Prism v5.0 using analysis of variance (ANOVA) and the Bonferroni test to compare the results, with differences being significant when $p < 0.05$.

In electron microscope, different conditions (control, OGD control, drug + OGD) were tested. One experiment (=1 optic nerve) was done for each condition. The experiment was done in the lab and then the samples sent to the technician for processing. The samples were re-named by different letters and micrographs were collected blindly. One whole transverse section of optic nerve was used for data collection. After classifying the glial cell injury following known injury scores (score 0, 1, 2, 3), the mean injury score was taken $\pm$ SEM. These data were then entered into GraphPad Prism v5.0 using analysis of variance (ANOVA) and the Bonferroni test to compare the results, with differences being significant when $p < 0.05$.

CHAPTER 7 : RESULTS AND DISCUSSION

7.1 Astrocyte Death During Ischaemia in nRON:

7.1.1 Introduction:

Astrocytes represent the predominant non-neuronal cell type in the CNS and perform essential and supporting functions in maintaining normal neuronal activity. Any factor that disturbs astrocyte survival, such as ischaemia, will therefore affect their supporting functions and threaten neuron function, including axonal functions like action potential conduction. Mid-gestation ischaemia is the leading cause of PVL, a form of white matter injury associated with 2 per 1000 live CP neonates (Fern, 1998). Therefore, identification of the mechanism of WM astrocyte injury in ischaemia is important for the design of therapeutic interventions to counter these important forms of brain injury.

Astrocyte sensitivity to ischaemia has been a matter of debate for several years. It was thought that astrocytes were highly resistant to ischaemia, as compared to neurons. *In vitro* studies have shown that astrocytes can tolerate ischaemia longer than neurons cultured from rat or mice hippocampus, cortex or whole brain (Lyons and Kettenmann, 1998, Zhao and Flavin, 2000, Goldberg and Choi, 1993, Haun et al., 1992, Juurlink and Hertz, 1993). Four to six hours of ischaemia are needed to produce significant levels of cultured astrocyte death (Goldberg and Choi, 1993). However, *in vitro* studies differ from the *in vivo* situation in that they have an unlimited extracellular space, which can dilute K^+ and reduce anoxic depolarisation as well as affecting ischaemic pH changes (Fern, 2001, Kraig and Chesler, 1990, Swanson et al., 1997, Giffard et al., 2000). These factors may mask the real effect of ischaemia on astrocytes *in situ*. However, hypoxic, acidic and ion-shifted Ringer (HAIR) solution, which mimics the ischaemic environment, can be used to address these concerns. Fifteen

minutes application of HAIR solution without glucose to P3-P6 cortical astrocyte culture resulted in 90% astrocyte death within 20 minutes of reperfusion (Bondarenko and Chesler, 2001b, Bondarenko and Chesler, 2001a). On the other hand, *in vivo* studies have established different models for ischaemia and have shown that astrocytes are highly sensitive to ischaemia. In these studies, global cerebral ischaemia for a short period (~10 minutes) was enough to cause neuronal death as well as to mediate a degree of astrocyte death in the hippocampus of an adult rat (Petito et al., 1998). Permanent MCAO of an adult rat shows astrocyte disintegration, nuclear pyknosis, and GFAP level decrease in the pre-optic area within the first 30 minutes of the onset of ischaemia (Garcia et al., 1993). Transient MCAO of an adult rat for 2 hours produced a decrease in GFAP levels in the core of the lesion in the cortex and basal ganglia over 4–16 hours (Chen et al., 1993). Not only GFAP, but also S100, vimentin, and Glutathione-S-Transferase Y_b (GST Y_b) astrocytic markers were shown to decrease after 3 hours of permanent MCAO of a hypertensive adult rat cortex (Liu et al., 1999). Similar findings have been reported by other *in vivo* studies (Schmidtkastrner et al., 1993, Davies et al., 1998, Martin et al., 1997, Yamashita et al., 1996). However, it has been argued that measuring GFAP level is not a sufficient way to estimate astrocyte numbers following ischaemia, as some studies have revealed that ischaemic swollen astrocytes lose their GFAP reactivity while they are still alive, and other studies have suggested overexpression of GFAP on disintegrating and/or swollen astrocytes (Petito and Halaby, 1993, Davies et al., 1998, Garcia et al., 1993, Schmidtkastrner et al., 1993). It seems that ultrastructural morphology or live imaging of astrocytes may be a more convenient way to study the effect of ischaemia on astrocytes (Davies et al., 1998, Fern, 1998, Garcia et al., 1993, Salter and Fern, 2008b, Shannon et al., 2007).

Whole mounted RON has been used to avoid the *in vitro* disadvantages and to gain easily handled white matter. Whole mounted P2 RON was exposed to an OGD environment for 80 minutes, and live imaging revealed that around 46.6% of Fura-2 loaded astrocytes died during the period of ischaemia (Fern, 1998). Another study used whole mounted P10 RON, and cell imaging found that Fura-2 loaded astrocyte death reached 21.8% following 45 minutes of OGD (Thomas et al., 2004). An electron microscope study of whole mounted P10 RON exposed to 60 minutes OGD showed a progressive astrocyte process and somata loss (Salter and Fern, 2008b). From these data, it is assumed that there is a small population of surviving astrocytes post-ischaemia, which is consistent with evidence showing some viable astrocytes within the core of ischaemia after MCAO (Thoren et al., 2005). These viable cells can be converted to reactive astrocytes, which is a main feature of the post-ischaemic brain and is characterised by an increase in cell number, volume and GFAP expression (Ridet et al., 1997, Davies et al., 1998). This issue has caused considerable confusion during studies of astrocytes in the post-ischaemic period, as some studies suggested that an early astrocytosis started 15 minutes after ischaemia (Petito, 1986) and others suggested a later onset of astrocytosis, initiated 24–48 hours after ischaemia (Davies et al., 1998). In the isolated mouse optic nerve, green fluorescent protein (GFP) expressing astrocytes revealed that astrocytes did not express any feature of astrocytosis within the first 180 minutes after ischaemia in neonatal whole mounted RON (Shannon et al., 2007).

It has been argued that the ischaemic injury sequence is quite different for different cell types in the CNS, and this debate has been the focus of research in this field for a number of years. The cellular mechanism of acute brain ischaemic injury is classified into two general stages: ionic disruption due to failure of ATP production,

followed by Ca^{2+} -mediated cell death (Ransom and Fern, 1997). In a previous study, Ca^{2+} -sensitive microelectrodes were used to monitor $[\text{Ca}^{2+}]_e$ and to correlate changes in $[\text{Ca}^{2+}]_e$ with anoxia-induced damage in adult white matter (Brown et al., 1998). Anoxia-mediated white matter injury was reduced by eliminating extracellular Ca^{2+} (Brown et al., 1998). Moreover, application of HAIR solution for 20 minutes on P3–P6 rat cortical astrocyte culture reduced the survival percentage of astrocytes to 3% within 20 minutes of perfusion. This was found to reach 94% by eliminating Ca^{2+} from the perfusion solution, indicating the important role of Ca^{2+} in astrocyte death (Bondarenko and Chesler, 2001a). It has been found that reperfusion after 20–40 minutes of HAIR solution triggers an accumulation of intracellular Na^+ , which triggers a Ca^{2+} influx via reversing Na^+ - Ca^{2+} exchanger in the cortical astrocytes culture (Bondarenko et al., 2005). VGCC had an important role in Ca^{2+} influx during *in vitro* ischaemia of Fura-2 isolated hippocampal astrocytes (Duffy and MacVicar, 1996). Electrophysiological studies have demonstrated the involvement of L-type and N-type Ca^{2+} channels in mediating anoxic injury in neonatal white matter (Fern et al., 1995a). Astrocytes are well equipped with Ca^{2+} channels, and a Ca^{2+} current was documented in cultured cortical astrocytes and optic nerve astrocytes (Verkhratsky et al., 1998, Barres et al., 1990a, Barres et al., 1989). Ischaemic injury in P10 RON astrocytes is mediated by acute swelling via a Na^+ - K^+ - Cl^- cotransporter, whereas it is dependent on Ca^{2+} influx via VGCCs in P0-P2 RON (Thomas et al., 2004, Fern, 1998). This is consistent with the significant damage to P10 mice optic nerve astrocyte processes induced by ischaemia even after removing the extracellular Ca^{2+} (Salter and Fern, 2008b). Replacement of extracellular Na^+ or Cl^- , or applying bumetanide, a Na^+ - K^+ - Cl^- cotransporter (NKCC) blocker, is found to protect astrocytes during up to 40 minutes of ischaemia, while replacement of extracellular HCO_3^- or the application of 500 μM of 4,

4-Diisothiocyanostilbene-2, 2-disulfonic acid (DIDS), a potent Cl^- transporter blocker, is found to protect astrocyte processes during up to 60 minutes of ischaemia in P10 mice or rat optic nerves (Salter and Fern, 2008b). Moreover, elimination of extracellular Na^+ or K^+ can be completely protective against ischaemic injury to both astrocyte somata and processes (Salter and Fern, 2008b). It has been shown that intracellular accumulation of Ca^{2+} in neonatal P0 RON astrocytes is mediated by a Ca^{2+} influx rather than the release of Ca^{2+} from intracellular stores or glutamate-gated channels (Fern, 1998). Ca^{2+} imaging was used to study the pathway of Ca^{2+} efflux in neonatal RON astrocytes, and it showed Ca^{2+} influx through L- and T-type VGCC, in which T-type VGCC plays the main role in the initial stage of ischaemia and cell death in the early onset of ischaemia (Fern, 1998).

7.1.2 Results:

7.1.2.1 aCSF Controls:

Under normal conditions, the majority of astrocytes in the optic nerve perfused with aCSF had stable $[Ca^{2+}]_i$ throughout the 100-minute experimental period. However, some cells died and showed an increase in $[Ca^{2+}]_i$ just prior to cell death. In Figure 7-1, a series of images of Fura-2 loaded cells collected at 510 nm following excitation at 360 nm images showed the viability of multiple cells every 20 minutes of the experiment. At 0 minutes, several cells could be identified (around 6 cells), most of which persisted until the end of the experiment (100 minutes). Two cells were taken as an example. Cell 1 persisted throughout the experiment, with some variations in dye intensity and a stable 340/380 ratio (excitation at 340 and 380 nm, corresponding to $[Ca^{2+}]_i$) and 360 intensity, as shown in the Cell-1 graph in Figure 7-1. Cell 2 was present at the beginning of the experiment but disappeared at 100 minutes. The Cell-2 graph shows an increase in $[Ca^{2+}]_i$ represented by an increase in the 340/380 ratio, followed by a drop in 360 intensity and cell death at around 90 minutes. The elevated $[Ca^{2+}]_i$ is an important finding preceded the astrocyte death as P0 RON astrocyte death is Ca^{2+} dependent (Fern, 1998). Moreover, a drop in 360 intensity, which revealed cell membrane breakdown and cell death, is double checked by the alive images which showed a sudden cell disappearance. Cells which died cannot be followed in alive images as they are disappeared and the field is moving and needs adjustment constantly. After cell death, the 340/380 ratio and 360 intensity are only indicative of the background fluorescence level. its sudden disappearance in alive images which is coincided with a drop in 360 The distribution of cell death in all control experiments is calculated in Figure 7-2. The percentage of the cells that died relative to total cell

number was plotted against time (10-minute intervals). Cells in the aCSF control started to die within 30 minutes, with a gradual increase in percentage until peaking at 80–90 minutes. In all, $4.5 \pm 1.4\%$ (24 of 492 cells, 17 nerves) of cells died in the aCSF control.

7.1.2.2 OGD Controls:

During ischaemia, increases in $[Ca^{2+}]_i$ levels were always observed prior to astrocyte death. In Figure 7-3 (left), a series of 360 images of Fura-2 loaded cells shows the viability of more than 10 cells throughout the 90-minute OGD experiment following the 10-minute baseline. At 0 minutes, multiple cells can be identified, which gradually die and disappear from the image over time, until only a few cells remain at 100 minutes. For example, Cell 1 was present at 0 minutes but disappeared after 50 minutes. In Cell 1, the 340/380 ratio peaked at around 50 minutes, followed by a sudden decline that coincided with cell death and a drop in 360 intensity to the background level, as shown in Figure 7-3. The incidence of cell death during OGD experiments is shown in Figure 7-4. The percentage of cell deaths relative to the total number of cells was plotted at 10-minute intervals. The cells started to die within the first 10 minutes of ischaemia (20 minutes of the experiment), which gradually increased to peak within 40 minutes of the experiment. In all, $56.5\% \pm 5.98\%$ (325 of 561 cells, 18 nerves) of cells died.

Figure 7-4 compares the aCSF and OGD conditions and shows a significantly higher incidence of cell death in the OGD than in the aCSF control ($56.5\% \pm 5.98\%$ as compared to $4.5 \pm 1.4\%$, respectively; $p < 0.05$). The percentage of cells that died during each 10 minutes is extremely high in the OGD control, compared with the aCSF control. On the other hand, in the aCSF controls there is a late onset of cell death (80 to

90 minutes), whereas the majority of cells that died in the OGD control had an early onset (20 to 60 minutes). Cells started to die within 20 minutes in the OGD experiment, compared with 30 minutes in the aCSF control.

7.1.2.3 Patterns of Ca^{2+} Influx:

As discussed previously, Ca^{2+} influx has been observed prior to the death of nRON astrocytes. It has been reported that these changes in intracellular Ca^{2+} have four different patterns, which were previously discussed by Fern (1998). The first pattern exhibits a rapid increase in $[\text{Ca}^{2+}]_i$ to reach a peak, followed by a rapid decline in 340/380 ratio that coincides with rapid cell death (Figure 7-6A). The second pattern shows a rapid increase in $[\text{Ca}^{2+}]_i$, which then plateaus before late cell death (Figure 7-6B). The third pattern shows a gradual increase in $[\text{Ca}^{2+}]_i$, peaking at a late onset and then followed by a drop in 340/380 ratio and cell death (Figure 7-6C). The last pattern is characterised by two peaks of $[\text{Ca}^{2+}]_i$ in which cell death coincides with the late peak (Figure 7-6D).

7.1.2.4 Cell Fading:

Almost all Fura-2-loaded astrocytes persisted during the 100-minute control experiments. However, around 0.9% of cells (6 of 610 cells) reached the point where they could not be followed. This category of cells is characterised by cell disappearance from the 360 images and by fairly stable 340/380 ratio and 360 intensity. Figure 7-5 (left) shows a series of 360 images of two Fura-2 loaded astrocytes in which Cell 1 disappeared at 60 minutes and Cell 2 at 80 minutes. When the 340/380 ratio graph (A) and 360 intensity graph (B) were double-checked, Cell 1 exhibited an increase in $[\text{Ca}^{2+}]_i$ with a drop in 360 intensity at around 60 minutes, whereas Cell 2 showed a

steady 340/380 ratio and 360 intensity. In conclusion, Cell 1 underwent cell death, whereas Cell 2 underwent fading. Fading cells were excluded from data analysis.

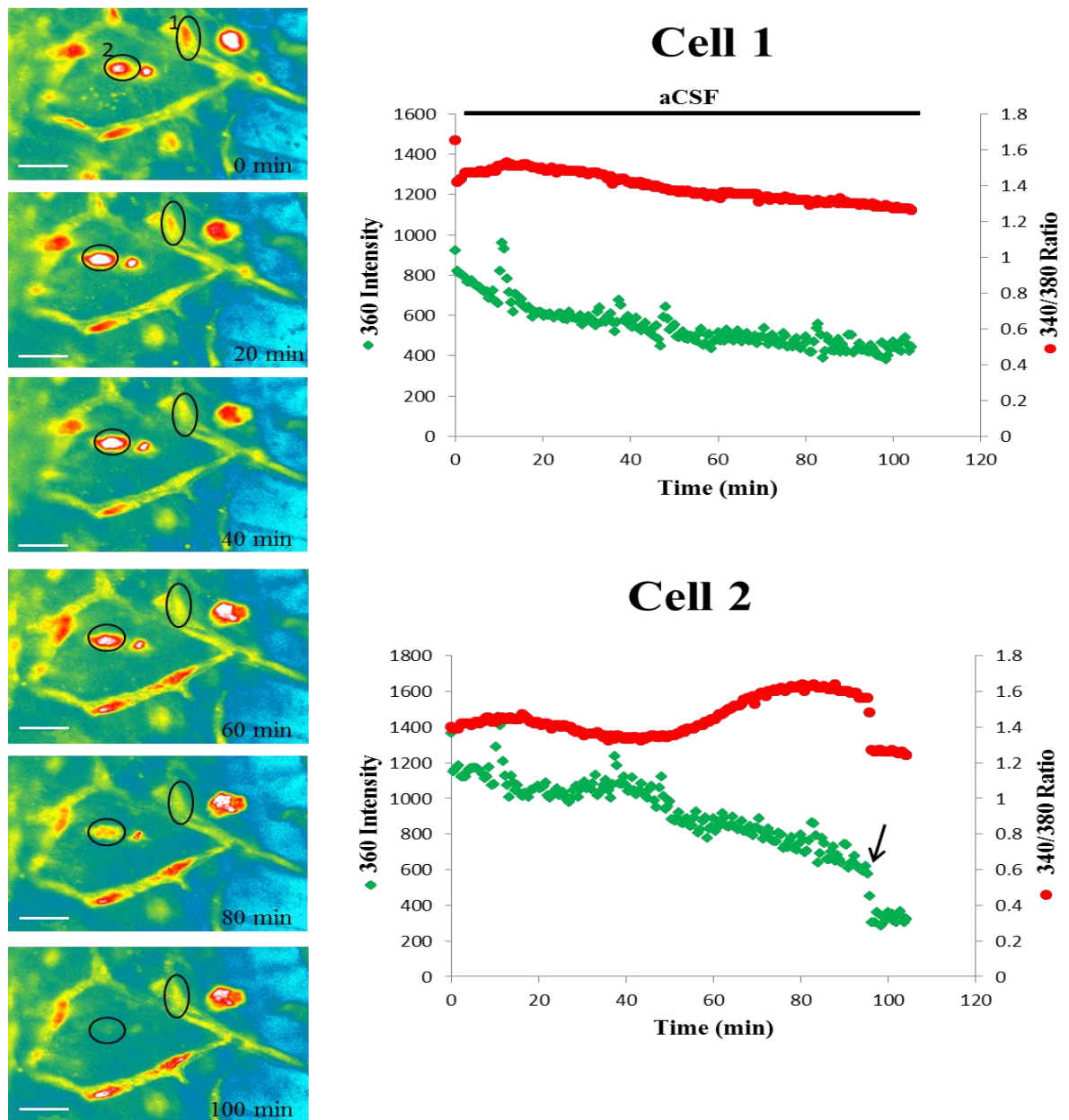


Figure 7-1: Representative data for Fura-2-loaded astrocytes in nRON under control conditions.

On the left, a series of 360 nm images taken at different times shows clearly recognisable Cells 1 and 2 (circled). Cell 1 was stable throughout the experiment (steady 340/380 ratio and 360 intensity), whereas Cell 2 died at around 90 minutes, with a sudden drop in 360 intensity (arrow) preceded by calcium influx (elevation of 340/380 ratio).

Scale bar = 4 μm .

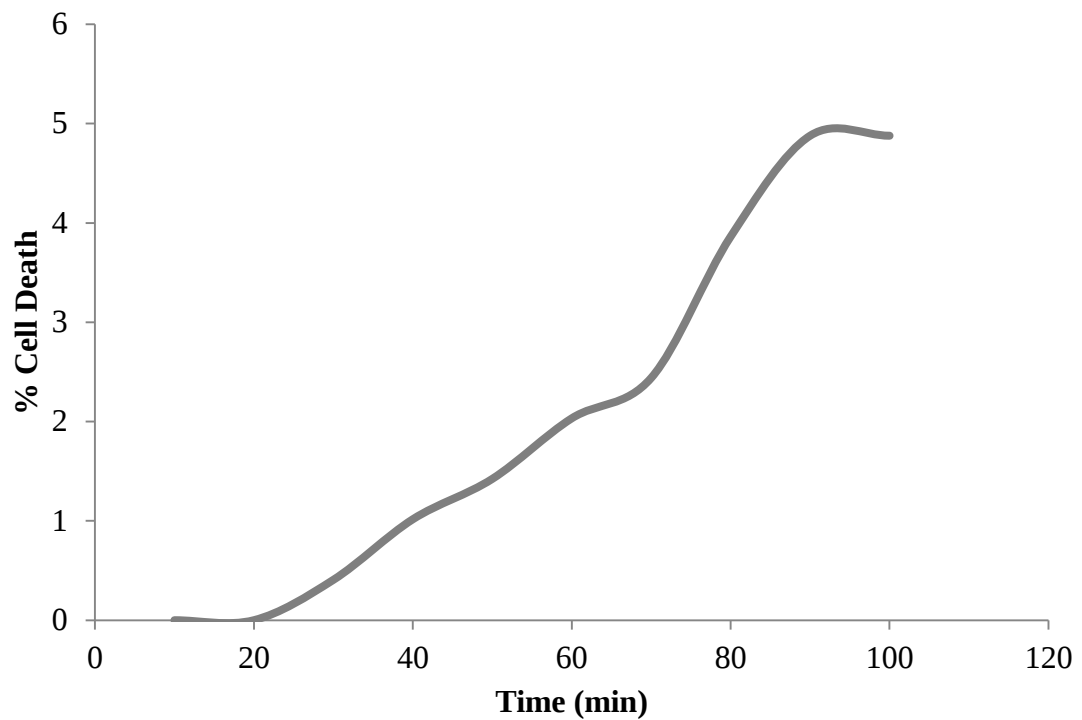


Figure 7-2: Percentage of cell death during aCSF control.

Cell-death percentage is plotted against time (20-minute intervals). Note that cell death in aCSF controls peaked with a late onset of around 80 minutes, and that levels of cell death were very low. N=17 experiments (17 nerves, 492 cells).

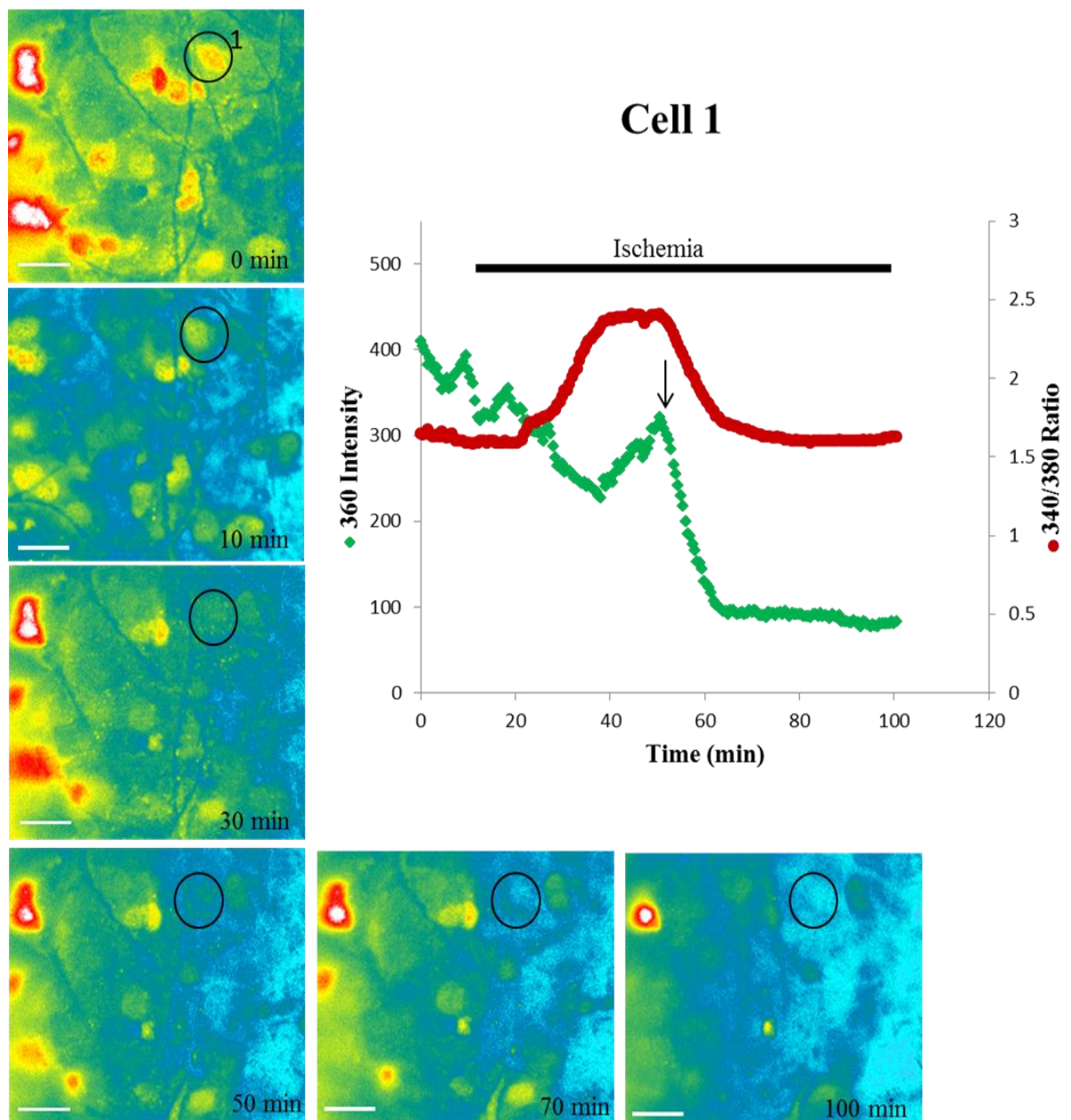


Figure 7-3: Representative data for Fura-2-loaded astrocytes in nRON under OGD conditions.

On the left, a series of images taken at different times shows clearly recognisable Cell 1 (circled). Cell 1 died at around 50 minutes, represented by a sudden drop in 360 intensity (arrow) that was preceded by calcium influx (elevated 340/380 ratio).

Scale bar = 4 μ m.

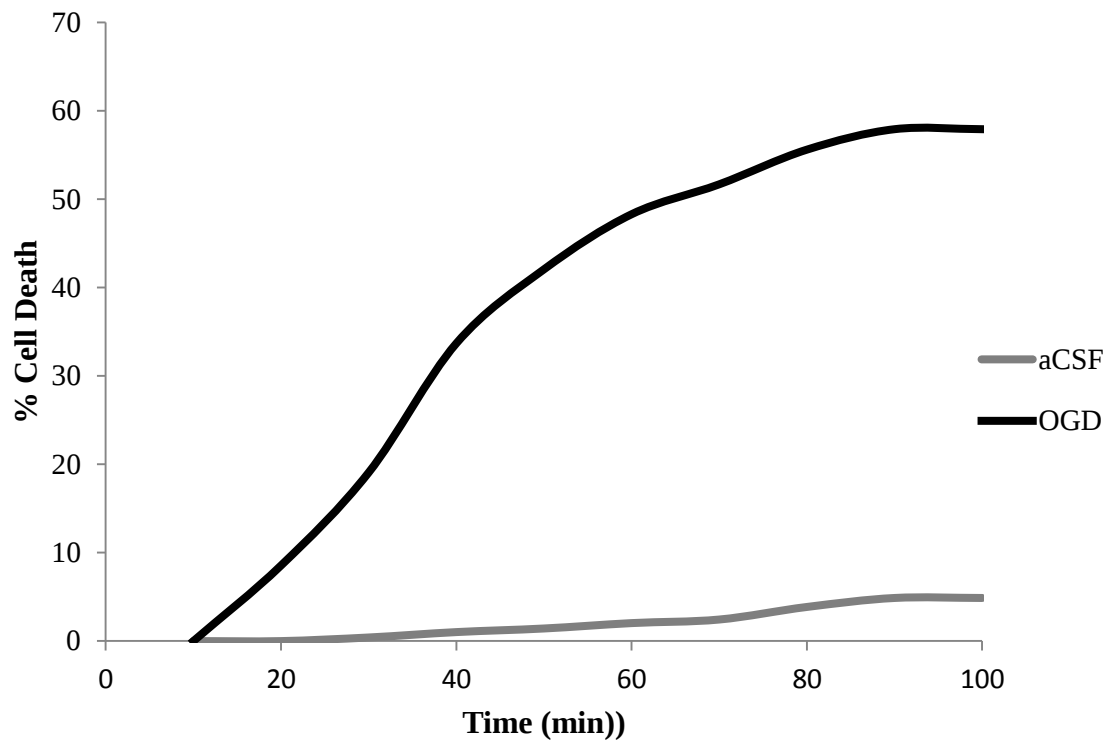


Figure 7-4: Percentage of cell death during OGD control.

The percentages of cell deaths are plotted at 20-minute intervals. Note that cells started to die at an early onset. Cell death under the OGD condition started within 20 minutes and gradually increased to peak at 40 minutes. N= 18 experiments (18 nerves, 561 cells).

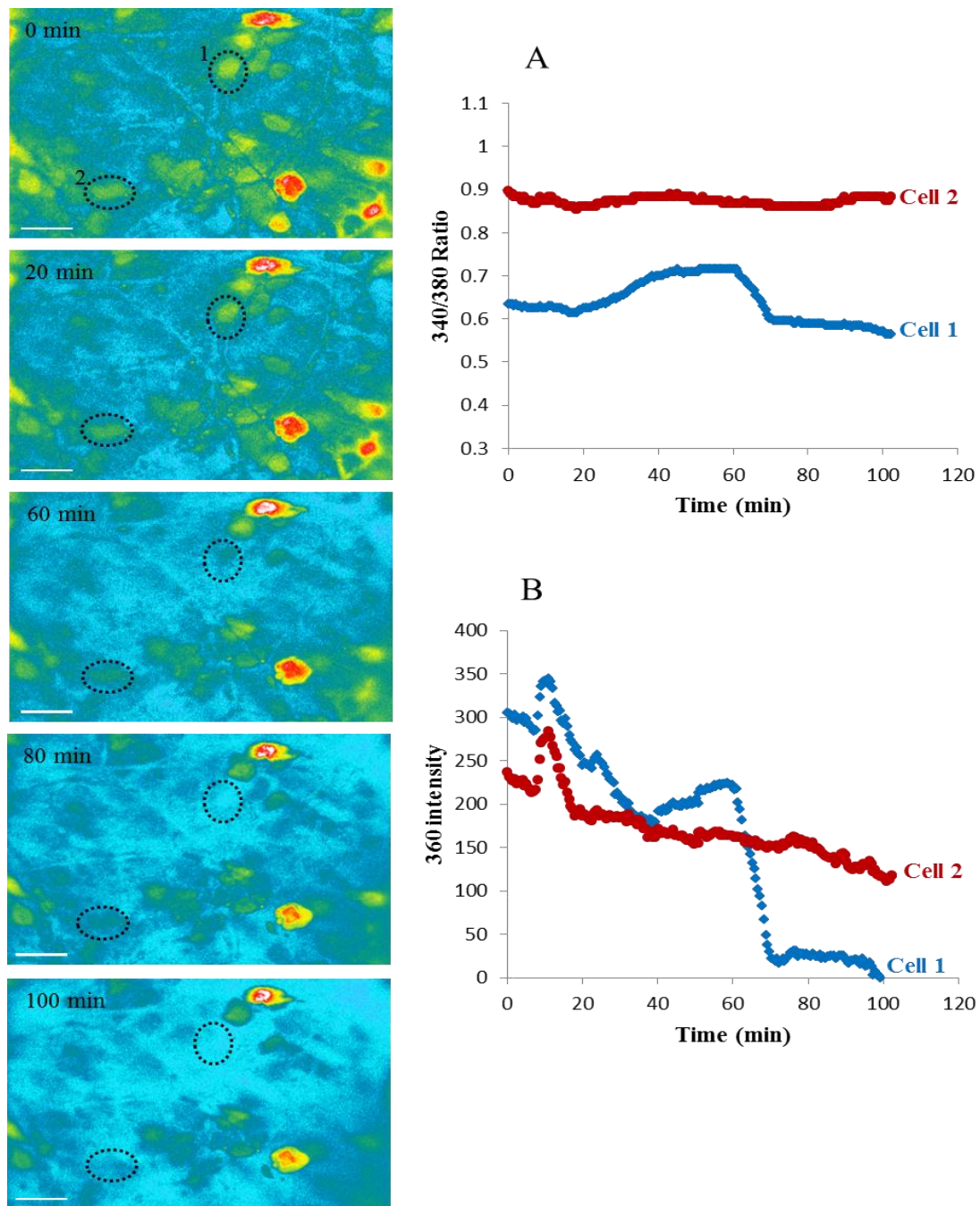


Figure 7-5: Representative data for fading of Fura-2-loaded astrocytes in nRON.

On the left, a series of 360 nm images taken at different times shows clearly recognisable Cells 1 and 2 (circled). Following both cells in the 360 images, Cell 1 disappeared at 60 minutes while Cell 2 disappeared at 100 minutes. (A) 340/380 ratios of both cells plotted against time show an increase in $[Ca^{2+}]_i$ in Cell 1, whereas Cell 2 maintains a stable ratio. (B) 360 intensity of both cells is plotted against time, showing a sudden drop in Cell 1 at 60 minutes, while Cell 2 maintains a steady 360 intensity. In conclusion, Cell 1 died at around 60 minutes while Cell 2 faded.

Scale bar = 4 μ m.

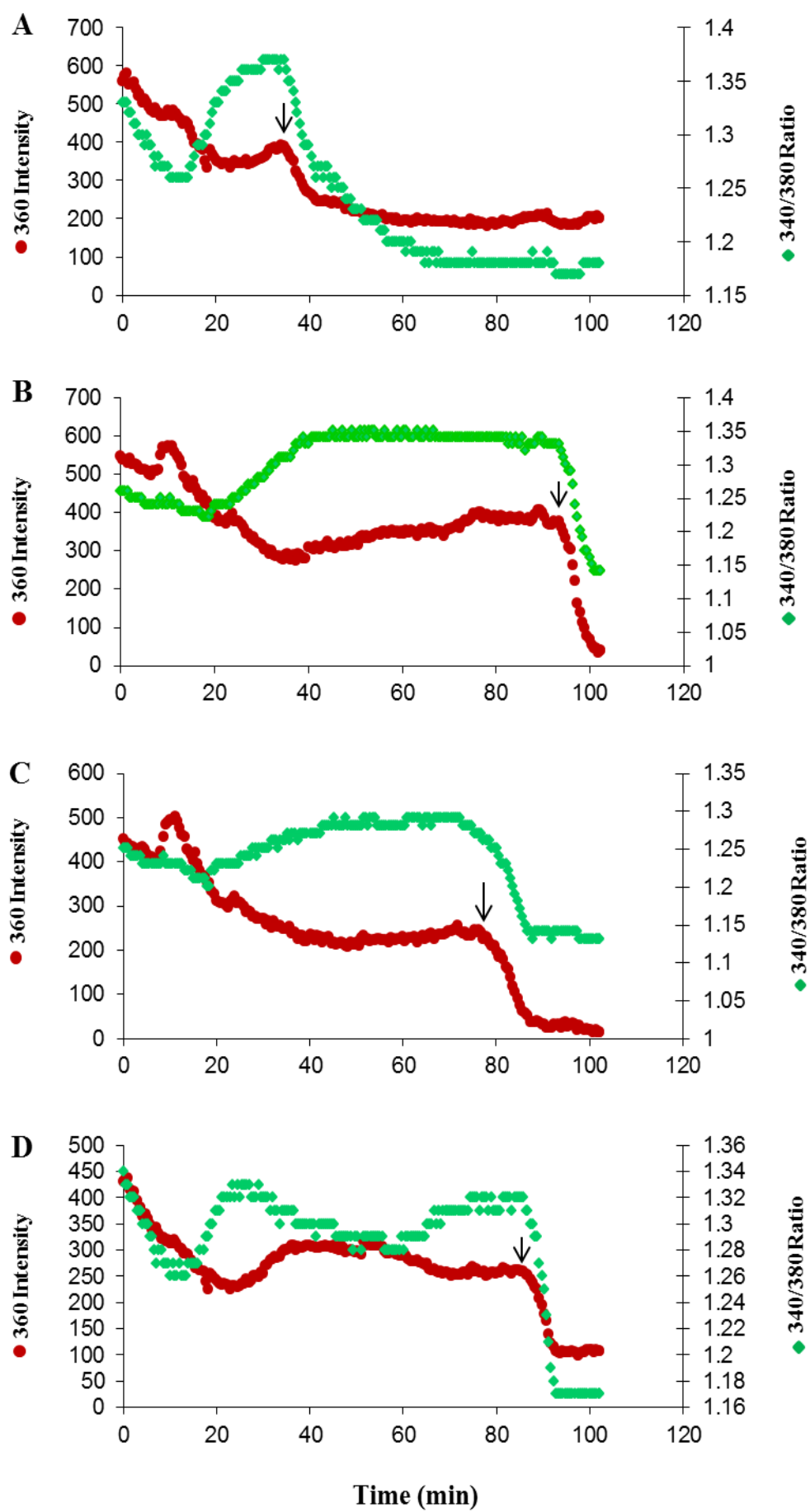


Figure 7-6: Four patterns of Ca^{2+} influx.

- (A) Ca^{2+} influx was rapid (represented by an increase in 340/380 ratio; green diamond) with a rapid decline coincident with cell death (arrow) represented by a drop in 360 intensity (red circle).
- (B) Ca^{2+} influx was rapid and maintained a plateau phase followed by late drop in both 340/380 ratio and 360 intensity, together with cell death (arrow).
- (C) Ca^{2+} influx was gradual and reached a peak at a late onset, which subsequently showed a drop in 340/380 ratio and cell death (arrow).
- (D) Ca^{2+} shows two peaks for influx, with cell death (arrow) occurring within the late peak.

7.1.3 Discussion:

7.1.3.1 Cell Identification:

The optic nerve is a white matter tract containing central axons and glial cells, and it is devoid of synapses (Stys, 1998). It has been documented that the majority of glial cells in P0 rat are astrocytes (Domowicz et al., 2011). This finding facilitated the use of Fura-2 AM to examine astrocytes in neonatal tissues. Confocal microscopy has previously shown a colocalisation of GFAP⁺ astrocytes and Fura-2 AM loaded cells, using a very similar protocol to the one I used, where all the Fura-2 AM (+) cells were GFAP⁺ (Fern, 1998). However, loading of Fura-2 was greater in cell bodies than in cell processes.

7.1.3.1 Ischaemia:

The mechanism of ischaemic injury in different CNS cells has been the focus of research activity for some time. Focusing on astrocytes, some differences have been documented in the mechanism of ischaemic injury in astrocytes in grey and white matter, despite both being dependent on Ca²⁺. By applying VGCC blockers and Ca²⁺ free solution, it has been suggested that ischaemic injury in white matter astrocytes is triggered by Ca²⁺ influx through VGCC, whereas grey matter astrocytes rely on Ca²⁺ release from intracellular sources (Duffy and MacVicar, 1996, Fern, 1998). Focusing on nRON astrocytes, ischaemic injury in P10 RON astrocytes is mediated by a Na⁺-K⁺-Cl⁻ cotransporter, while it is Ca²⁺-dependent in P0–P2 RON (Thomas et al., 2004, Fern, 1998).

The present study reveals that P0 nRON astrocytes are highly vulnerable to ischaemia. Astrocytes began to die within the first 10 minutes of ischaemia, and

significant cell death was detected within 30 minutes of ischaemia, which is consistent with the findings of previous work (Fern, 1998). Around 42% of all astrocytes died within the first 40 minutes of ischaemia, reaching $56.5\% \pm 5.98\%$ after 90 minutes of OGD. All astrocytes showed an increase in $[Ca^{2+}]_i$ prior to death, indicating that cell death is Ca^{2+} -dependent, as suggested previously (Fern, 1998, Bondarenko and Chesler, 2001a). Four patterns of $[Ca^{2+}]_i$ were noted during the OGD experiments. It has been suggested that these different patterns of $[Ca^{2+}]_i$ are related to different types of VGCC, because the early peak of Ca^{2+} influx is mediated by the T-type VGCC, whereas the late peak is mediated by the L-type (Fern, 1998). This was consistent with culture astrocytes which demonstrated their expression to both L- and T-type Ca^{2+} channels (Verkhratsky et al., 1998, Barres et al., 1989, Corvalan et al., 1990). Moreover, P2 nRON astrocyte tissue prints revealed well-supplemented astrocytes by both L- and T-type VGCC, in which the T-type is expressed more than the L-type (Barres et al., 1990a). Ischaemia mediates astrocyte depolarisation, which promotes a gradual and slow Ca^{2+} influx via VGCC (Duffy and MacVicar, 1996).

7.2 The Protective Effect of Picrotoxin in P0 RON Ischaemia:

7.2.1 Introduction:

Neurons as well as glial cells play a role in conducting and processing neuronal network information. Glial cells and neurons also express many of the same neurotransmitter receptors. Research over the last two decades has focused on glutamate receptors. However, it has been shown that, other than glutamate, multiple neurotransmitter receptors—such as GABA—are expressed in glial CNS, and these have become a topic of research interest.

The GABA_A receptor is an ionotropic receptor that is selectively permeable to the anion Cl[−] (Bormann, 2000). GABA has a dual effect: an inhibitory neurotransmitter in adult CNS, and an excitatory neurotransmitter in immature CNS (Ben-Ari et al., 2007). Several studies reported the depolarisation effect of GABA in astrocytes (Fraser et al., 1995, Israel et al., 2003, Meier et al., 2008, Macvicar et al., 1989), as astrocytes are well equipped with GABA_A receptors (Fraser et al., 1995, Fraser et al., 1994). This effect is thought to be related to the high level of intracellular Cl[−] in immature CNS cells, which reverse the Cl[−] driving force (Squire L.R., 2008, Ben-Ari et al., 2007). The onset of the excitatory to inhibitory shift depends on Cl[−] homeostasis, which differs according to several factors such as sex, species, brain site and cell type (Ben-Ari et al., 2007). A number of studies have generated some controversy regarding the exact onset of the E_{GABA}/I_{GABA} switch in rat neurons. These studies are in general agreement that the excitatory effect of GABA occurs during P0 to P5, and the E_{GABA}/I_{GABA} switch takes place during the second postnatal week (Tyzio et al., 2007, Ben-Ari et al., 1997, Khazipov et al., 2004).

A large body of evidence has revealed the presence of the GABA_A receptor in cultured astrocytes in the hippocampus (Fraser et al., 1995, Blankenfeld and Kettenmann, 1991), retina (Clark and Mobbs, 1992), Bergmann glia of cerebellar slices (Riquelme et al., 2002, Muller et al., 1994), acutely isolated astrocytes (Fraser et al., 1994), cultured spinal cord (Rosewater and Sontheimer, 1994), isolated neonatal rat spinal cord (Pastor et al., 1995), and nRON (Sakatani et al., 1992, Lake, 1992, Ochi et al., 1993). In nRON, it is found that GABA transmitter expression is localised to the macroglia somata (Lake, 1992). GABA immuno-electron microscopy studies revealed the presence of GABA transmitter in pre-myelinated axons and in the cytoplasm and processes of glial cells in developing rat optic nerves, but not in those of adults (Sakatani et al., 1992). Confocal immunostaining and high-pressure liquid chromatography (HPLC) studies have revealed the transient expression of GABA in GFAP⁺ astrocytes in early neonatal RON, which attenuated after the third postnatal week (Ochi et al., 1993). GABA receptor is found in type-2 (fibrous) astrocytes as well as in O-2A progenitor cells and oligodendrocytes of P0-P7 RON cultures (Barres et al., 1990b). The GABA transporter (GAT 1-3) mRNA and the GABA_A receptor mRNA have been detected in nRON (Domingues et al., 2010, Howd et al., 1997).

Several articles have demonstrated the depolarising effect of GABA on nRON. The sucrose gap membrane potential recording of developing RON found that application of muscimol, a GABA_A agonist, mediates a depolarisation effect that can be blocked by bicuculline, a GABA_A antagonist, whereas baclofen, a GABA_B agonist, had no effect (Sakatani et al., 1992). This study provided evidence that the depolarisation effect of GABA in neonatal RON is mediated by GABA_A. However, GABA_A-induced depolarisation could be due to the direct activation of axonal GABA_A receptor, or it could be mediated by the activation of glial GABA_A producing

depolarisation of axons. The effect of GABA_A on astrocytes was a matter of debate because astrocytes are not excitable cells, until Butt and Jennings established the action of GABA_A receptor on neonatal RON astrocytes, which were recognised morphologically after intracellular injection of horseradish peroxidase (Butt and Jennings, 1994b, Butt and Jennings, 1994a). Electrophysiological studies have revealed a similar depolarisation effect following GABA and muscimol application, but not after baclofen, which can be blocked by bicuculline (Butt and Jennings, 1994b, Butt and Jennings, 1994a). This data suggests that GABA_A receptors are expressed by nRON astrocytes, but not GABA_B receptors, which is consistent with evidence from several other studies (Kettenmann and Schachner, 1985, Macvicar et al., 1989, Walz and MacVicar, 1988, Butt and Tutton, 1992, Hosli et al., 1990, Magoski et al., 1992, Mudrickdonnon et al., 1993). Moreover, nRON astrocytes showed a single-phase depolarisation after increasing the $[K^+]_o$ to around 10 mM, which proved the direct action of GABA on the GABA_A receptor of nRON astrocytes (Butt and Jennings, 1994b). This is consistent with a direct effect of GABA on the GABA_A receptor of immature astrocyte cultures (Kettenmann and Schachner, 1985, Kettenmann et al., 1987) and on astrocytes in isolated tissues (Clark and Mobbs, 1992, Macvicar et al., 1989). However, the GABA effect on axons cannot be ignored, as electrophysiology has revealed a depression in action potential conduction following GABA application and has suggested the direct action of GABA on the axonal GABA_A receptor of nRON axons and on the neonatal rat spinal dorsal column (Constantinou and Fern, 2009, Sakatani et al., 1991a, Sakatani et al., 1992, Honmou et al., 1993, Simmonds, 1983). However, it has been suggested that the action of GABA on glial cells is mediated by extracellular K^+ released from axons (Hosli et al., 1981). Contrary to that suggestion, GABA_A-mediated astrocyte depolarisation leads to K^+ release, and this could be the

cause of axonal depolarisation (Kettenmann and Schachner, 1985, Macvicar et al., 1989). However, some evidence reveals that although GABA induces an increase in $[K^+]_e$, it is insufficient to wholly account for GABA-induced depolarisation in either astrocytes or axons (Butt and Jennings, 1994a, Honmou et al., 1993). This is consistent with a study that showed that the rise in $[K^+]_e$ mediated by GABA was not enough to produce the effect on the CAP as compared to those mediated by GABA and which suggested that $[K^+]_e$ induced by GABA had only a secondary role in GABA effect on nRON (Sakatani et al., 1994).

The effect of GABA on nRON is dose-dependent, and it attenuates with age, consistent with a transient presence of GABA in nRON (Sakatani et al., 1992, Butt and Jennings, 1994a) and in maturing myelin deficient RON (Lim et al., 1993). Focusing on the nRON astrocyte, it has been shown that the depolarisation effect of GABA on the peak and amplitude of the action potential diminishes with age, starting from the largest effect at P1–4 and declining such that there is no effect at P21–25 (Butt and Jennings, 1994a). This is consistent with the GABA immune electron microscopy and confocal immunostaining of developing RON which revealed a reduction in GABA immunoreactivity with age (Ochi et al., 1993, Sakatani et al., 1992). However, PCR performed in developing and adult RON revealed the presence of GAT1-3 mRNA and GABA_A receptor subunit mRNA in both neonatal and adult RON (Howd et al., 1997, Domingues et al., 2010), although sub-unit expression levels were found to change with maturation. GABA-induced depolarisation in P10 nRON astrocytes is found to be dose-dependent, with the highest response observed for 10 mM GABA > 1 mM > 0.5 mM > 0.1 mM GABA (Butt and Jennings, 1994a). 100 μ M GABA was shown to be sufficient to suppress the CAP of P10 nRON (Constantinou and Fern, 2009). However, depolarisation produced by GABA is accompanied by increased $[Ca^{2+}]_i$ (Perea et al.,

2009, Fraser et al., 1995, Meier et al., 2008), and it is suggested that astrocyte excitability via Ca^{2+} influx via VGCC following GABA_A depolarisation could modulate neuronal activity (Meier et al., 2008, Perea et al., 2009).

It is widely accepted that during ischaemia, GABA accumulates in the synaptic cleft (Globus et al., 1991, Phillis et al., 1994, Shuaib et al., 1994, Ravindran et al., 1994). It has been shown that GABA_A receptors are sensitive to a reduction in ATP (Gyenes et al., 1988, Harata et al., 1997, Stelzer et al., 1988). During ischaemia, the failure of Na^+/K^+ -ATPase leads to a depolarisation of the cell membrane; this potentiates the opening of voltage-gated Ca^{2+} and Na^+ channels and thus increases the influx of Ca^{2+} and Na^+ ions in presynaptic terminals (Schwartz-Bloom and Sah, 2001). A high level of $[\text{Ca}^{2+}]_i$ in the presynaptic terminals enhances GABA and glutamate release from vesicles to the synaptic cleft (Schwartz-Bloom and Sah, 2001). Activation of reactive oxygen species (ROS) also promotes GABA efflux and inhibits its uptake (Sah and Schwartz-Bloom, 1999, Rego et al., 1996, Saransaari and Oja, 1998). An increased level of intracellular Na^+ in presynaptic terminals reverses GABA transporters and therefore increases GABA accumulation in extracellular space (Schwartz-Bloom and Sah, 2001), while evidence suggests that GABA can also leak from injured nerve terminals (Burke and Taylor, 1992, Phillis et al., 1994). Moreover, GABA can be secreted through Ca^{2+} -dependent or Ca^{2+} -independent vesicular excretion, which is mediated by depolarisation (Schwartz-Bloom and Sah, 2001). Extracellular GABA binds to the GABA_A receptors, whereas glutamate binds to NMDA and AMPA receptors. GABA_A receptor activation allows Cl^- influx in adults and accumulation in post-synaptic terminals. On the other hand, activation of AMPA receptors increases Na^+ influx and initiates membrane depolarisation. Intracellular Ca^{2+} accumulation occurs following activation of NMDA receptors, and through voltage-

gated Ca^{2+} ions as well as some Ca^{2+} permeable AMPA receptors. Accumulation of $[\text{Ca}^{2+}]_i$ in post-synaptic terminals activates phospholipase A_2 and its metabolites (arachidonic acid, prostaglandins and thromboxane), which inhibit GABA receptors. A high level of $[\text{Ca}^{2+}]_i$ generates ROS, which also inhibit GABA receptor function.

With respect to white matter, the cellular components of WM could affect the reaction to ischaemia and the WM response to ischaemia via neurotransmitter release (Levine et al., 1993). Microdialysis studies have revealed a rise in extracellular GABA levels following WM ischaemia (Shimada et al., 1993). In addition, it has been shown that astrocyte swelling and ATP reduction following prolonged ischaemia can mediate the release of EAAs from astrocytes (Kimelberg, 2005, Kimelberg et al., 1990, Mongin and Kimelberg, 2002). GABA_A receptor activation is found to produce a partial nerve block in nRON (Constantinou and Fern, 2009, Sakatani et al., 1992, Sakatani et al., 1991b). This is accompanied by axon depolarisation and an increase in the $[\text{K}^+]_e$ (Constantinou and Fern, 2009, Sakatani et al., 1994, Simmonds, 1983). Prolonged exposure (80 minutes) of GABA was enough to produce a significant fall in the CAP in nRON, which was prevented by picrotoxin, a GABA_A blocker (Constantinou and Fern, 2009). However, this depression in CAP was reversible upon washout, which demonstrates that GABA is unlikely to cause damage, but the chronic effect of GABA on axon conduction is showed to be a slower conduction velocity (Sakatani et al., 1993). That means that blocking the GABA_A receptor during long exposure to GABA, as in case of ischaemia, might be protective.

The role of GABA_A receptors during hypoxia has been studied in neonatal (P2–P9) isolated hemisected spinal cord and spinal dorsal column axons (Levine et al., 1993). The study found that isolated dorsal column (WM) can tolerate prolonged hypoxia (120 minutes), while the hemicord (WM + GM) was very sensitive to hypoxia,

which produced a depression in CAP. Moreover, it has been found that bicuculline blocks this hypoxic conduction failure and shows a neuroprotective effect against hypoxia (Levine et al., 1993). However, this study, done in P2-P9 rats, involved a preparation in the pre-myelination period (P0-P4), which is highly tolerant to 60 minutes of anoxia, aglycemia or both, and in an early myelination period (P5-P20), which is partially tolerant to 60 minutes of anoxia or aglycemia but not both (Fern et al., 1998). This suggests a greater effect of ischaemia (hypoxia + aglycemia) on WM than hypoxia alone. Eighty minutes of ischaemia was enough to produce around 46.6% astrocyte death in P0 RON (Fern, 1998). Several studies have focused on adult grey matter ischaemia and have revealed that the application of GABA_A and/or GABA_B agonists can be protective against ischaemic damage (Xu et al., 2008, Cozzi et al., 2002, Ito et al., 1999, Kulinskii and Mikhel'son, 2000). With respect to RON, GABA_B receptor activation is shown to be protective against ischaemic injury in adult RON, but there has been no previous attempt to study the effect of GABA_A receptor activation on P0 RON during ischaemia (Fern et al., 1994, Fern et al., 1995b). GABA_A activation may play a protective role in adults, as it mediates hyperpolarisation, while it may be damaging in neonates, as it mediates depolarisation, and this is particularly important in immature WM due to high GABA expression. In this section, I study the effect of GABA on the Fura-2 AM loaded nRON astrocytes and examine the effect of picrotoxin, a GABA_A antagonist, during 90 minutes of ischaemia.

7.2.2 Results:

7.2.2.1 GABA Agonist Application:

Astrocyte viability was assessed via 360 intensity and the 340/380 ratio, and was stable when 100 μ M of GABA was applied in aCSF during the 100-minute experiment. 100 μ M GABA was applied for 90 minutes following a 10-minute baseline perfusion of aCSF, resulting in a total cell death of $1.9\% \pm 1.2\%$ (2 of 134 cells, 6 nerves). The total number of cell deaths during GABA application in the aCSF experiment was not significantly different to that of aCSF alone ($1.9\% \pm 1.2\%$ compared to $4.5 \pm 1.4\%$, respectively; $p > 0.05$). On the other hand, perfusion of 100 μ M GABA + aCSF for 10 minutes followed by 90 minutes of 100 μ M GABA + OGD showed an increase in cell death compared to aCSF controls and reached $21.7\% \pm 7.1\%$ (36 of 166 cells; 6 nerves), which was not significantly different from the GABA + aCSF ($21.7\% \pm 7.1\%$ compared to $1.9\% \pm 1.2\%$, respectively; $p > 0.05$). However, GABA perfusion during OGD significantly reduced the total number of cell deaths compared to OGD without GABA ($21.7\% \pm 7.1\%$ compared to $56.5\% \pm 5.98\%$, respectively; $p < 0.05$).

All cells that died during GABA experiments exhibited an increase in $[Ca^{2+}]_i$ prior to death. In Figure 7-7, a series of 360 images of Fura-2-loaded cells was taken during the GABA + OGD experiment and showed the viability of two cells (Cell 1 and Cell 2, circled) throughout the experiment. Focusing on Cells 1 and 2 in the 360 images, it appears that Cell 1 died at around 50 minutes, which is consistent with the drop (arrow) in 360 intensity that is preceded by a Ca^{2+} influx (increase in 340/380 ratio). Conversely, Cell 2 was identified in 360 images throughout the experiment and had stable 360 intensity and 340/380 ratio.

Studying the distribution of cell death during GABA + OGD experiments revealed that early cell death started at 20 minutes and gradually increased to peak at 40 minutes, as shown in Figure 7-8. The distribution of cell death in GABA + OGD is similar to that in OGD, as both produce early cell death—beginning at around 20 minutes and peaking at 40 minutes—despite the higher percentage of cell death during each 10-minute interval in the OGD control.

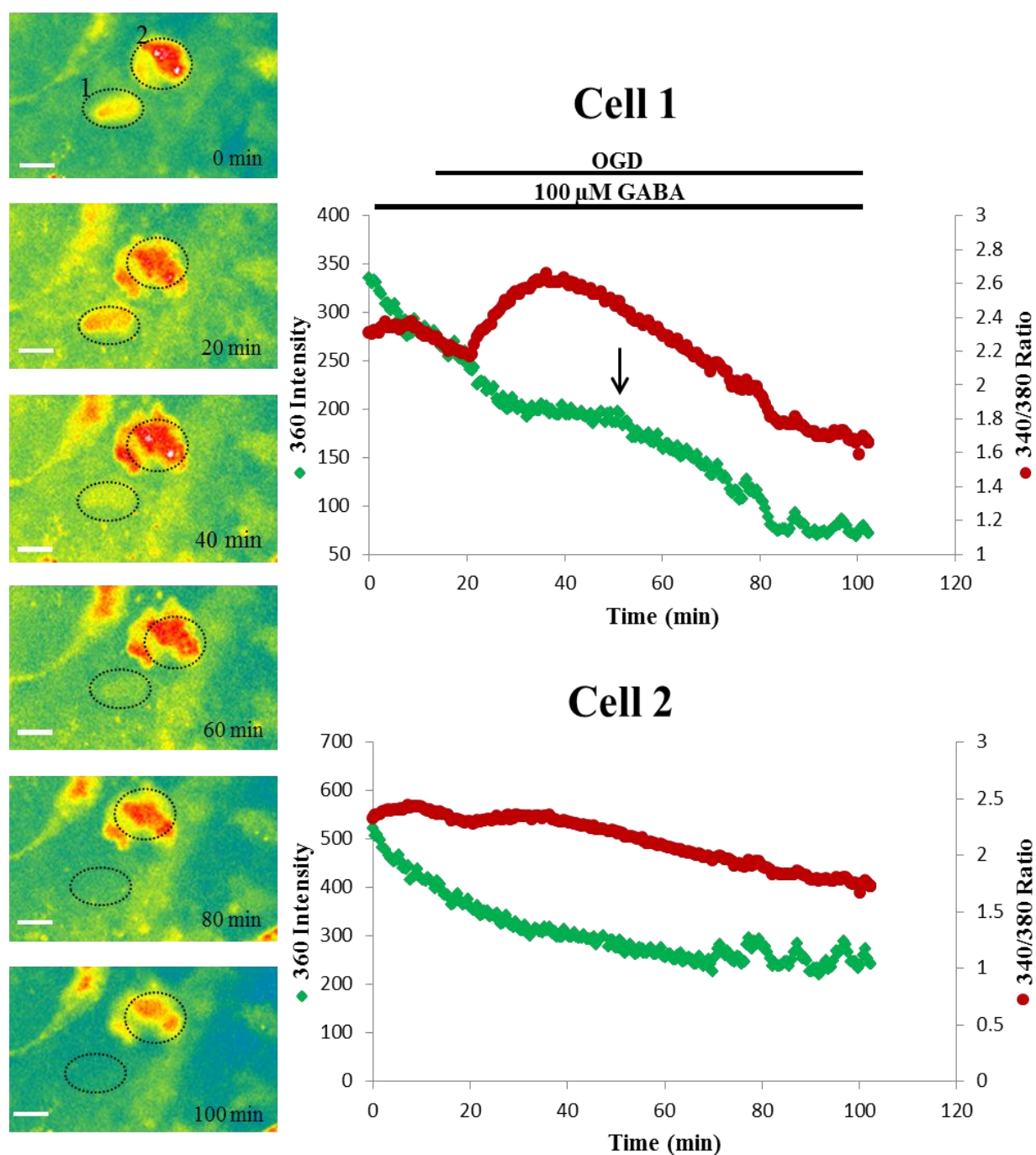


Figure 7-7: Representative data for Fura-2-loaded astrocytes in nRON during OGD + GABA.

On the left, a series of 360 nm images taken at different times shows clearly recognisable Cells 1 and 2 (circled). Cell 2 was stable throughout the experiment (steady 340/380 ratio and 360 intensity), whereas Cell 1 died at around 50 minutes, with a sudden drop in 360 intensity (arrow) preceded by calcium influx (elevation in 340/380 ratio).

Scale bar = 2 μ m.

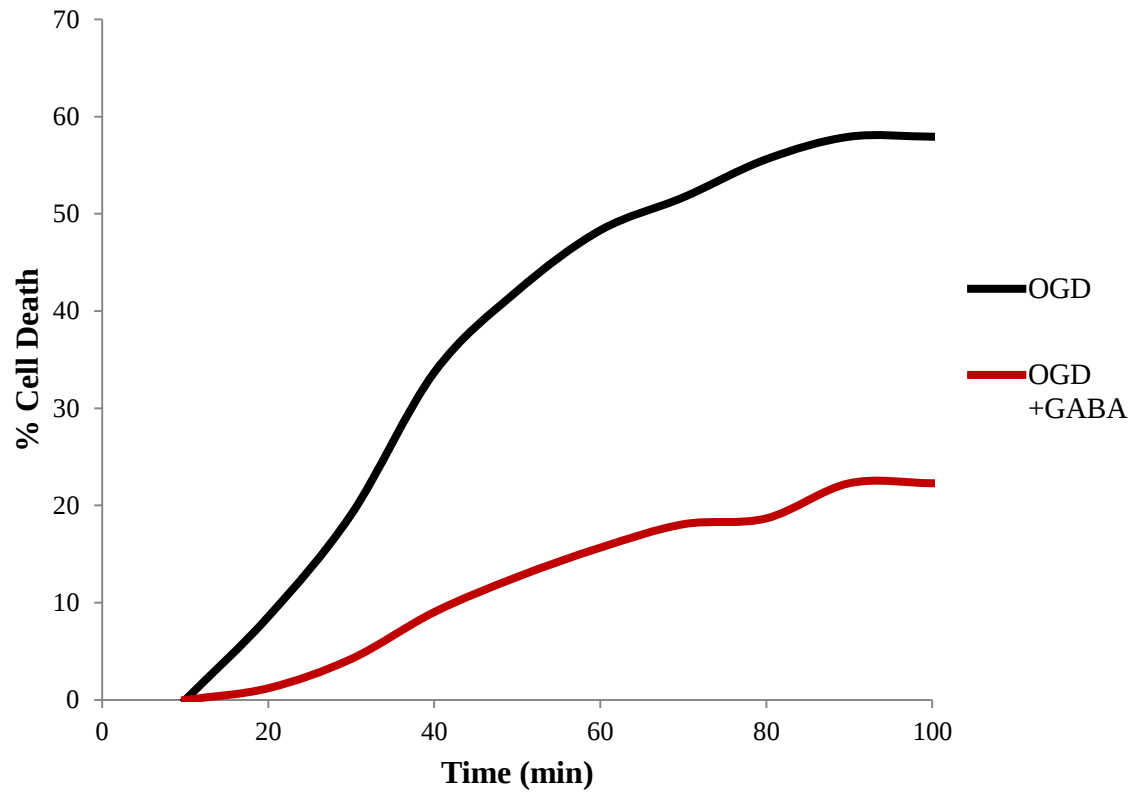


Figure 7-8: Percentage of cell death during OGD + 100 μ M GABA.

The percentage of cell death is plotted at 20-minute intervals. Note that cells started to die with OGD application. The cell death in GABA + OGD started within the initial 20 minutes and gradually increased. N=6 experiments (6 nerves, 166cells).

7.2.2.2 GABA Antagonist (Picrotoxin) Application:

Perfusion with 100 μ M picrotoxin, a non-competitive GABA_A antagonist, in aCSF did not harm the astrocytes in nRON, and the total percentage of cell death reached $3.7\% \pm 2.5\%$ (6 of 173 cells in 90 minutes, 6 nerves). Results from applying 100 μ M picrotoxin + aCSF did not differ significantly from the aCSF control ($3.7\% \pm 2.5\%$ compared to $4.5 \pm 1.4\%$, respectively; $p > 0.05$). Moreover, 100 μ M GABA was added with 100 μ M picrotoxin in aCSF, that showed a stable 340/380 ratio and 360 intensity and again did not significantly change the percentage of cell death ($2.8\% \pm 1.8\%$; 3 of 104 cells in 90 minutes, 4 nerves).

Perfusion of 100 μ M picrotoxin in aCSF for 10 minutes, followed by 90 minutes of 100 μ M picrotoxin in OGD, reduced the total percentage of cell death from that found in OGD alone ($17.9\% \pm 5.5\%$; 28 of 156 cells, 6 nerves). 300 μ M picrotoxin was examined for any additional effect, and the total percentage of cell death was $30.0\% \pm 9.6\%$ (46 of 155 cells, 5 nerves). Both 100 μ M and 300 μ M picrotoxin showed significantly lower total percentage of cell death compared to the OGD control ($17.9\% \pm 5.5\%$ and $30\% \pm 9.6\%$, compared to $56.5\% \pm 5.98\%$, respectively; $p < 0.05$).

All cells that died during picrotoxin + aCSF or picrotoxin + OGD showed a Ca^{2+} rise prior to death. In Figure 7-9, a series of 360 images of Fura-2-loaded cells was taken through the picrotoxin + OGD experiment, and this series expressed the viability of two cells (Cell 1 and Cell 2; circled) throughout the 100-minute experiment. Both cells are identifiable in the initial image (0 minute image). Following the progression of these cells during the experiment suggests that Cell 1 persisted throughout the experiment, with a steady 360 intensity and 340/380 ratio, whereas Cell 2 disappeared

at 70 minutes, which is consistent with the drop (arrow) in 360 intensity and the increase in 340/380 ratio prior to cell death.

Picrotoxin 100 μ M played a role not only in reducing the total percentage of cell death during OGD but also in postponing the initial onset of cell death until 50 minutes; see Figure 7-10. Cells started to die at 50 minutes, which was also the peak in cell death incidence, and then gradually declined to zero at 100 minutes.

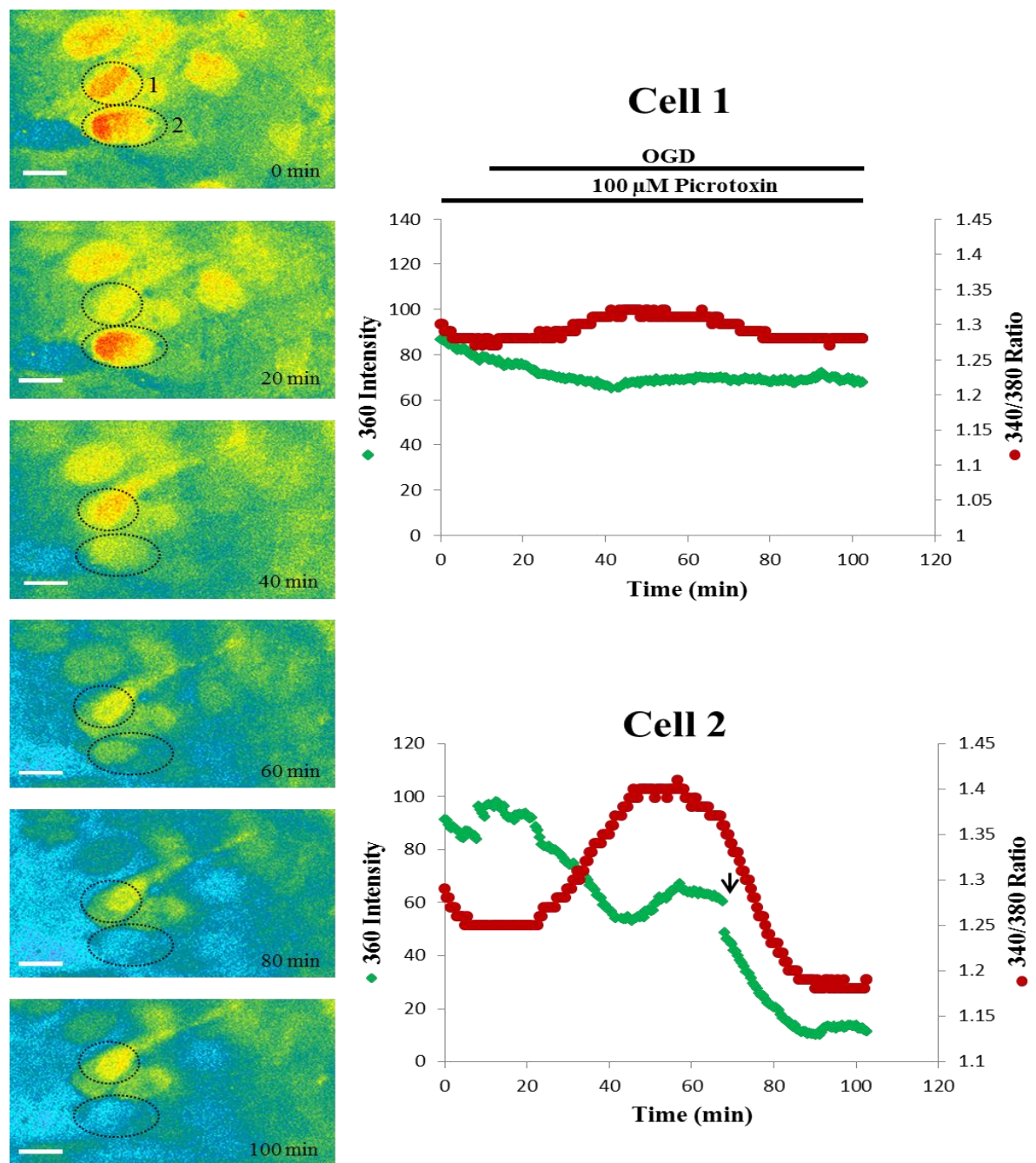


Figure 7-9: Representative data for Fura-2-loaded astrocytes in nRON for picrotoxin + OGD.

On the left, a series of 360 nm images taken at different times shows clearly recognisable Cells 1 and 2 (circles). Cell 1 was stable throughout the 100-minute experiment (steady 340/380 ratio and 360 intensity), whereas Cell 2 died at around 70 minutes, with a sudden drop in 360 intensity (arrow) preceded by calcium influx (elevation in 340/380 ratio). Scale bar = 2μ m.

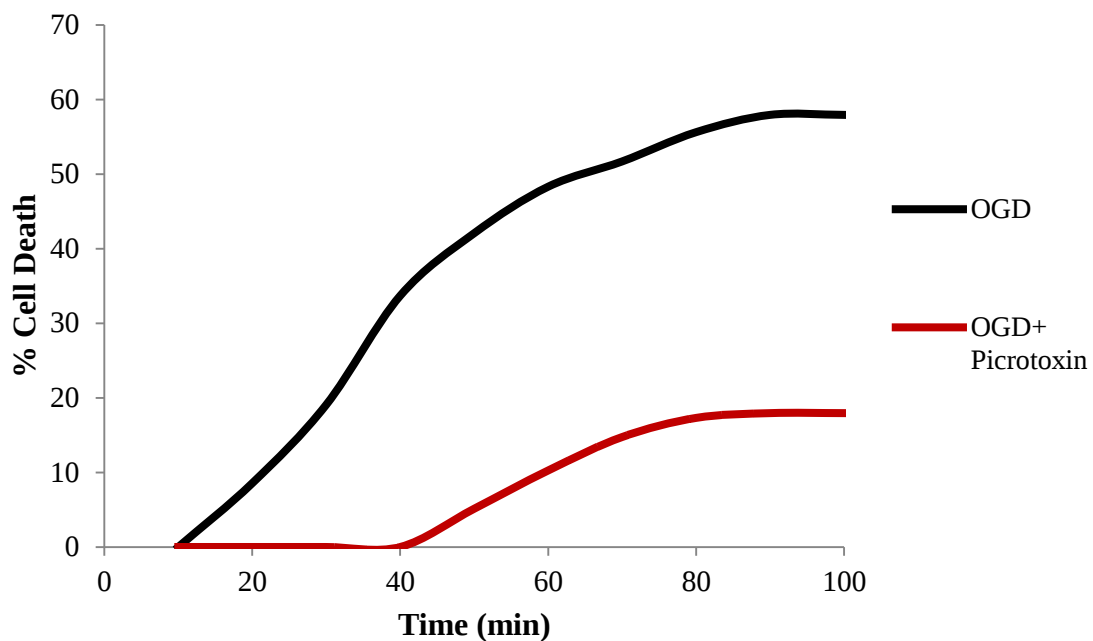


Figure 7-10: Percentage of cell death during OGD + 100 μ M picrotoxin.

Percentage of cell death plotted at 20-minute intervals. Note that cells started to die at a later onset of 50 minutes, after which cell death gradually declined. N=6 experiments (6 nerves, 156 cells).

7.2.3 Discussion:

Astrocytes in nRON are well equipped with GABA_A receptors (Lake, 1992, Barres et al., 1990b, Domingues et al., 2010, Sakatani et al., 1992, Ochi et al., 1993). Several studies have provided evidence of the depolarising action of GABA_A activation on nRON (Sakatani et al., 1992), astrocytes (Butt and Jennings, 1994b, Butt and Jennings, 1994a) and axons (Constantinou and Fern, 2009, Sakatani et al., 1991b). The transient presence of GABA in nRON has been documented in several studies (Lake, 1992, Ochi et al., 1993, Sakatani et al., 1992). GABA_A activation in nRON mediates a partial nerve block (Sakatani et al., 1992, Sakatani et al., 1991b). Long exposure (80 minutes) of GABA on nRON produces a fall in CAP, which is reversible upon washout (Constantinou and Fern, 2009). Picrotoxin blocks the effect of GABA on nRON and prevents CAP suppression (Constantinou and Fern, 2009). The application of bicucullin, a GABA_A antagonist, has been found to protect spinal cord axons against hypoxia (Levine et al., 1993).

In the current study, the application of 100 μ M GABA for 90 minutes of aCSF revealed a small percentage of astrocyte death that was not significantly different from the aCSF control. Moreover, the application of 100 μ M GABA for 10 minutes in aCSF, followed by 90 minutes in OGD, significantly reduced the total percentage of cell death compared to the OGD control. This protective effect may be due to receptor desensitisation relieving GABA_A receptor activation during OGD, which might contribute to injury. Rapid desensitisation was reported to be characteristic of GABA_A receptors in nRON (Sakatani et al., 1992, Bormann and Clapham, 1985). In neonatal RON, it was found not only that GABA application produced this type of rapid desensitisation, but that muscimol, a GABA_A agonist, produced the same effect

(Sakatani et al., 1992). Agonist protection via receptor desensitisation has been reported previously for GluR in P10 RON (Wilke et al., 2004). Despite the protective role of GABA during OGD (apparently due to receptor desensitisation), cells started to die at a very early onset of 20 minutes, reaching maximum cell death at 40 minutes, in a distribution similar to that of the OGD control. All cells that died during GABA experiments were associated with an increase in $[Ca^{2+}]_i$ which is consistent with Ca^{2+} -dependent astrocyte death during ischaemia (Bondarenko and Chesler, 2001a, Fern, 1998).

Picrotoxin, a non-competitive GABA antagonist, binds to the M2 segment of the GABA_A receptor and blocks the Cl^- channel (Zorumski and Isenberg, 1991). It has been found that picrotoxin inhibits the effect of GABA_A in nRON (Sakatani et al., 1991b, Constantinou and Fern, 2009, Fraser et al., 1994). In the present study, 100 μ M picrotoxin was first tested during aCSF and was found to result in cell death that was not significantly different from that of the aCSF control. On the other hand, 100 μ M picrotoxin application was found to significantly reduce the total percentage of cell death during 90 minutes of OGD. This is consistent with the protective role of bicucullin during rat spinal cord hypoxia (Levine et al., 1993). A larger 300 μ M dose of picrotoxin was used to test the maximum level of protection provided by picrotoxin. However, the results showed that 300 μ M picrotoxin did not provide any additional protection for nRON astrocytes during OGD. Moreover, 100 μ M picrotoxin not only reduced the total percentage of cell death in OGD but also postponed the initial onset of cell death from 20 minutes to 50 minutes in comparison to the OGD control. Cells started to die at 50 minutes, at a sudden peak level, which was followed by a gradual decline in the percentage of cell death with time, to reach 0% at 100 minutes. All cells that died during the picrotoxin experiment revealed an increased level of $[Ca^{2+}]_i$, which

is consistent with Ca^{2+} -dependent astrocyte death at this age (Fern, 1998, Bondarenko and Chesler, 2001a). It has been reported that the early Ca^{+2} influx and astrocyte death in P0 RON is mediated by T-type VGCC, while the late one is more likely produced by L-type VGCC (Fern, 1998). In the present study, picrotoxin application was showed to abolish the early death of astrocytes during ischaemia. This could be due to the indirect blocking effect of picrotoxin on T-type VGCC, which therefore eliminated early astrocyte death during ischaemia.

7.3 The Protective Effect of Strychnine in P0 RON

Ischaemia:

7.3.1 Introduction:

The glycine receptor is a pentameric receptor that allows Cl^- conductance via its central pore. Due to the high $[\text{Cl}^-]_i$ in neonates, glycine mediates Cl^- efflux following Cl^- gradients (Verkhratsky and Steinhauser, 2000). Glycine therefore mimics GABA in its dual action as an inhibitory neurotransmitter in adults and as an excitatory in neonates (Lynch, 2004, Betz and Laube, 2006). Glycine can also mediate depolarisation in adult CNS via activation of the NMDA receptor (Johnson and Ascher, 1987). However, it has been found that glycine does not significantly change the CAP mediated by NMDA in neonatal dorsal column axons (Matsumoto et al., 2005).

Glycine receptors are distributed throughout the CNS (Rajendra et al., 1997). Several studies have focused on glycine distribution at light levels by glycine receptor immunoreactivity and strychnine autoradiography (White et al., 1990, Todd et al., 1996, Vandenpol and Gorcs, 1988) or by immuno-electron microscopy (Triller et al., 1985). These studies revealed high expression of glycine receptors in the spinal cord and medulla, less expression in the midbrain, hypothalamus and thalamus, and no detectable glycine receptor in the higher brain (Rajendra et al., 1997, Lynch, 2004). PCR and immunohistochemistry have revealed a high glycine receptor expression in the cerebellum (Garcia-Alcocer et al., 2008). In the spinal cord, the foetal glycine receptor is usually an $\alpha 2$ -homomeric receptor, while it is an $\alpha 1\beta$ -heteromeric receptor in adults (Becker et al., 1988). The $\alpha 2$ glycine subunit is thought to be linked to an embryonic subunit (Akagi et al., 1991), while $\alpha 4$ and β glycine subunits are heavily expressed in white matter during all developmental stages (Belachew et al., 1998,

Laube et al., 2002). However, the neonatal rat spinal cord and auditory system express not only the $\alpha 2$ subunit but are also abundant of $\alpha 1$ and β subunits, and the switch to the adult type takes place around P20 (Becker et al., 1988, Friauf et al., 1997, Watanabe and Akagi, 1995). Focusing on glial cells, glycine receptors are detected in glial cells of rat spinal cord, and PCR has revealed the expression of $\alpha 1$ and β but not of the $\alpha 2$ or $\alpha 3$ subunits (Verkhratsky and Steinhauser, 2000, Kirchhoff et al., 1996). In addition, mRNAs of different glycine receptor subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, and β) were detected in different age ranges (P0, P11 and adult) of RON glial cells and revealed that glial cells of P0 RON express $\alpha 2$ and $\alpha 4$ and strongly express β subunits of the glycine receptor (Domingues et al., 2010).

Functional glycine receptors have been reported in different parts of the CNS, such as cerebral cortex, spinal cord and optic nerve (Kilb et al., 2008, Constantinou and Fern, 2009, Pastor et al., 1995, Simmonds, 1983). Glycine receptor activation has been found to produce a depolarisation in isolated RON (Simmonds, 1983). Glycine depolarisation in nRON is dose-dependent, where the maximum depolarisation achieved by glycine application was seven times greater than that achieved by muscimol application (Simmonds, 1983). Long exposure of glycine (80 minutes) produced a significant fall in CAP of P10 RON, which is reversible after washout (Constantinou and Fern, 2009). This depolarisation produced by glycine receptor activation in P10 RON was successfully blocked by strychnine (Constantinou and Fern, 2009). The effect of glycine receptor activation on astrocytes has been studied in neonatal spinal cord using a patch-clamp technique which revealed glycine-mediated membrane currents in all astrocytes and was blocked by strychnine (Pastor et al., 1995).

The level of extracellular glycine increased after ischaemia in both grey and white matter (Shimada et al., 1993, Phillis and O'Regan, 2003, Phillis et al., 1997,

Kennedy et al., 2002, Kim et al., 2000, Hagberg et al., 1985, Oja and Saransaari, 2009, Luccini et al., 2010). Common carotid and vertebral arteries occlusion for 10–30 minutes was sufficient to increase 30-fold the level of extracellular hippocampus taurine (Hagberg et al., 1985). High-performance liquid chromatography and amino acid analyser revealed a significant rise in the glycine vitreous level after optic nerve ischaemia (Kim et al., 2000). Internal capsule (WM) microdialysis demonstrated an increase in the level of extracellular taurine, a strychnine-sensitive glycine-receptor agonist, after global brain ischaemia induced by multiple extracranial vessel occlusions (Shimada et al., 1993). In the adult brain, it is found that glycine has a protective effect against ischaemic injury through its inhibitory effect against neuronal depolarisation (Lu et al., 2012, Petrat et al., 2012, Tanabe et al., 2010). As glycine is an excitatory neurotransmitter in the developing brain, an elevated level and long exposure of glycine receptor agonists (glycine or taurine) might mediate damage. Blocking glycine receptor is found to be protective against ischaemic injury in the developing brain (Marret et al., 1999, Gellert et al., 2011). However, these studies used kynurenic acid or kynurenic acid analogue to block the glycine site of the NMDA receptor (Gellert et al., 2011, Marret et al., 1999). There has been no previous attempt to study the effect of blocking the strychnine-sensitive glycine-receptor during developmental white matter ischaemia. Electrophysiology revealed that strychnine blocked the depolarisation effect of glycine in nRON and prevented CAP suppression (Constantinou and Fern, 2009). My experiments studied the effect of blocking the strychnine-sensitive glycine-receptor on Fura-2-loaded P0 RON astrocytes.

7.3.2 Results:

7.3.2.1 Glycine Receptor Agonist (Glycine) Application:

Some cell death was observed during 100 μ M glycine perfusion with a normal oxygenated aCSF, which reached around $2.2\% \pm 1.7\%$ (2 of 89 cells, 5 nerves). However, this is not significantly different from cell death in the aCSF control ($2.2\% \pm 1.7\%$ compared to $4.5 \pm 1.4\%$, respectively; $p > 0.05$). On the other hand, glycine application in OGD solution reduces total cell death to $15.4\% \pm 4.7\%$ (27 of 165 cells, 5 nerves), and this reduction is highly significant compared to the OGD control ($15.4\% \pm 4.7\%$ compared to $56.5\% \pm 5.98\%$, respectively; $p < 0.05$).

An increase in $[Ca^{2+}]_i$ was documented before all cell death during glycine experiments, and representative cells are shown in Figure 7-11. A series of 360 images is shown (left) representing two astrocytes loaded with Fura-2 AM at different times during the experiments. At 0 minutes, multiple cells are present, two of which are selected and marked within circles (Cells 1 and 2). Following the progression of these two cells throughout the experiment suggests that Cell 1 persisted until the end of the experiment, which is consistent with the steady 360 intensity and 340/380 ratio; in comparison, Cell 2 disappeared at around 55 minutes, which was confirmed by the drop in 360 intensity (arrow) preceded by $[Ca^{2+}]_i$ (an increase in 340/380 ratio).

The percentage of cell death during each 10-minute interval of the experiment is plotted against time in Figure 7-12. Cells started to die at 40 minutes, followed by two waves (generations) of cell death: the first peak was around 50–60 minutes, while the larger peak occurred at around 80 minutes. Comparing this distribution with the OGD control suggests the role of glycine in postponing cell death from 20 minutes in the

OGD control to 40 minutes, and its role also in delaying the main peak of cell death from 40 minutes in the OGD control to 80 minutes.

7.3.2.2 Glycine Receptor Antagonist (Strychnine) Application:

Strychnine 2 μ M (a potent competitive glycine receptor antagonist) perfusion with aCSF shows some cell death, reaching $1.34\% \pm 0.9\%$ (2 of 149 cells, 6 nerves). Strychnine does not harm cells, as the percentage of cell death observed for strychnine does not differ significantly from that found in the aCSF control ($1.34\% \pm 0.9\%$ compared to $4.5 \pm 1.4\%$, respectively; $p < 0.05$). On the other hand, 2 μ M strychnine application in combination with OGD solution is found to produce around $10.8\% \pm 3.1\%$ cell death (17 of 146 cells, 6 nerves). Strychnine at 2 μ M significantly reduces the total percentage of cell death compared to the OGD control ($10.8\% \pm 3.1\%$ compared to $56.5\% \pm 5.98\%$, respectively; $p < 0.05$). A larger dose of strychnine of about 5 μ M was perfused with OGD solution and was found to produce $17.3\% \pm 8.0\%$ cell death (27 of 156 cells, 5 nerves). Application of 5 μ M strychnine with OGD significantly reduced the total percentage of cell death ($17.3\% \pm 8.0\%$ compared to $56.5\% \pm 5.98\%$, respectively; $p < 0.05$). However, 5 μ M strychnine does not produce more protection than a 2 μ M concentration ($17.3\% \pm 8.0\%$ compared to $10.8\% \pm 3.1\%$, respectively).

All cells that died during strychnine experiments exhibited an increase in $[Ca^{2+}]_i$ before the onset of death. Representative cells are shown in Figure 7-11. A series of 360 images was collected at different times during the experiment (left), and two cells (Cells 1 and 2) were circled on the initial image (0 minutes). Following these two cells during the experiment confirmed the persistence of Cell 1 throughout the 100-minute experiment, which is consistent with the steady 360 intensity and 340/380 ratio; Cell 2

disappeared at around 75 minutes, which was confirmed by the drop (arrow) in 360 intensity, which was preceded by a rise in $[Ca^{2+}]_I$ (increase in 340/380 ratio).

Focusing on the percentage cell death during each 10-minute interval showed that cells started to die at 40 minutes, followed by two peaks in cell death, the first at around 50–60 minutes, with the second around 80 minutes, as shown in Figure 7-14. Comparing the distribution of cell death in the OGD control with that for 2 μ M strychnine + OGD showed that strychnine helped to postpone cell death from 20 minutes in the OGD control to 40 minutes, and that it delayed the large peak of cell death from 40 to 80 minutes.

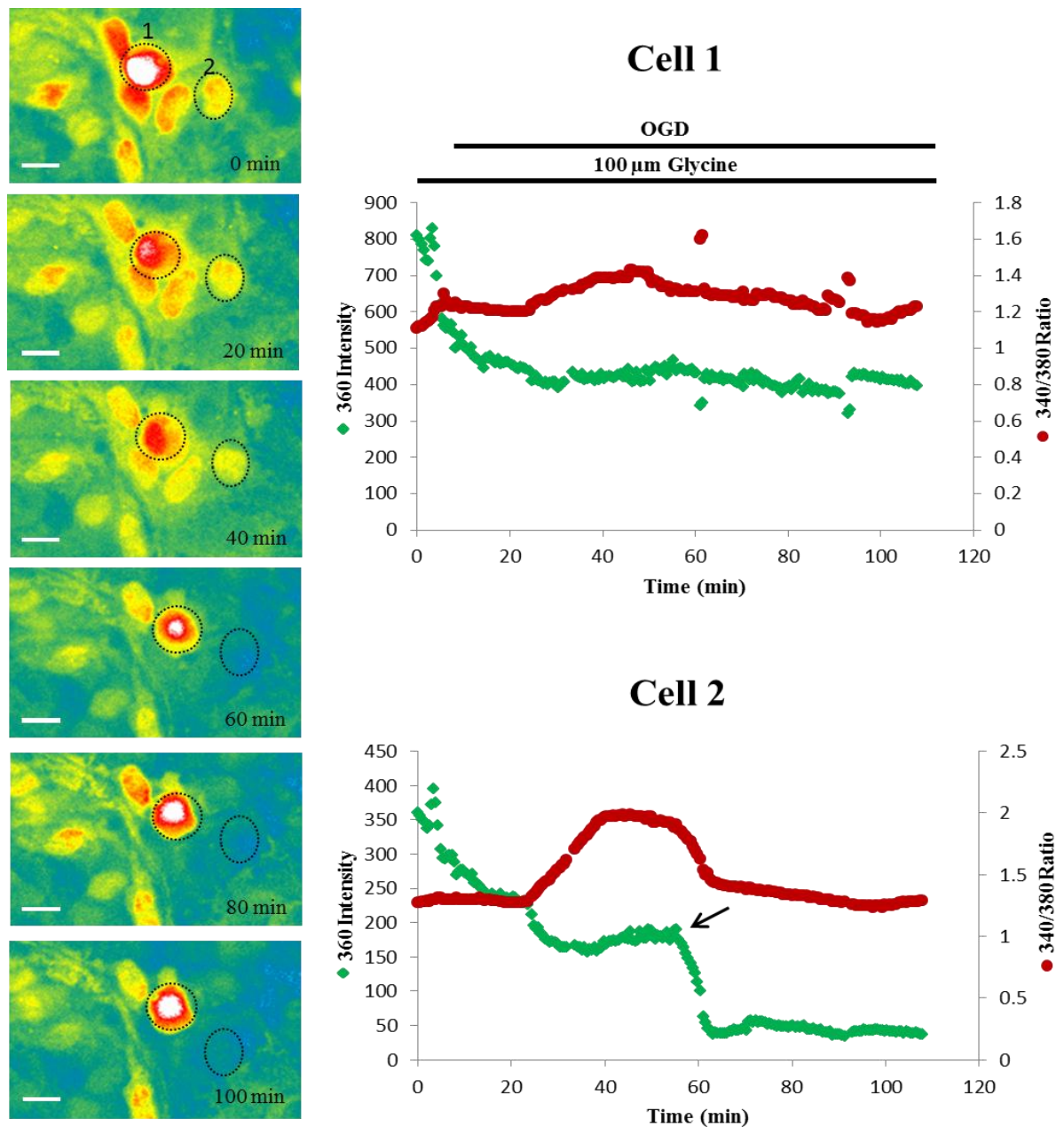


Figure 7-11: Representative data for Fura-2-loaded astrocytes in nRON during OGD + glycine.

On the left, a series of 360 nm images taken at different times shows clearly recognisable Cells 1 and 2 (circled). Cell 1 was stable throughout the 100-minute experiment (steady 340/380 ratio and 360 intensity); Cell 2 died at around 55 minutes, with a sudden drop in 360 intensity (arrow) preceded by calcium influx (elevation in 340/380 ratio).

Scale bar = 2 μ m.

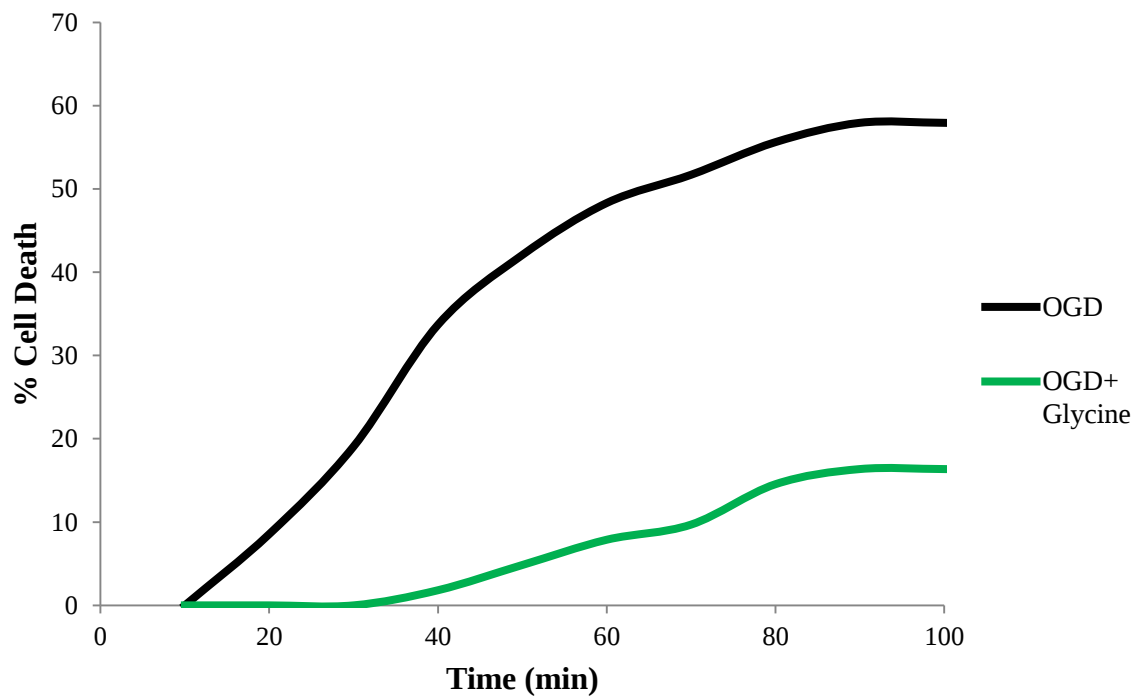


Figure 7-12: Percentage of cell death during OGD + 100 μ M glycine.

The percentage of cell death is plotted at 20-minute intervals. Note that cells started to die at a later onset of 40 minutes and reached a peak at around 80 minutes. N=5 experiments (5 nerves, 165 cells).

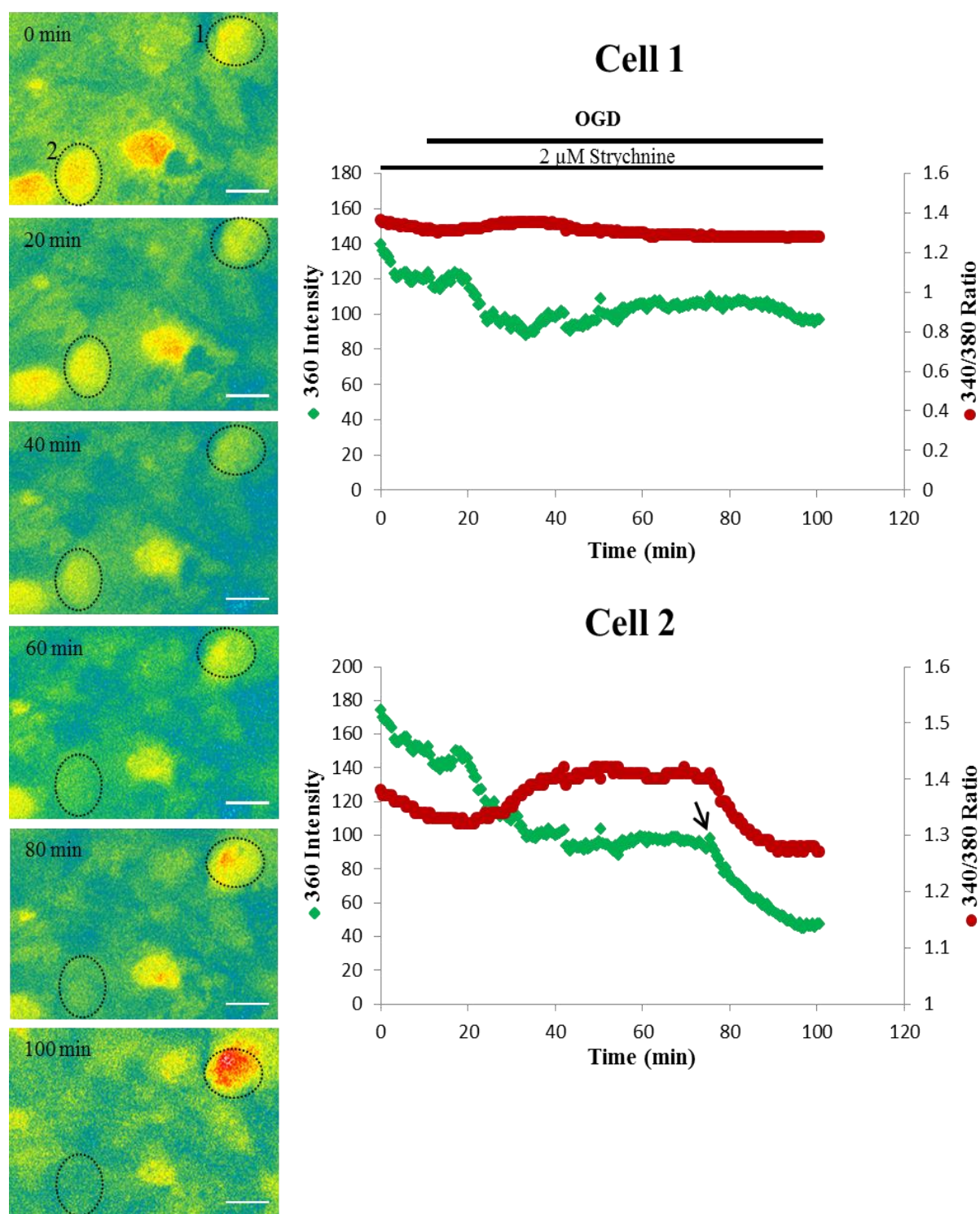


Figure 7-13: Representative data for Fura-2-loaded astrocytes in nRON during OGD + strychnine.

On the left, a series of 360 nm images taken at different times shows clearly recognisable Cells 1 and 2 (circled). Cell 1 was stable throughout the 100-minute experiment (steady 340/380 ratio and 360 intensity); Cell 2 died at around 75 minutes, with a sudden drop in 360 intensity (arrow) preceded by calcium influx (elevation in 340/380 ratio).

Scale bar = 2 μ m.

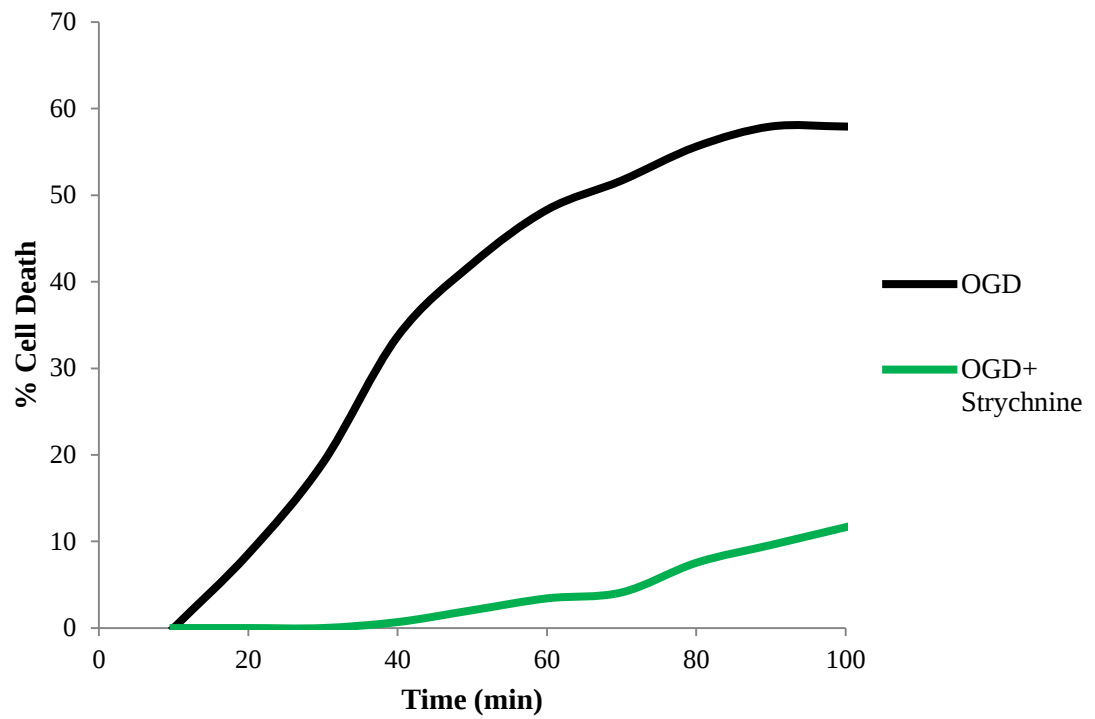


Figure 7-14: Percentage of cell death during 2 μ M Strychnine + OGD.

Percentage of cell death is plotted at 20-minute intervals. Note that cells started to die at a later onset of 40 minutes and reached a peak at 80 minutes. N=6 experiments (6 nerves, 146 cells).

7.3.2.3 A Combination of Strychnine and Picrotoxin:

A combination of 100 μM picrotoxin and 2 μM strychnine was examined during the OGD experiment, and cell death was found to reach $31.7\% \pm 7.4\%$ (53 of 167 cells, 5 nerves). Cell death associated with the combination of 100 μM picrotoxin and 2 μM strychnine was significantly lower than that observed for the OGD control ($31.7\% \pm 7.4\%$ compared to $56.5\% \pm 5.98\%$, respectively; $p < 0.05$), but the protective effect of the drugs was not additive.

All cells that died during the application of both 100 μM picrotoxin and 2 μM strychnine showed an increase in $[\text{Ca}^{2+}]_i$ just prior to the onset of death. A representative example of cell death is shown in Figure 7-15. Following Cells 1 and 2 throughout the 360 images, it appeared that Cell 1 persisted until the end of the 100-minute experiment, which is consistent with the steady pattern of 360 intensity and 340/380 ratio; Cell 2 died at around 45 minutes, as indicated by the drop in 360 intensity (arrow) preceded by $[\text{Ca}^{2+}]_i$ increase (represented by an increase in 340/380 ratio).

The distribution of cell death during the 100 μM picrotoxin and 2 μM strychnine OGD experiment was examined by plotting the percentage of cell death for each 10-minute interval of the experiment, as shown in Figure 7-16. Cells started to die at 30 minutes, and the distribution of cell death had a more random pattern, with multiple peaks at 30, 60 and 80 minutes. Comparing the onset of cell death of the OGD control and OGD combined with both picrotoxin and strychnine, at 10-minute intervals, shows that the combination of picrotoxin and strychnine postpones the initial onset of cell death from 20 minutes, as in OGD, to 30 minutes.

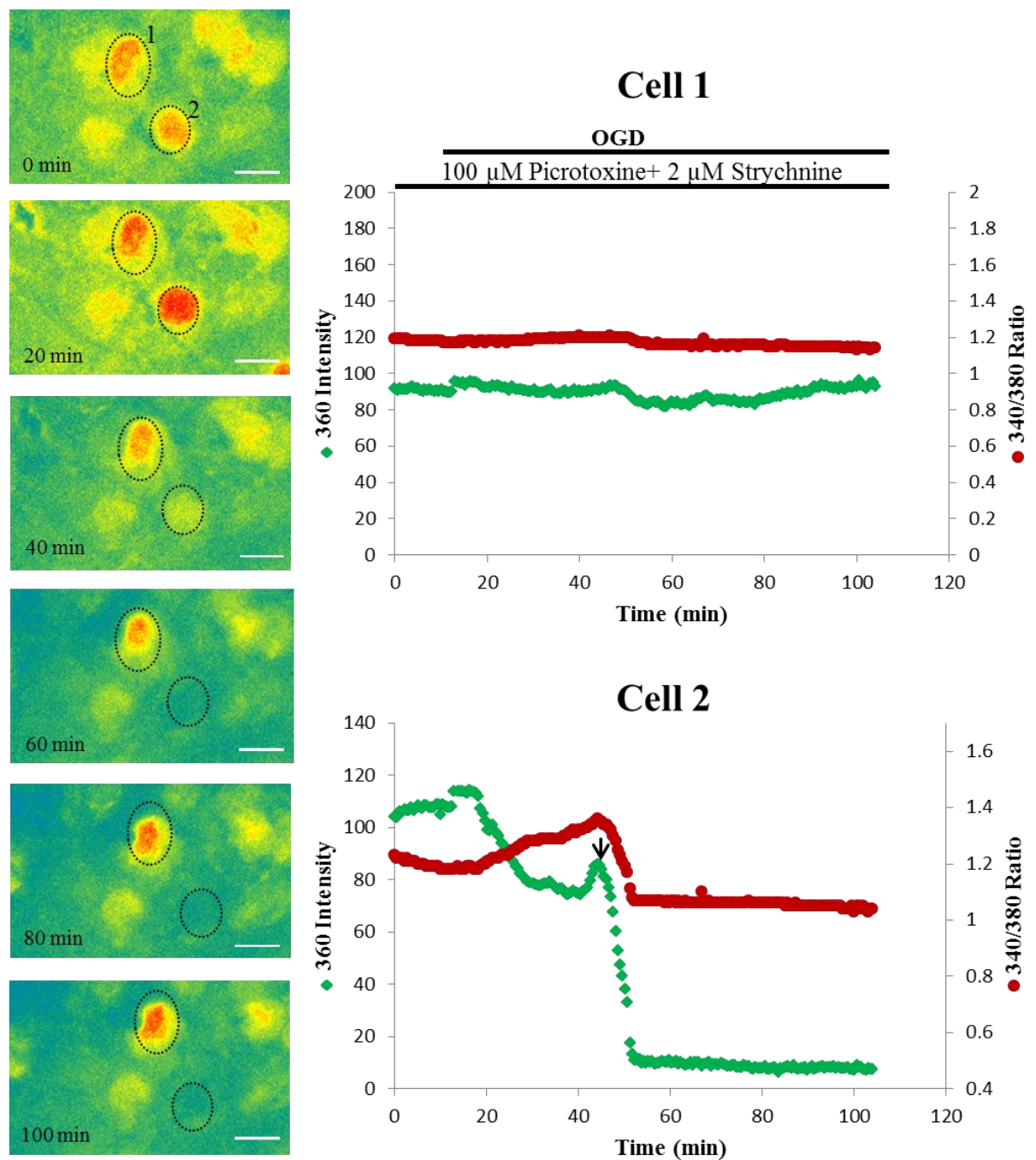


Figure 7-15: Representative data for Fura-2-loaded astrocytes in nRON during OGD + picrotoxin and strychnine.

On the left, a series of 360 nm nRON images taken at different times shows clearly recognisable Cells 1 and 2 (circled). Cell 1 was stable throughout the experiment (steady 340/380 ratio and 360 intensity); Cell 2 died at around 45 minutes, with a sudden drop in 360 intensity (arrow) preceded by calcium influx (elevation in 340/380 ratio).

Scale bar = 2 μ m.

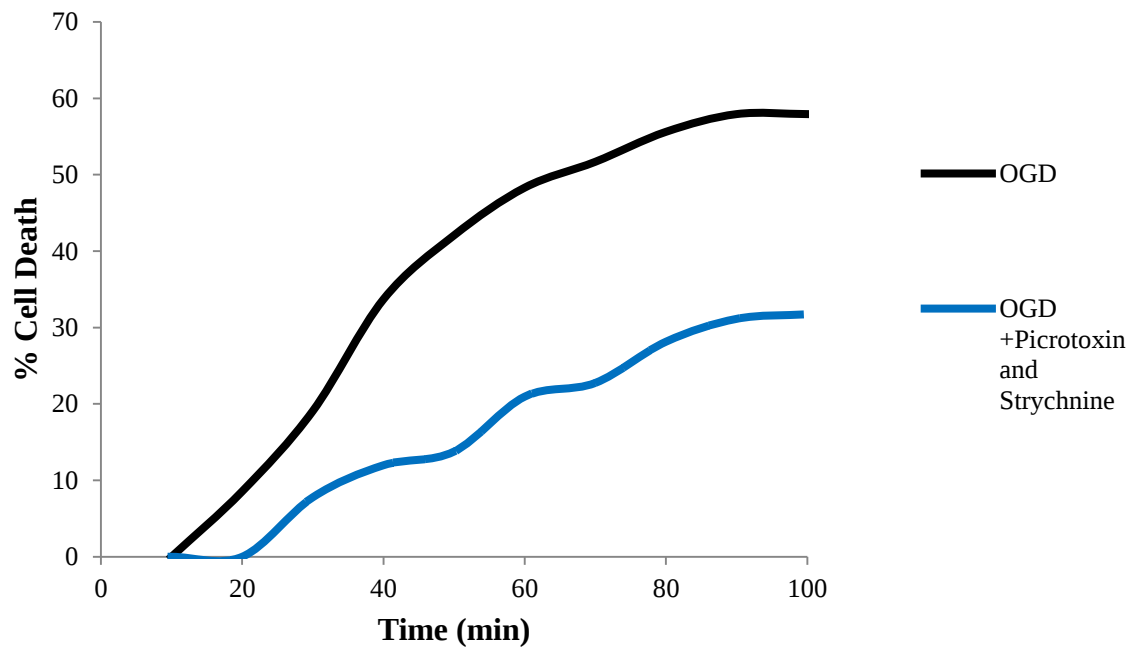


Figure 7-16: Percentage of cell death during OGD +100 μ M picrotoxin and 2 μ M strychnine.

Percentage of cell death is shown at 20-minute intervals. Note that cells started to die at 30 minutes, followed by a random pattern of cell death. N=5 experiments (5 nerves, 167 cells).

7.3.3 Discussion:

Multiple studies reported a glycine depolarisation effect in different parts of the CNS, such as cerebral cortex, spinal cord and optic nerve (Kilb et al., 2008, Constantinou and Fern, 2009, Pastor et al., 1995, Simmonds, 1983). In neonatal RON, glycine produced a significant reduction in CAP, which was reversed after washout (Constantinou and Fern, 2009). A patch clamp study revealed that all astrocytes in neonatal rat spinal cord depolarised after glycine application (Pastor et al., 1995). The extracellular level of glycine is elevated during ischaemia (Phillis and O'Regan, 2003, Kim et al., 2000, Phillis et al., 1997, Hagberg et al., 1985). Common carotid artery occlusion for 10–30 minutes was sufficient to increase the level of extracellular taurine (Hagberg et al., 1985). Blocking the glycine site of NMDA by kynurenic acid was protective during developmental brain ischaemia (Gellert et al., 2011, Marret et al., 1999). There has been no previous attempt to study the effect of blocking a strychnine-sensitive glycine-receptor on nRON.

In the present study, 100 μ M glycine application in aCSF for 100 minutes did not significantly alter the total percentage of cell death compared to an aCSF control. 100 μ M glycine application with OGD for 90 minutes, after 10 minutes in aCSF, significantly reduced the total percentage of cell death compared to an OGD control. This reduction in the responsiveness of the glycine receptor could be due to desensitisation, which is a known property of the glycine receptor (Wang and Lynch, 2011, Papke et al., 2011, Kraushaar and Backus, 2002). However, it is suggested that the rapid and dramatic changes in $[Cl^-]_i$ produced by glycine or GABA play a critical role in the apparent desensitisation (Karlsson et al., 2011). Studying the distribution of cell death of glycine in OGD revealed that glycine postpones initial cell death to 40 minutes compared to 20 minutes in OGD. Moreover, cells started to die at a low rate,

which gradually increased to peak at around 80 minutes rather than at 40 minutes, as observed in OGD. The representative cell death is shown in Figure 7-11, and it reveals that cell death was preceded by an increase in $[Ca^{2+}]_i$, which is consistent with the Ca^{2+} -dependent astrocyte death of P0 RON (Fern, 1998).

Strychnine has been used to antagonise the effect of glycine in different parts of the CNS, such as cerebral cortex, spinal cord and optic nerves (Belachew et al., 1998, Constantinou and Fern, 2009, Kawasaki et al., 2004). Electrophysiology of P10 RON revealed that 2 μ M strychnine prevented the reduction in CAP associated with glycine (Constantinou and Fern, 2009). In this study, the application of 2 μ M strychnine to aCSF solution produced no effect on astrocytes. However, 2 μ M strychnine significantly reduced the total percentage of cell death compared to the OGD control. A larger dose of strychnine of 5 μ M was applied to reveal any maximum effect threshold. However, 5 μ M was found to reduce the total percentage of cell death during OGD, but it had no additional effect over that of 2 μ M strychnine. Strychnine 2 μ M not only reduces the total percentage of cell death during OGD, but it also postpones the initial onset of cell death to 40 minutes rather than 20 minutes, as in OGD. Moreover, cells started to die at a very low percentage and peaked later, at 80 minutes instead of the 40 minutes observed in OGD. All cells that died during the strychnine experiment followed the Ca^{2+} -dependent cell death pathway, as shown in the representative data in Figure 7-13 (Fern, 1998).

It is known that strychnine is a potent competitive glycine receptor blocker. However, it has been shown that strychnine also competitively antagonises the $\alpha 7$ α -Btx-sensitive nicotinic receptor (Matsubayashi et al., 1998). To avoid any possibility that strychnine produced a protective effect due to a nicotinic antagonising effect, α -

bungarotoxin was tested during OGD in the next section. It has been argued that picrotoxin, a GABA_A antagonist, inhibits the glycine receptor ion channel as an allosteric antagonist for the glycine receptor (Bowery and Smart, 2006). However, it is well established that picrotoxin is useful in the case of homomeric glycine receptor (Bowery and Smart, 2006). P0 RON glial cells have been shown to heavily express β subunits (Domingues et al., 2010), which rules out the possibility of picrotoxin interference with P0 RON glycine receptor in this case.

It has been shown that GABA_A and glycine receptors can coexist in the same cell or synapse, such as in the spinal cord (Rajendra et al., 1997, Bohlhalter et al., 1994, Furuyama et al., 1992, Todd et al., 1996). This is thought to play some role in controlling their actions (Bohlhalter et al., 1994, Furuyama et al., 1992). As shown in this study, both 2 μ M strychnine and 100 μ M picrotoxin have a protective effect on P0 RON astrocytes during OGD. So it is possible that GABA_A and glycine receptors are localised in the same astrocyte and that therefore, a combination of both antagonists might be more potent than each of them separately. A combination of 2 μ M strychnine and 100 μ M picrotoxin was used to study the maximum protection level that can be achieved by these antagonists. This study revealed that the co-application of both 2 μ M strychnine and 100 μ M picrotoxin to P0 RON during OGD significantly reduced the total percentage of cell death. However, a combination of both antagonists did not produce any additional protection compared to the effect of each antagonist individually. In addition, the antagonist combination postponed initial cell death to 30 minutes compared to 20 minutes in OGD. It seems that there were two generations of cell death, with the first peak at 30 minutes and the next peak at 60 minutes. Again, a combination of both antagonists did not provide any advantages in terms of the distribution of cell death compared with using each antagonist separately. All cells that

died during OGD combined with picrotoxin and strychnine followed the Ca^{2+} -dependent cell death pathway, as previously established (Fern, 1998); see Figure 7-15. The difference in the results for the application of strychnine and picrotoxin separately and in combination might be due to the different mechanisms of action of each antagonist. A study of rabbit retinal ganglion cells suggested that strychnine and picrotoxin have an opposing action (Caldwell et al., 1978). There are no previous studies of the mechanism of strychnine or picrotoxin antagonist action in white matter. Therefore, the possibility of contrary actions of strychnine and picrotoxin on RON astrocytes cannot be excluded.

7.4 Role of Nicotine in Ischaemia:

7.4.1 Introduction:

Acetylcholine, a broadly distributed neurotransmitter in the CNS, binds to both nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs). NACHRs are found in the pre- and post-synaptic membrane and in extra-synaptic sites (Abreu-Villaca et al., 2011). They are permeable to such cations as Na^+ , K^+ and Ca^{2+} (Abreu-Villaca et al., 2011). Two types of nAChRs are identified: homomeric ($\alpha 7$ - $\alpha 9$) α -bungarotoxin nAChRs (α -BTX nAChRs), which are highly permeable to Ca^{2+} , and heteromeric ($\alpha 2$ - $\alpha 6$ and $\beta 2$ - $\beta 4$) non- α -BTX nAChRs, which have a lower permeability to Ca^{2+} than the α -BTX nAChRs (Fucile, 2004, Steinlein and Bertrand, 2008). In the mammalian brain, $\alpha 7$ is the predominant subtype of homomeric nAChRs, while it is $\alpha 4\beta 2$ in heteromeric nAChRs (Rahman et al., 2008). The distribution of nAChRs is expanded to non-neuronal areas such as immune cells, cancer cells, airway epithelial cells, urinary bladder cells, vascular endothelial cells, keratinocytes and reproductive organs (Kawashima and Fujii, 2008).

The optic nerve is made from glial cells and the extended axons of retinal ganglion (Bolton and Butt, 2005). It has been found that during postnatal development, a spontaneous action potential is generated from retinal ganglion cells and is propagated along the optic nerve, which plays a role in synaptic system development and functional maturation (Fawcett et al., 1984, Mooney et al., 1996, Shatz and Stryker, 1988, Sretavan et al., 1988). This retinal wave is known to be mediated by the cholinergic system during this period of development and subsequently is produced by the glutamatergic system (Feller et al., 1996, Penn et al., 1998, Bansal et al., 2000, Zhou and Zhao, 2000). More precisely, it has been found that nAChRs (especially $\alpha 3$

and $\beta 2$ nAChR subunits) are critical for retinal waves in the developmental period of mice between E16 and P11, while ionotropic glutamate receptor takes this role between P11 and P14 (Bansal et al., 2000). Acetylcholine is secreted from starburst amacrine cells (SACs) which binds to nAChRs of surrounding SACs and propagate a wave through gap junctions (Ford and Feller, 1995). All of this gives the cholinergic system a significant role in the development of the visual pathway and synaptic circuit.

NACHR subunits are detected in the rat CNS at early embryonic periods (Tribollet et al., 2004, Zoli et al., 1995). For example, $\alpha 4$ subunit mRNA is detected in the rat spinal cord as early as E11 and in the cerebral cortex at E17-19 (Zoli et al., 1995). $\beta 2$ subunit mRNA are homogenously distributed all over the CNS at E11-12 and slightly decline after E13-15 (Zoli et al., 1995). Rat $\alpha 7$ subunits are detected in hippocampus at E12 and in spinal cord at E16 (Tribollet et al., 2004). In the rat optic nerve, only the $\alpha 4$ subunit mRNA has been detected, showing moderate expression at E15, a low level at P0 and total absence at P4 (Zoli et al., 1995). However, it has been found that not only the $\alpha 4$ subunit is expressed in P0 RON glial cells but that there is also a low level of $\beta 2$ expression (Domingues et al., 2010). This is consistent with the expression mainly of $\alpha 4$ and $\beta 2$ subunits in P1 retina (Moretti et al., 2004). The nAChR subunits ($\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$) mRNA expression in RON glial cells increase with age, with some differences found in P11 and adults, as shown in (Domingues et al., 2010). However, astrocytes are found to respond to $\alpha 7$ nAChR activation and to be blocked by α -BTX application in hippocampus culture of new-born rats (Sharma and Vijayaraghavan, 2001). PCR and immunocytochemistry of P7 corpus callosum O2A progenitor cell culture have clarified that they express $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ nAChRs (Rogers et al., 2001). By using immunoprecipitation and radioligand binding, it has been observed that there is a wide range of nAChR subunits in adult

white matter, such as $\alpha 4$, $\alpha 6$, $\beta 2$ and $\beta 3$ subunits, and only low levels of $\alpha 2$, $\alpha 5$ and $\alpha 7$ nAChR subunits (Gotti et al., 2005, Cox et al., 2008).

Functional nAChRs have been examined in the neonatal CNS. It has been established that nAChR activation in the pre- and post-synaptic membrane mediates Ca^{2+} influx (Abreu-Villaca et al., 2011). However, it has been found that nAChRs are also able to mediate Ca^{2+} influx in non-synaptic areas, as demonstrated in the developing frog optic tectum (Edwards and Cline, 1999). Moreover, nAChR activation through nicotine application evokes Ca^{2+} influx in dye-loaded axons of developing RON (Zhang et al., 2004). This Ca^{2+} influx in P2–P6 nRON produced by nAChR activation was blocked by d-tubocurarine (curare) and mecamylamine, but not by α -BTX, which ruled out the possibility of the presence of functional $\alpha 7$ nAChRs in nRON axons (Zhang et al., 2004). This is consistent with the electrophysiological studies which revealed that nAChR activation suppresses the CAP and it shows the blocking effect of mecamylamine to nAChRs of P10 RON (Constantinou and Fern, 2009). It has been suggested that Ca^{2+} influx mediated by nAChR activation in nRON is produced through a direct activation of axonal nAChRs but not glial cells (Zhang et al., 2004). However, this suggestion results from examining the ability of glial neuroactive substances (ATP and glutamate) to mediate Ca^{2+} influx in nRON axons. It was found that an axonal Ca^{2+} influx following application of BzATP and ATP, both ATP analogues, and kainite, a glutamate analogue, was significantly less than that produced by nicotine (Martin, 1992, Zhang et al., 2004). It is suggested that this is mediated by two independent pathways: through differences in membrane conductance produced by Ca^{2+} influx, mainly through nAChRs and not VGCC, and through a suppression of axonal excitability mediated by cation influx (Zhang et al., 2004). By contrast, it has been suggested that glial cells have a role in mediating axonal conduction failure after

prolonged exposure to nicotine (Constantinou and Fern, 2009). Electrophysiology has revealed that nAChR activation suppresses the CAP and produces a non-reversible conduction block (Constantinou and Fern, 2009). Electron microscope examination of P10 RON after prolonged exposure (80 minutes) of nicotine have revealed severe oligodendrocyte and astrocyte injury, while axons morphology was preserved (Constantinou and Fern, 2009). This suggests that glial cells are the underlying cause of the non-reversible conduction block. Nicotine affects the nRON CAP in a dose-dependent manner, in which 50 μ M has the highest effect as compared to lower doses (Zhang et al., 2004). This is consistent with the nicotine dose-dependent effect of fluo-3 AM loaded cultured cortical astrocytes (Oikawa et al., 2005). Moreover, it is found that a Ca^{2+} influx mediated by nAChRs is attenuated with age, in which the Ca^{2+} response at P1 is 10 times greater than that at P30 (Zhang et al., 2004).

It is suggested that glial cells release ACh as they have choline acetyltransferase (Wessler et al., 1997, Lan et al., 1996). Moreover, glial cells express nAChRs, as reported, in astrocytes (Sharma and Vijayaraghavan, 2001, Oikawa et al., 2005) and in corpus callosum O-2A progenitor cells (Rogers et al., 2001). Functional nAChRs have been examined in hippocampal and cortical astrocytes, in which $\alpha 7$ nAChRs had played a role (Sharma and Vijayaraghavan, 2001, Oikawa et al., 2005). A whole cell voltage clamp study on hippocampal astrocytes revealed a current following the application of ACh, a nAChR agonist, and of atropine, a mAChR antagonist (Sharma and Vijayaraghavan, 2001). This current was completely blocked by $\alpha 7$ nAChR antagonists, which are methyllycaconitine (MLA) and α -BTX (Sharma and Vijayaraghavan, 2001). Neonatal hippocampal astrocytes loaded with fluo-3 dye revealed a rise in $[\text{Ca}^{2+}]_i$ after nAChR activation, which was completely abolished by MLA and α -BTX (Sharma and Vijayaraghavan, 2001). The source of the Ca^{2+} rise

inside the hippocampal astrocytes is suggested to be from a Ca^{2+} influx through permeable nAChRs, which trigger Ca^{2+} release from intracellular sources (Sharma and Vijayaraghavan, 2001). By contrast, it is suggested that a Ca^{2+} rise in embryonic (E19) cortical cultured astrocytes is via Ca^{2+} influx through L-type VGCC but not via Ca^{2+} release from cytosolic sources (Oikawa et al., 2005). The nAChR activation of embryonic cortical astrocytes increases the $[\text{Ca}^{2+}]_i$ in fluo-3 loaded astrocytes, which is blocked by dihydro- β -erythroidine, an $\alpha 4\beta 2$ antagonist, and by MLA in addition to nifedipine, an L-type VGCC blocker, and by EGTA, a free Ca^{2+} chelator (Oikawa et al., 2005).

The link between nicotine exposure and CNS injury has been established in several studies. It has been shown that maternal smoking and early prenatal exposure to nicotine produces selective white matter deficits (Abdel-Rahman et al., 2005, Froen et al., 2002, Jacobsen et al., 2007). Microdialysis studies of striatum and brainstem of one-week-old piglets revealed that early exposure to nicotine during hypoxemia could injure the cell membranes and worsen the metabolism of the immature brain, which is represented by increased glycerol and lactate levels, respectively, in the extracellular space (Froen et al., 2002). It is known that the level of extracellular ACh is increased after continuous bilateral closure of common carotid arteries (Yamamuro et al., 1996). Electron microscopic studies of nRON reveal that long exposure to nicotine mediates glial cell injury, in which astrocyte injury is characterised by swollen mitochondria, diffuse vacuolisation, process dissolution, and cell membrane destruction, all of which resemble the features of glial ischaemic injury (Constantinou and Fern, 2009, Thomas et al., 2004, Wilke et al., 2004). It has been shown that astrocytes are more susceptible to injury after a long nicotine exposure than are oligodendrocytes (Constantinou and

Fern, 2009). There have been no previous attempts to study the effect of long nicotine exposure on white matter glial cells, especially astrocytes, during ischaemia.

7.4.2 Results:

7.4.2.1 NACHR Agonist (Nicotine) Application:

The cholinergic agonist nicotine (50 μ M) perfusion with aCSF produced a small percentage of cell death, at $4.9\% \pm 2.2\%$ (6 of 104 cells, 5 nerves). This percentage of cell death is not significantly different from the cell death during the aCSF control ($4.9\% \pm 2.2\%$ as compared to $4.5 \pm 1.4\%$, respectively; $p>0.05$). On the other hand, 50 μ M nicotine perfusion with OGD solution produced $29.4\% \pm 7.3\%$ cell death (61 of 194 cells, 7 nerves). Nicotine did not significantly affect the total percentage of cell death during OGD ($29.4\% \pm 7.3\%$ as compared to $56.5\% \pm 5.98\%$, respectively; $p>0.05$).

All cells that died during the nicotine experiment experienced a rise in $[Ca^{2+}]_i$ just before the onset of cell death. Figure 7-17 represents an example of cell death during the 100-minute nicotine experiment. A series of 360 images was taken every 20 minutes during the experiment, representing some Fura-2 AM loaded astrocytes. Two astrocytes (Cell 1 and Cell 2) were chosen in the 0-minute 360 image. Following both cells at different times, it appears that Cell 1 died at around 80 minutes, confirmed by the drop in 360 intensity (arrow), which was preceded by a rise in $[Ca^{2+}]_i$ (a rise in 340/380 ratio), while Cell 2 persisted until the end of the experiment, which was consistent with the steady pattern of 360 intensity and the 340/380 ratio.

Focusing on the distribution of cell death during the nicotine experiment shows that cells started to die as early as 20 minutes into the experiment and reached a peak at around 40 minutes; see Figure 7-18. It seems that nicotine did not affect the distribution of cell death as compared to the OGD control, as both of them show an early cell death at around 20 minutes, with cell death peak at around 40 minutes.

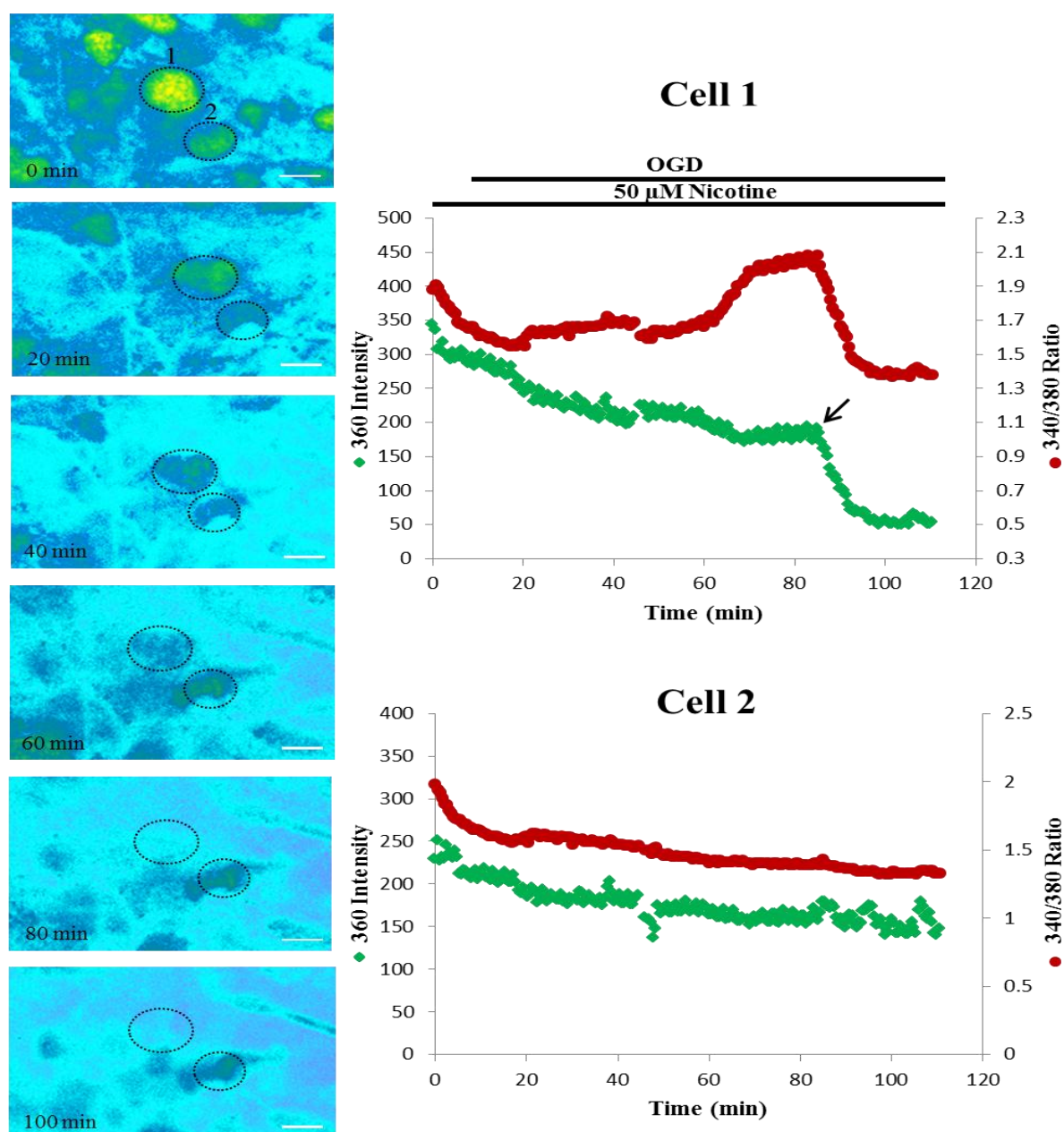


Figure 7-17: Representative data of Fura-2 loaded astrocytes in nRON during OGD + nicotine.

On the left, a series of 360 nm images taken at different times clearly show recognisable Cells 1 and 2 (in circles). Cell 1 died at around 80 minutes, with a sudden drop in 360 intensity (arrow) preceded by calcium influx (elevation in 340/380 ratio), while Cell 2 was stable throughout the 100-minute experiment (steady 340/380 ratio and 360 intensity).

Scale bar = 2 μ m.

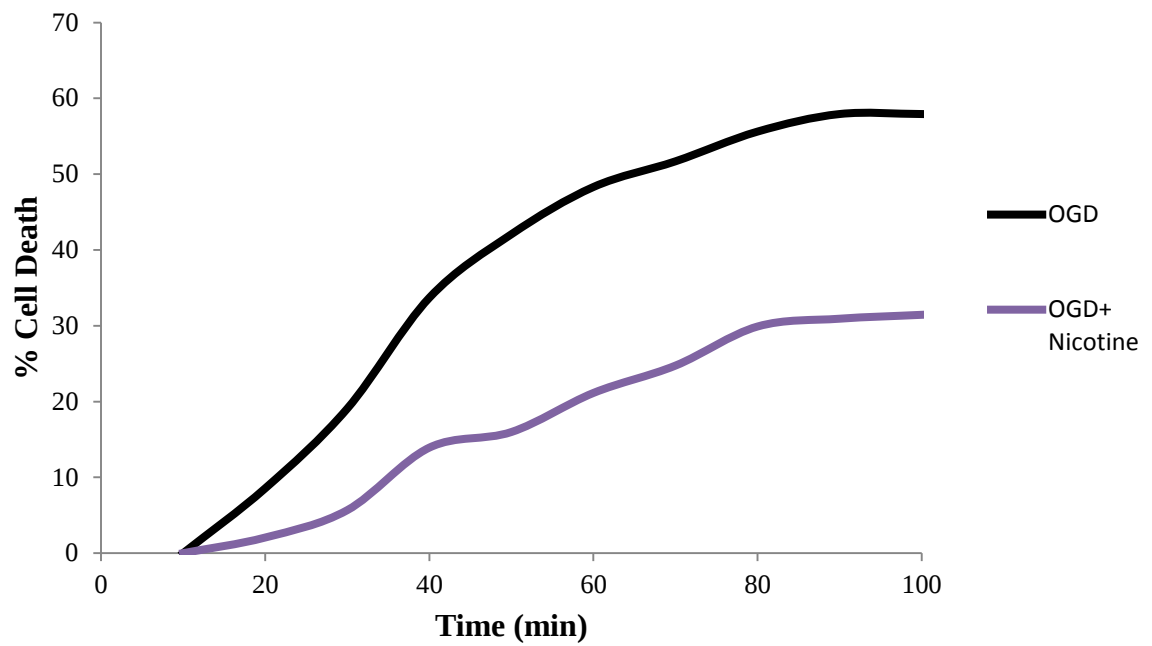


Figure 7-18: Percentage of cell death during 50 μ M nicotine + OGD.

The percentage of cell death for each 20 minutes of the experiment is plotted against time. Note that cells start to die at 20 minutes and reach a peak at 40 minutes. N=7 experiments (7 nerves, 194 cells).

7.4.2.2 NACHR Antagonist (Mecamylamine) Application:

Perfusion of the cholinergic nicotinic antagonist mecamylamine (10 μ M) with aCSF exhibits some cell death, at around $8.9\% \pm 8.2\%$ (11 of 136 cells, 5 nerves). However, this percentage of cell death does not differ from that achieved by aCSF control ($8.9\% \pm 8.2\%$ as compared to $4.5 \pm 1.4\%$, respectively; $p>0.05$). On the other hand, applying the same concentration of mecamylamine (10 μ M) with OGD solution produced a greater percentage of cell death, reaching around $51.2\% \pm 11.6\%$ (87 of 170 cells, 6 nerves). A more concentrated dose of mecamylamine (30 μ M) was used and produced a similar percentage of cell death to the previous dose of about $50.4\% \pm 10.4\%$ (102 of 202 cells, 6 nerves). However, the cell death produced by both 10 μ M and 30 μ M mecamylamine did not significantly differ from that mediated by OGD alone ($51.2\% \pm 11.6\%$ and $50.4\% \pm 10.4\%$, respectively, as compared to $56.5\% \pm 5.98\%$; $p>0.05$).

A rise in the $[Ca^{2+}]_i$ was documented with all cells that died during the mecamylamine experiments. Figure 7-19 shows an example of the viability of some of the cells during this experiment. Two Fura-2 AM loaded cells (Cell 1 and Cell 2) were selected in the 360 image at 0 minutes. Following these two cells through different times, we can conclude that Cell 1 persisted until the end of the experiment, which was consistent with the stable pattern of 360 intensity and 340/380 ratio, while Cell 2 died at around 40 minutes, demonstrated by the drop in 360 intensity (arrow), which is preceded by an increase in $[Ca^{2+}]_i$ (represented by a rise in the 340/380 ratio).

Figure 7-20 plots the percentage of cell death for every 10 minutes of the 10 μ M mecamylamine experiment against time, and the plot documents the beginning of cell death at around 20 minutes, reaching a peak at around 30 minutes. The distribution of

cell death throughout the 10 μ M mecamylamine experiment is quite similar to that of the OGD control, as both of them show an early cell death at 20 minutes. However, the application of 10 μ M mecamylamine seems to move the peak of cell death from 40 minutes in the OGD control to 30 minutes in this experiment.

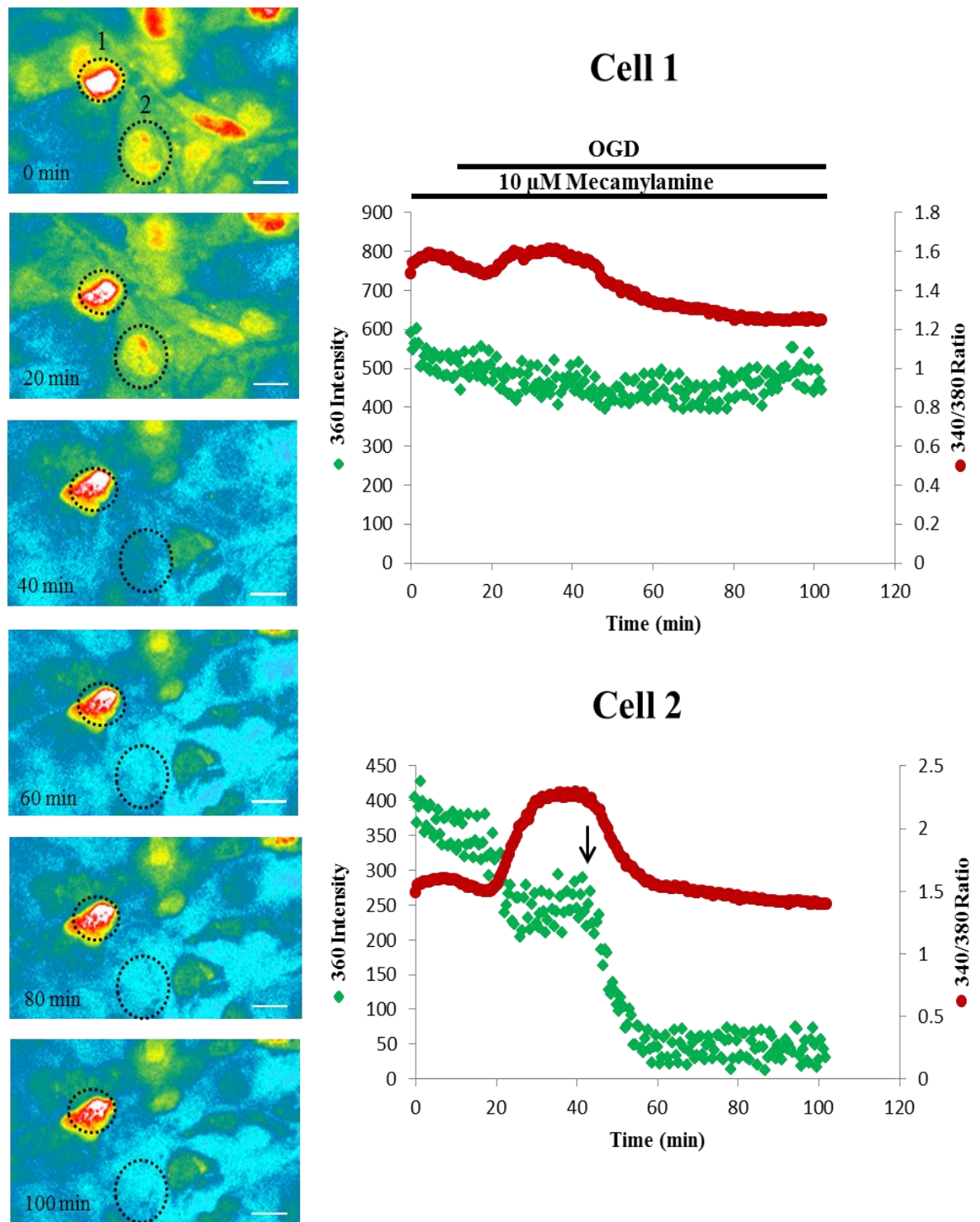


Figure 7-19: Representative data of Fura-2 loaded astrocytes in nRON during OGD + mecamylamine.

On the left, a series of 360 nm images taken at different times which clearly show Cells 1 and 2 (in circles). Cell 1 was stable throughout the 100-minute experiment (steady 340/380 ratio and 360 intensity), while Cell 2 died at around 40 minutes, with a sudden drop in 360 intensity (arrow) preceded by a calcium influx (elevation in 340/380 ratio). Scale bar = 2 μ m.

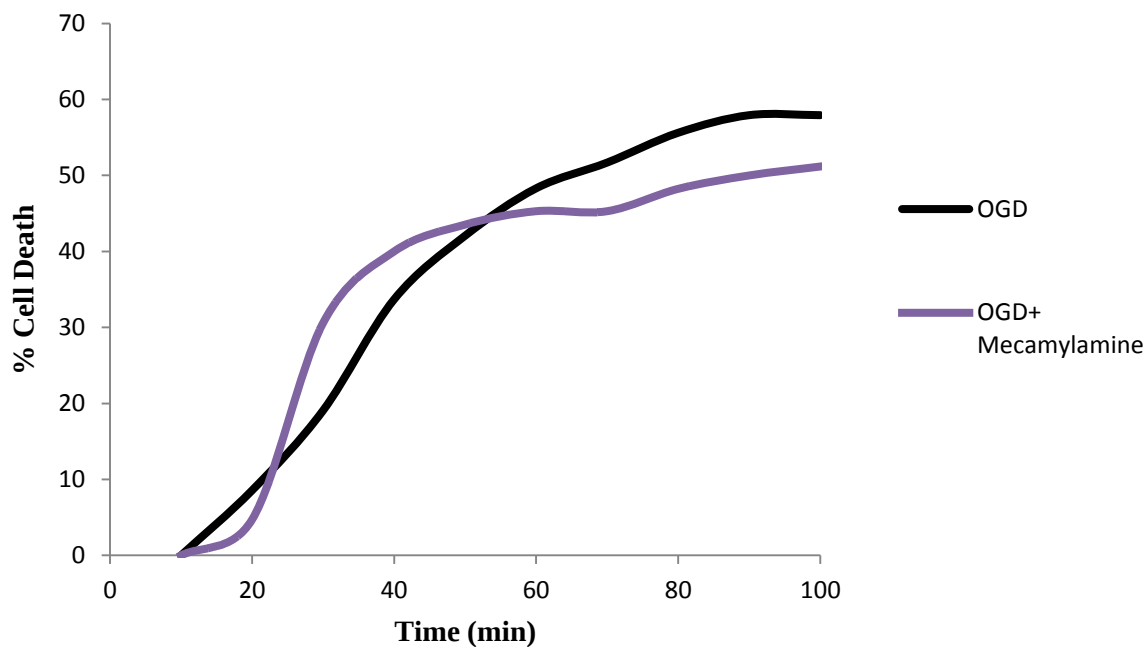


Figure 7-20: Percentage of cell death during 50 μ M Mecamylamine + OGD. The percentage of cell death for each 20 minutes of the experiment is plotted against time. Note that cells started to die at 20 minutes and reached a peak at 30 minutes. N=6 experiments (6 nerves, 170 cells).

7.4.2.3 NACHR Antagonist (α -bungarotoxin) Application:

Perfusion of a high affinity nicotinic blocker α -bungarotoxin (100 nM) with aCSF produced a small percentage of cell death, calculated as $1.9\% \pm 1.2\%$ (3 of 173 cells, 5 nerves). Bungarotoxin did not harm the cells, as the total percentage of cell death produced by bungarotoxin in aCSF does not significantly differ from that of the aCSF control ($1.9\% \pm 1.2\%$ as compared to $4.5 \pm 1.4\%$, respectively; $p>0.05$). On the other hand, perfusion of 100 nM α -bungarotoxin with OGD solution produced around $59.5\% \pm 8.2\%$ cell death (117 of 195 cells, 6 nerves) through the 100-minute experiment. The total cell death during bungarotoxin in OGD is not significantly different from that of the OGD control ($59.5\% \pm 8.2\%$ as compared to $56.5\% \pm 5.98\%$, respectively; $p>0.05$).

All cells that died during the bungarotoxin experiment exhibited a rise in $[Ca^{2+}]_i$ immediately before the onset of death. An example is shown in Figure 7-21, representing two Fura-2 AM loaded cells (Cell 1 and Cell 2). Following both cells through a series of 360 images shows that Cell 1 died at around 50 minutes, demonstrated by the drop (arrow) in 360 intensity which is preceded by a rise in $[Ca^{2+}]_i$ (a rise in 340/380 ratio), while Cell 2 existed until the end of the experiment, consistent with the stable 360 intensity.

Cells started to die in the first 10 minutes of the OGD + bungarotoxin experiment, followed by a wave of cell death which reached a peak at 50 minutes, as shown in Figure 7-22. It seems that bungarotoxin does not improve the distribution of cell death during OGD as compared to the OGD control, as both of them had an early onset of cell death (10 and 20 minutes, respectively) and an early peak of cell death (50 and 40 minutes, respectively).

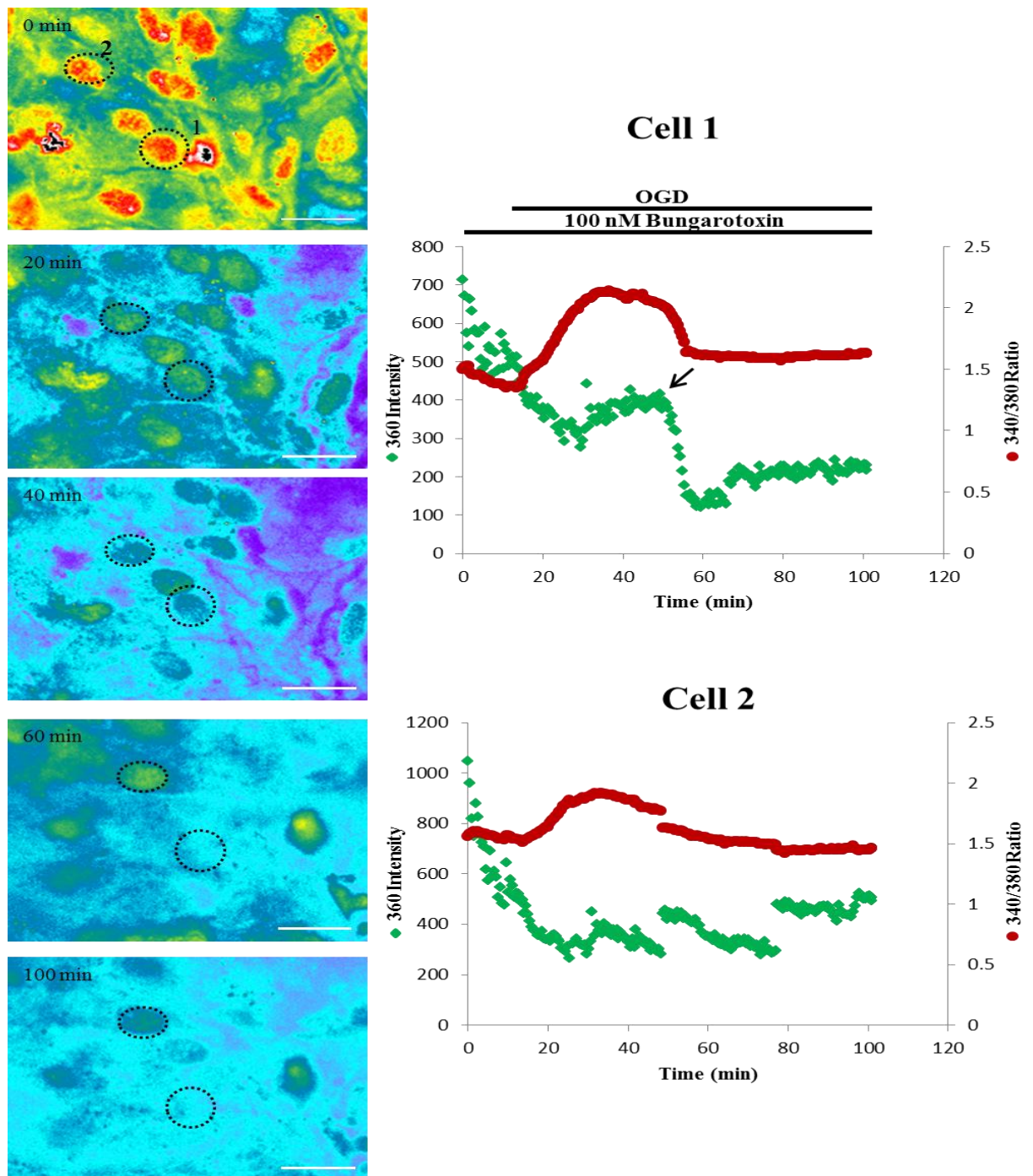


Figure 7-21: Representative data of Fura-2 loaded astrocytes in nRON during OGD + bungarotoxin.

On the left, a series of 360 nm images taken at different times, clearly showing Cells 1 and 2 (in circles). Cell 1 died at around 50 minutes with a sudden drop in 360 intensity (arrow) preceded by a calcium influx (elevation of 340/380 ratio), while Cell 2 was stable throughout the 100-minute experiment (steady 340/380 ratio and 360 intensity). Scale bar = 2 μ m.

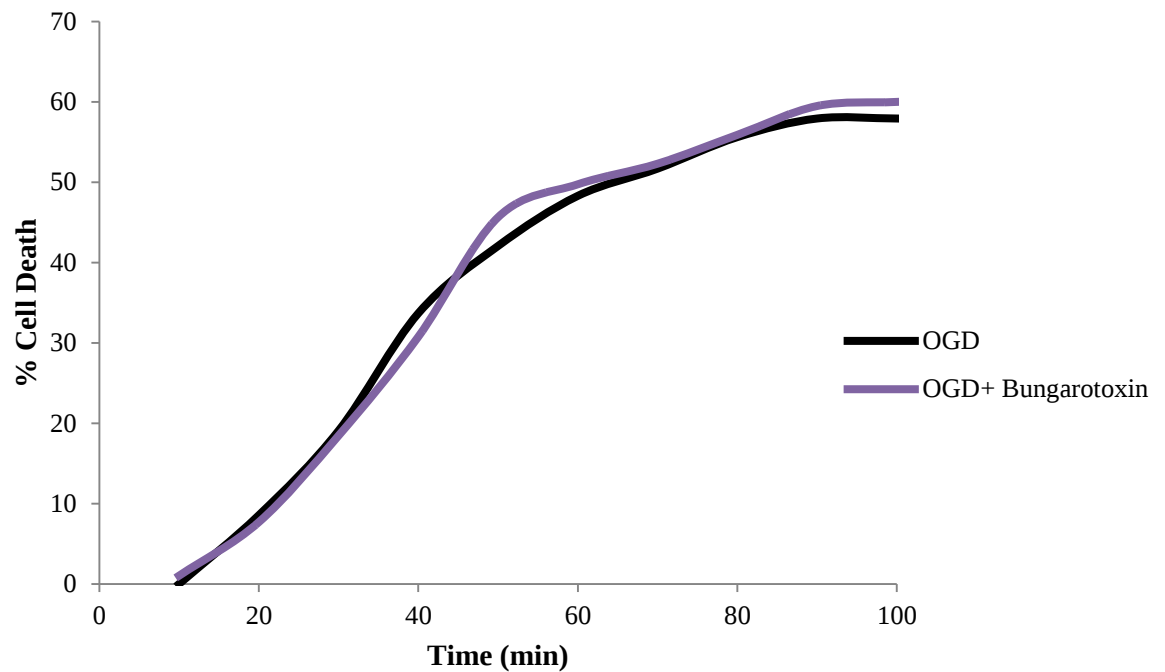


Figure 7-22: Percentage of cell death during 100 nM bungarotoxin + OGD. The percentage of cell death for each 20 minutes of the experiment is plotted against time. Note that cells start to die during the first 10 minutes, with death gradually increasing with time, to reach a peak at 50 minutes. N=6 experiments (6 nerves, 195 cells).

7.4.3 Discussion:

NAChRs are widely distributed in mammalian CNS (Swanson et al., 1987, Tribollet et al., 2004, Zoli et al., 1995). NAChR subunits are detected in neonatal and adult RON (Cox et al., 2008, Constantinou and Fern, 2009, Zoli et al., 1995). Functional nAChRs are examined in nRON (Constantinou and Fern, 2009, Zhang et al., 2004). It is found that activation of a nicotinic receptor produces a partial CAP block in nRON (Zhang et al., 2004, Constantinou and Fern, 2009). The response of nicotine, an nAChR agonist, is dose-dependent, with the greatest action found to be produced by 50 μ M (Zhang et al., 2004). Mecamylamine is effective in blocking the nicotine action in nRON, but not α -bungarotoxin (Constantinou and Fern, 2009, Zhang et al., 2004). However, this response of nicotine seen in RON is thought to be mediated by glial cells (Constantinou and Fern, 2009). Astrocytes represent around 70% of all cells present in P0 RON (Vaughn, 1969). It is found that astrocytes express nAChRs (Sharma and Vijayaraghavan, 2001, Sharma and Vijayaraghavan, 2002, Hosli et al., 2001). Hippocampal astrocytes express the α 7 subtype of nAChR, which is sensitive to α -Btx (Sharma and Vijayaraghavan, 2001, Sharma and Vijayaraghavan, 2002). On the other hand, α 4 and β 2 nAChR subunits mRNA were detected on glial cells of P0 RON (Domingues et al., 2010). However, mRNA expression does not reflect the presence of a functional receptor, but some data reveals that an increase in the ChAT mRNA level is followed by enzyme activity after 2 days (Holler et al., 1996). It has been shown that maternal smoking can produce white matter injuries (Abdel-Rahman et al., 2005, Froen et al., 2002, Jacobsen et al., 2007). Long exposure to nicotine mediates a conduction block and glial injuries which mimic ischaemic injuries (Constantinou and Fern, 2009, Zhang et al., 2004). ACh is known to rise in the extracellular space during ischaemia (Yamamuro et al., 1996). In the current study, we studied the effect of nAChR

activation on P0 RON astrocytes during ischaemia. Both $\alpha 4\beta 2$ nAChR antagonist (mecamylamine) and $\alpha 7$ nAChR antagonist (α -bungarotoxin) were tested on nRON astrocytes.

In the present study, nicotine perfusion with aCSF solution for 100 minutes did not significantly alter the total percentage of cell death compared to an aCSF control. NACHRs are ion channels that are expected to desensitise after exposure to an agonist such as nicotine (Yamodo et al., 2010, McNERNEY et al., 2000, Fenster et al., 1999b, Fenster et al., 1999a). However, 50 μ M nicotine perfusion with OGD solution for 90 minutes after 10 minutes in aCSF did not change the total percentage of cell death compared to an OGD control. Studying the distribution of cell death of OGD + nicotine revealed that it has a similar distribution to that of OGD alone, in which cells started to die as early as 20 minutes and peaked at 40 minutes.

Mecamylamine is a non-selective and non-competitive antagonist for nAChRs (Bacher et al., 2009). It is a potent blocker for $\alpha 4\beta 2$ and $\alpha 3\beta 2$ nAChR subunits but not for the $\alpha 7$ subunit (Papke et al., 2001a). It has been shown that 10 μ M mecamylamine blocks the Ca^{2+} influx mediated by nAChR activation in P2–P6 RON (Zhang et al., 2004). Moreover, electrophysiology clarified the blocking effect of 10 μ M mecamylamine to nAChRs in P10 RON (Constantinou and Fern, 2009). In the current study, 10 μ M mecamylamine perfusion for 100 minutes with aCSF solution did not alter the total percentage of cell death as compared to aCSF controls. 10 μ M mecamylamine perfusion with OGD solution for 90 minutes produced a large percentage of cell death, which is not significantly different from that of OGD alone. A larger dose of mecamylamine, of 30 μ M, was examined, and it revealed a similar cell death percentage to the 10 μ M mecamylamine. Studying the distribution of cell death

during OGD + 10 μ M mecamylamine revealed that early cell death started at 20 minutes and peaked at about 30 minutes. These results showed that mecamylamine did not help in protecting P0 RON astrocytes during ischaemia.

It has been well established that cortical and hippocampal astrocytes express functional nAChRs which are of the $\alpha 7$ homomeric type (Sharma and Vijayaraghavan, 2001, Oikawa et al., 2005). A whole cell-voltage clamp on neonatal hippocampal astrocytes revealed an astrocyte current produced following ACh application, which is blocked by α -BTX (Sharma and Vijayaraghavan, 2001). The high molecular weight of α -bungarotoxin does not interfere with crossing and binding to the receptor (Zhang et al., 2004). In the current study, 100 nM of α -BTX was perfused with aCSF for 100 minutes and produced a small percentage of cell death, which is not significantly different from that of the aCSF control. Moreover, α -BTX perfusion with OGD solution for 90 minutes produced a large percentage of cell death, which is similar to that of OGD alone. That showed that not only mecamylamine but also α -BTX did not protect P0 RON astrocytes from ischaemic injury. Representative cell death is shown following nicotine, mecamylamine, and α -BTX application, and revealed that cell death was preceded by an increase in $[Ca^{2+}]_i$, which is consistent with the Ca^{2+} -dependent astrocyte death of P0 RON (Fern, 1998).

To sum up, the current study showed that nicotine application did not mediate astrocyte injury when perfused with aCSF solution. This is could be due to nAChR desensitisation. However, nicotine application during OGD did not reduce the total percentage of cell death as expected in the case of receptor desensitisation. Neither mecamylamine nor α -BTX protects P0 astrocytes from ischaemic injury. These results suggest the absence of functional nAChRs in P0 RON astrocytes. This is consistent with the low level of nAChR subunit expression in P0 RON (Zoli et al., 1995,

Domingues et al., 2010). Immunoprecipitation studies reveal low levels of nAChR subunits at the P0 retina (Moretti et al., 2004). Immunoreactivity of the $\alpha 7$ nAChR subunit is shown to be absent in P0 neonatal cerebellum, while it starts to appear at P3 (delToro et al., 1997).

7.5 Role of Norepinephrine in Ischaemia:

7.5.1 Introduction:

Norepinephrine (noradrenaline) is a widely distributed neurotransmitter that binds to α_1 , α_2 , β_1 and β_2 adrenergic receptors (Odowd et al., 1989, Nicoll et al., 1990). Each subunit is subdivided into three groups: α_{1A} , α_{1B} , α_{1D} , α_{2A} , α_{2B} , α_{2C} , β_1 , β_2 and β_3 (Nicholas et al., 1996). It is well established that different types of adrenoceptors are expressed in white matter (Dawidek and Robinson, 1993, Happe et al., 2004, Venugopalan et al., 2006). An autoradiographic study revealed the presence of β_2 adrenoceptors in the human optic nerve (Dawidek and Robinson, 1993) and α_1 and β_2 receptors in the adult rat optic nerve (Mantyh et al., 1995). In the developing brain, autoradiography has revealed the expression of α_2 adrenoceptors in developing rat white matter, such as the anterior commissure, corpus callosum and cerebellar peduncle (Happe et al., 2004). Studying adrenoceptor development in the rat brain revealed the transient presence or decreased level of expression of α_2 adrenoceptors during the postnatal period (Happe et al., 2004). This is consistent with the autoradiography study that suggested the transient expression of α_2 adrenoceptors in the corpus callosum and anterior commissure of the developing brain (Sanders et al., 2005).

It has been found that white matter glial cells express both α and β adrenoceptors (Mantyh et al., 1995, Venugopalan et al., 2006, Domingues et al., 2010). Autoradiographic studies revealed that glial cells express α_1 and α_2 adrenoceptors in the white matter area of the adult rat spinal cord (Venugopalan et al., 2006). Immunohistochemistry suggested a colocalisation between GFAP astrocytes and α_2 adrenoceptors in the adult RON (Nikolaeva et al., 2009). Moreover, autoradiography and GFAP immunoreactivity revealed the expression of β_2 adrenoceptors by astrocytes

in the adult RON (Mantyh et al., 1995). PCR of different RON ages revealed glial cell mRNA expression of $\alpha 2$ and $\beta 2$ at P0 RON and $\alpha 1$, $\alpha 2$, and β at P11 and adult RON (Domingues et al., 2010).

Functional adrenoceptors have been studied in white matter. Autoradiography was used to measure the activity of G-protein coupling following NE application in the white matter of adult rat spinal cord (Venugopalan et al., 2006). This study revealed the role of NE in activating GTP γ S (guanosine 5'-O-(γ -[35S]thio)triphosphate) through activating α adrenoceptors, as this activation was blocked by phentolamine, an α adrenoceptor antagonist, but not by propranolol, a β adrenoceptor antagonist (Venugopalan et al., 2006). A similar technique was used in the corpus callosum and anterior commissure of developing rat brain, and it showed that epinephrine application activated GTP γ S binding, which was blocked by RX821002, an $\alpha 2$ adrenergic blocker (Sanders et al., 2005). Electrophysiological studies of P10 RON revealed that long NE exposure (80 minutes) produced a decline in CAP and a non-reversible conduction block (Constantinou and Fern, 2009). This effect of NE on P10 RON was blocked by phentolamine or a combination of a phentolamine and propranolol, but not propranolol alone, revealing the main role of α AR (Constantinou and Fern, 2009). Moreover, electron microscopic studies of P10 RON following 80 minutes of NE exposure revealed significant glial cell damage and intact axons (Constantinou and Fern, 2009). This suggests that glial cells underlie the irreversible conduction block following NE long exposure. Moreover, the effect of NE was studied in nRON (<P3), and showed that NE raised the CAP amplitude in a dose-dependent manner, in which 10^{-4} NE produced higher CAP amplitude than 10^{-6} NE (Honmou and Young, 1995). NE action on nRON was blocked by atenolol hydrochloride, a $\beta 1$ adrenoceptor blocker, but not by phentolamine, an α adrenoceptor blocker (Honmou and Young, 1995). Moreover,

application of isoproterenol hydrochloride, a β adrenoceptor agonist, raised the CAP amplitude in nRON, which was blocked by propranolol hydrochloride (Honmou and Young, 1995). It has been shown that application of methoxamine hydrochloride, an $\alpha 1$ adrenoceptor agonist, or clonidine hydrochloride, an $\alpha 2$ adrenoceptor agonist, had no effect on the CAP of nRON (Honmou and Young, 1995). These studies suggested that β adrenoceptors play a main role in nRON (<P3) axons, while α adrenoceptors play this role in nRON (P10) axons (Honmou and Young, 1995, Constantinou and Fern, 2009). The NE effect on nRON is diminished by eliminating $[Ca^{+2}]_e$ or applying Cd^{+2} (cadmium hydrochloride), which suggests the role of NE in mediating Ca^{2+} influx (Honmou and Young, 1995).

Functional adrenoceptors have also been studied in astrocytes. Adrenoceptors are known to stimulate multiple ion channels, such as Ca^{2+} and K^+ channels in astrocytes (Roy and Sontheimer, 1995, Salm and McCarthy, 1990). Therefore, adrenoceptor expression by astrocytes is likely, as astrocytes play an important role in extracellular ion homeostasis. NE is found to elevate the $[Ca^{2+}]_I$ in Fura-2 loaded astrocytes of cerebral culture via activation of $\alpha 1$ and/or $\alpha 2$ adrenoceptors (Salm and McCarthy, 1990). A patch clamp study of astrocyte/neuronal culture of neonatal (P1-P2) cerebral cortex reported an outward current following isoproterenol perfusion (Muyderman et al., 2001). Moreover, it has been noted that isoproterenol or clonidine perfusion mediates a rise in $[Ca^{2+}]_I$ that was blocked by eliminating extracellular Ca^{2+} , suggesting the important role of α and β adrenoceptors in astrocyte Ca^{2+} influx (Muyderman et al., 2001). NE perfusion was demonstrated to produce an intracellular Ca^{2+} current in a calcium-orange loaded astrocyte culture of adult hippocampus (Duffy and Macvicar, 1995). This Ca^{2+} signal also was found to be mediated by phenylephrine (PE), an $\alpha 1$ adrenoceptor agonist, but not by clonidine, and Ca^{2+} transients were

completely blocked by prazosin, an $\alpha 1$ adrenoceptor blocker, but not by yohimbine, an $\alpha 2$ adrenoceptor antagonist (Duffy and Macvicar, 1995). This study suggested that the PE-mediated $[Ca^{2+}]_I$ increase is more likely due to intracellular Ca^{2+} release than to Ca^{2+} influx, as eliminating the extracellular Ca^{2+} did not block the Ca^{2+} transients following PE application (Duffy and Macvicar, 1995).

It is well established that the level of extracellular NE increases during ischaemia (Bhardwaj et al., 1990, Globus et al., 1989, Perego et al., 1992, Richards et al., 1993, Uchihashi et al., 1998). In addition, it has been shown that long exposure to NE produces glial injury to P10 RON, which mimics the features of ischaemic injuries (Constantinou and Fern, 2009). This is consistent with the glial injury following oxidative stress produced by over-activation of adrenoceptors in rat brain culture (Khorchid et al., 2002). Electrophysiological studies of adult RON subjected to one hour of OGD and one hour of reperfusion revealed that NE application worsened CAP recovery, while reserpine, an irreversible vesicular monoamine transporter antagonist, and desipramine or nisoxetine, NE transporter antagonists, improved CAP recovery (Nikolaeva et al., 2009). Interestingly, application of $\alpha 2$ adrenoceptor agonists UK14,304 (5' -BROMO-6- [2-imidazolin-2-yl-amino] -quinoxaline) and medetomidine increased CAP recovery, as did the application of $\alpha 2$ blockers BRL44408 (2-[(4,5-dihydro- 1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1 H-isoindole) and atipamezole, indicating receptor desensitisation (Nikolaeva et al., 2009). The $[Ca^{+2}]_I$ level was increased in the Fluo-4 loaded axons of adult RON after 30 minutes of OGD and was blocked by nisoxetine, atipamezole and desipramine (Nikolaeva et al., 2009). Other studies demonstrated the protective effect of tetrodotoxin (TTX), a Na^+ channel inhibitor, by inhibiting NE release during spinal cord ischaemia (Sumiya et al., 2001, Uchihashi et al., 1998). However, there has been

no previous attempt to study the effect of adrenoceptor activation on white matter astrocytes. In this section, I study the effect of NE on Fura-2 AM -loaded nRON astrocytes and examine the effect of picrotoxin, a GABA_A antagonist, during 90 minutes of ischaemia.

7.5.2 Results:

7.5.2.1 Adrenergic Receptor Agonist (Norepinephrine) Application:

Perfusion of 100 μ M of adrenoceptors agonist norepinephrine with aCSF resulted in around $3.1\% \pm 2.9\%$ cell death (4 of 129 cells, 5 cells) in 100 minutes of experiments. Cell death produced during the norepinephrine aCSF experiments did not differ significantly from that produced during an aCSF control ($3.1\% \pm 2.9\%$ as compared to $4.5 \pm 1.4\%$, respectively; $p>0.05$). On the other hand, 100 μ M norepinephrine perfusion during OGD produced around $54.8\% \pm 7\%$ cell death (103 of 188 cells, 6 nerves). Cell death produced during norepinephrine in OGD did not differ significantly from that produced by OGD alone ($54.8\% \pm 7\%$ as compared to $56.5\% \pm 5.98\%$, respectively; $p>0.05$).

Cell death during the norepinephrine experiment experienced a rise in $[Ca^{2+}]_i$ just before the onset of death. Figure 7-23 presents an example of two Fura-2 AM loaded cells (Cell 1 and Cell 2). Following these two cells through different times in 360 images, it can be seen that Cell 1 died at around 50 minutes, indicated by the drop (arrow) in 360 intensity preceded by $[Ca^{2+}]_i$ influx, while Cell 2 existed throughout the experiment, consistent with the steady 360 intensity and 340/380 ratio.

The percentage of cell death is plotted every 10 minutes during the norepinephrine OGD experiment, as seen in Figure 7-24. The cells started to die at 20 minutes, followed by a wave of cell death, with peaks at 30 and 40 minutes. The distribution of cell death during the norepinephrine OGD experiment resembles the distribution in OGD alone, as both conditions show an early cell death at around 20 minutes, and the peak of cell death is found at around 40 minutes.

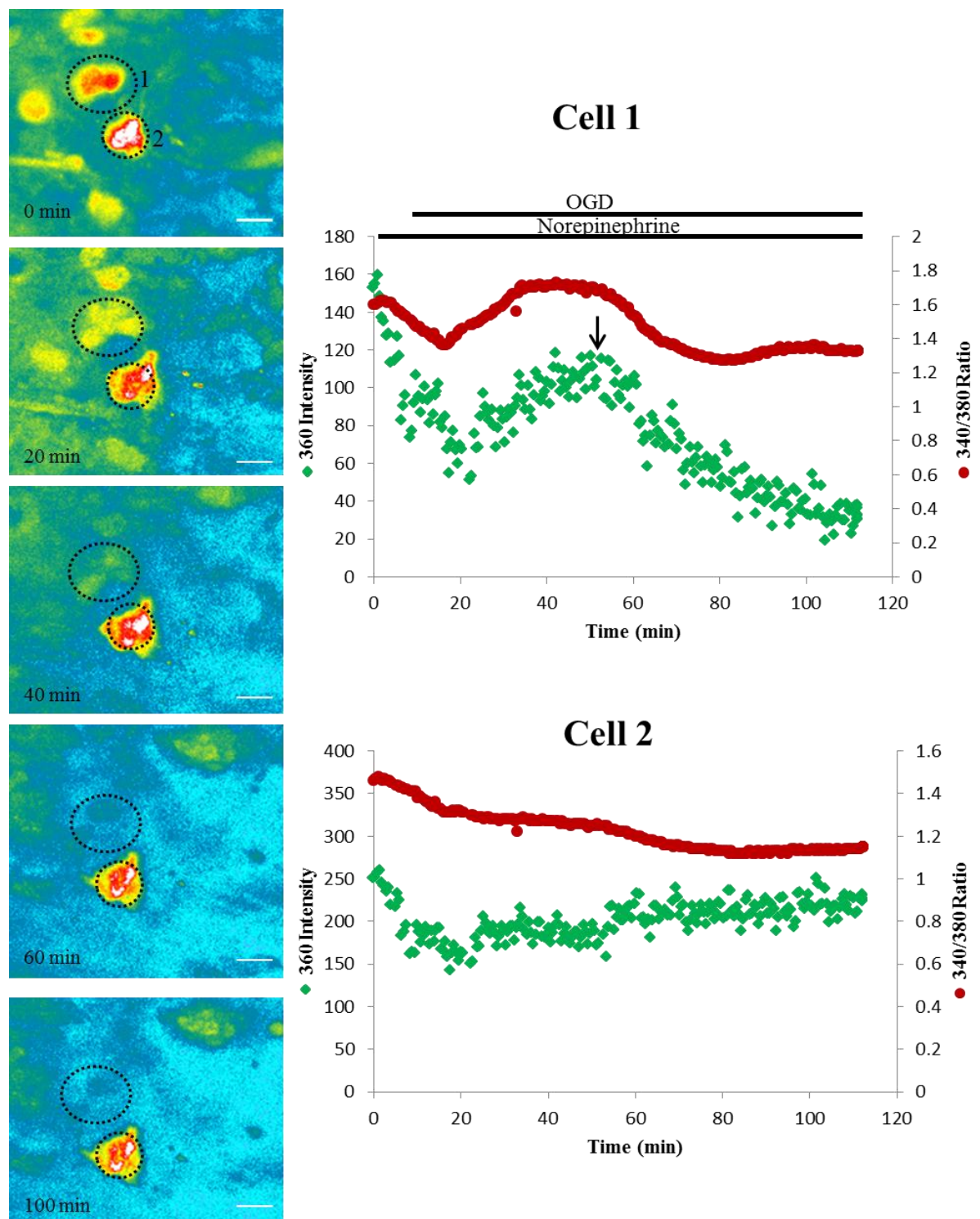


Figure 7-23: Representative data of Fura-2 loaded astrocytes in nRON during OGD + Norepinephrine.

On the left, a series of 360 nm images taken at different times, clearly showing Cells 1 and 2 (in circles). Cell 1 died at around 50 minutes with a sudden drop in 360 intensity (arrow) preceded by a calcium influx (elevation of 340/380 ratio), while Cell 2 was stable throughout the 100-minute experiment (steady 340/380 ratio and 360 intensity). Scale bar = 2 μ m.

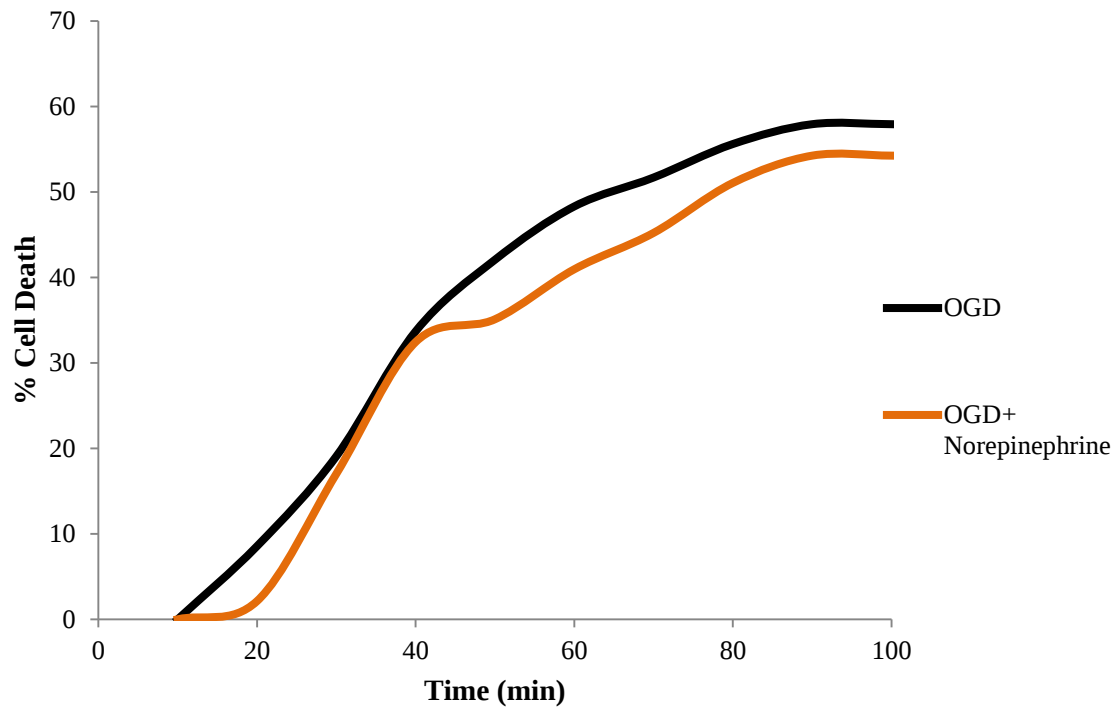


Figure 7-24: Percentage of cell death during 100 μ M Norepinephrine + OGD. The percentage of cell death for each 20 minutes of the experiment is plotted against time. Note that cells start to die at 20 minutes to reach a peak at 30 and 40 minutes. N=6 experiments (6 nerves, 188 cells).

7.5.2.2 Adrenergic Receptor Antagonist (Propranolol and Phentolamine)

Application:

A combination of an α adrenoceptor blocker, phentolamine (10 μ M), and a β adrenoceptor blocker, propranolol (10 μ M), was applied to aCSF, and it produced around $2.2\% \pm 1.3\%$ cell death (3 of 138 cells, 5 nerves). However, this percentage of cell death produced by phentolamine and propranolol does not differ significantly from that produced by aCSF control ($2.2\% \pm 1.3\%$ as compared to $4.5 \pm 1.4\%$, respectively; $p > 0.05$). On the other hand, phentolamine and propranolol perfusion during OGD produced around $41.17\% \pm 9.5\%$ (65 of 161 cells, 5 nerves) cell death. Cell death produced during norepinephrine in OGD did not differ significantly from that produced by OGD alone ($41.17\% \pm 9.5\%$ as compared to $56.5\% \pm 5.98\%$, respectively; $p > 0.05$).

Cells that died during phentolamine and propranolol experiment exhibited a rise in $[Ca^{2+}]_i$ before the onset of cell death. Figure 7-25 shows a series of 360 images collected during phentolamine and propranolol OGD experiment. At 0 minute, two Fura-2 AM cells were chosen (Cell 1 and Cell 2; circled). Following these two cells, it can be seen that Cell 1 died at around 50 minutes, indicated by the drop (arrow) in 360 intensity which is preceded by a rise in $[Ca^{2+}]_i$ (an increase in 340/380 ratio), while Cell 2 existed until the end of the experiment (100 minutes), shown by the stable 360 intensity and 340/380 ratio.

The percentage of cell death every 10 minutes during phentolamine and propranolol OGD experiment is plotted against time in Figure 7-26. During the phentolamine and propranolol OGD experiment, the cells started to die at 30 minutes, followed by a peak of cell death at around 40 minutes. It appears that phentolamine and propranolol postpone cell death to 30 minutes, compared with the 20 minutes found in

OGD alone. However, the peak of cell death is similar in the phentolamine and propranolol OGD experiments and the OGD control, found to be around 40 minutes.

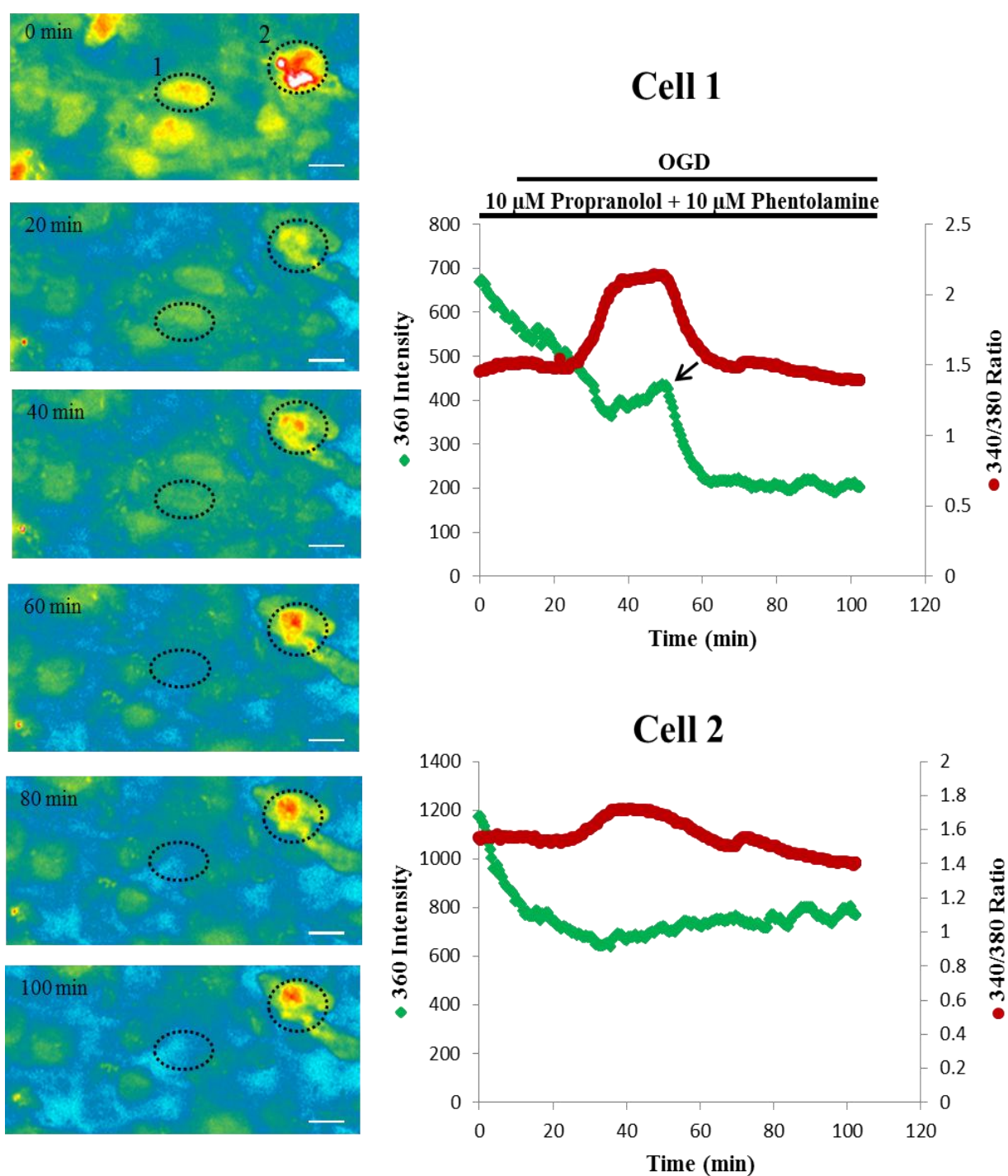


Figure 7-25: Representative data of Fura-2 loaded astrocytes in nRON during OGD + propranolol and phentolamine.

On the left, a series of 360 nm images taken at different times, clearly showing Cells 1 and 2 (in circles). Cell 1 died at around 50 minutes with a sudden drop in 360 intensity (arrow) preceded by a calcium influx (elevation of 340/380 ratio), while Cell 2 was stable throughout the 100-minute experiment (steady 340/380 ratio and 360 intensity) Scale bar = 2 μ m.

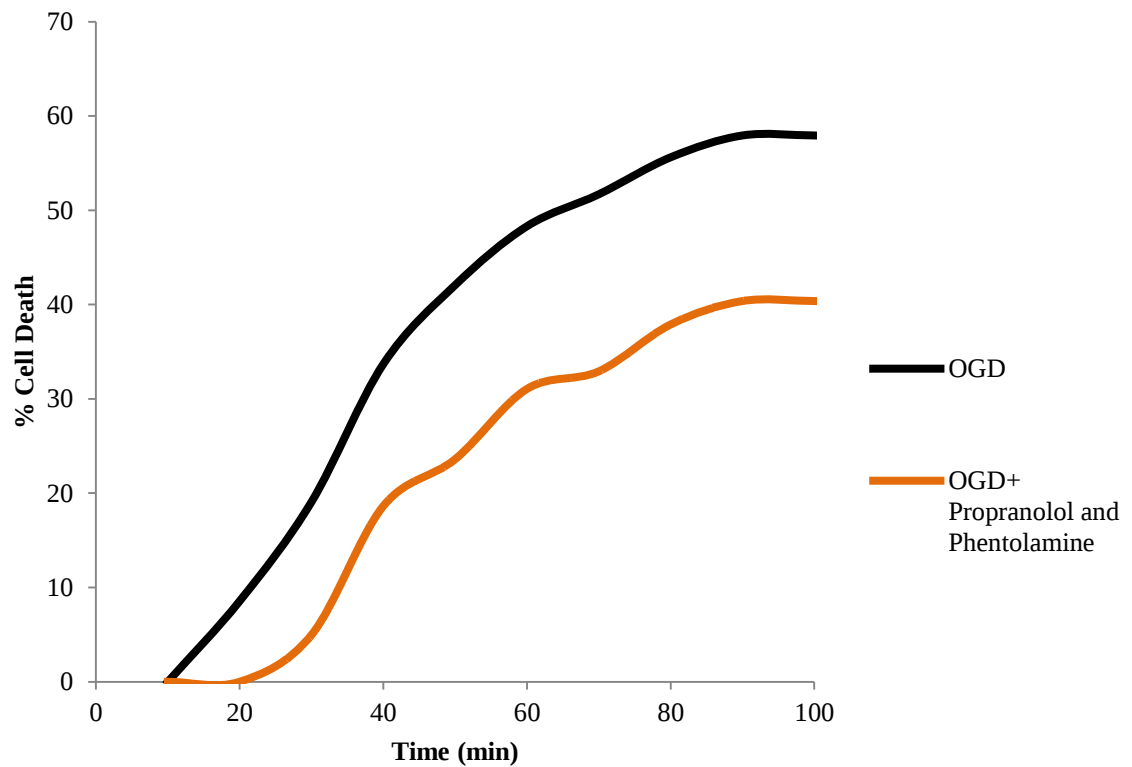


Figure 7-26: Percentage of cell death during 10 μ M propranolol +10 μ M phentolamine + OGD.

The percentage of cell death for each 20 minutes of the experiment is plotted against time. Note that cells start to die at 30 minutes to reach a peak at 40 minutes. N=5 experiments (5 nerves, 161 cells).

7.5.3 Discussion:

White matter is now known to express different types of adrenoceptors. Autoradiography has reported the expression of β_2 , α_1 , and α_2 adrenoceptors in different areas of white matter, such as the optic nerve, corpus callosum, anterior commissure and cerebellar peduncle (Dawidek and Robinson, 1993, Happe et al., 2004, Mantyh et al., 1995). However, adrenoceptors are transiently expressed during the postnatal period of developing white matter (Happe et al., 2004, Sanders et al., 2005). Astrocytes are shown to express α_1 , α_2 , and β adrenoceptors in white matter regions (Mantyh et al., 1995, Nikolaeva et al., 2009, Domingues et al., 2010). Functional adrenoceptors have been described in different regions of white matter (Honmou and Young, 1995, Sanders et al., 2005, Venugopalan et al., 2006, Constantinou and Fern, 2009) and astrocytes (Duffy and Macvicar, 1995, Muyderman et al., 2001, Salm and McCarthy, 1990). It has been established that the level of extracellular NE increases during ischaemia (Bhardwaj et al., 1990, Globus et al., 1989, Perego et al., 1992, Richards et al., 1993, Uchihashi et al., 1998). Other studies have described the effect of long NE exposure on conduction block and glial cell injuries (Constantinou and Fern, 2009, Khorchid et al., 2002). However, it has been shown that applying adrenoceptor blockers, such as propranolol and phentolamine, can inhibit the irreversible NE-mediated injury (Constantinou and Fern, 2009). During the recovery period after one hour of ischaemia, it has been shown that adrenoceptor blockers and NE transporter inhibitors can improve recovery outcomes (Nikolaeva et al., 2009). Na^+ channel inhibitors are shown to be protective if taken before the onset of ischaemia to limit NE release (Sumiya et al., 2001, Uchihashi et al., 1998).

In the present study, NE perfusion with aCSF solution for 100 minutes did not significantly alter the total percentage of cell death compared to an aCSF control. 90

minutes of 10 μ M NE perfusion with OGD solution after 10 minutes in aCSF produced a large percentage of cell death that is not significantly different from that of the OGD control. Studying the cell death distribution of the OGD + NE experiment revealed that it had a similar distribution to that of OGD alone, in which cells started to die as early as 20 minutes and peaked at 40 minutes.

Both propranolol, a non-selective competitive β ADR antagonist, and phentolamine, a non-selective competitive α ADR antagonist, have been used to block both α and β ADRs (Madison and Nicoll, 1986, Nicoll et al., 1990, Kang et al., 2000). Electrophysiology showed that propranolol blocked isoproterenol-induced excitability in (<P3) nRON (Honmou and Young, 1995). Moreover, atenolol has been also found to inhibit NE-mediated excitability in (<P3) nRON, while phentolamine has no significant effect (Honmou and Young, 1995). In P10 RON, it has been shown that propranolol, or a combination of propranolol and phentolamine, can block the irreversible conduction block, while phentolamine has no significant effect (Constantinou and Fern, 2009). Despite the insignificant role of α adrenoceptors in neonatal RON axons, some studies have revealed significant effects of both α and β adrenoceptors in astrocytes (Duffy and Macvicar, 1995, Muyderman et al., 2001, Salm and McCarthy, 1990). For this reason, I used a combination of propranolol and phentolamine in the current study. In these experiments, 10 μ M propranolol + 10 μ M phentolamine perfusion for 100 minutes with aCSF solution did not alter the total percentage of cell death compared to aCSF controls. 10 μ M propranolol + 10 μ M phentolamine perfusion with OGD solution for 90 minutes produced a large percentage of cell death, which did not differ significantly from that of OGD alone.

To sum up, the current study revealed that NE perfusion did not induce any astrocyte injury when perfused with aCSF for 100 minutes. One explanation could be receptor desensitisation. However, NE application during OGD did not reduce cell death as expected in the case of receptor desensitisation. Moreover, a combination of both propranolol and phentolamine did not protect the astrocytes from ischaemic cell death. These results suggested the absence of functional adrenoceptors in P0 RON astrocytes, which could be due to the transient presence of adrenoceptors and decreased level in the postnatal period (Happe et al., 2004, Sanders et al., 2005). However, these results did not exclude the NE action on P0 RON axons as it might be damaging for axons.

7.6 Ultrastructural Analysis of P0 RON Ischaemic Injury:

7.6.1 Introduction:

The morphology of the optic nerve, a model for axon-glial interactions, has been intensively studied at the ultrastructural level (Hildebrand and Waxman, 1984, Peters and Vaughn, 1967, Skoff et al., 1976a, Skoff et al., 1976b, Skoff et al., 1980, Tennekoon et al., 1977, Vaughn, 1969, Waxman et al., 1992, Butt et al., 1994c). In the early post-natal period, axons are approximately 0.2–0.3 μm in diameter, unmyelinated and separated into fascicles by glial cell processes (Peters and Vaughn, 1967). In transverse sections of optic nerve, axons appear as circles filled with microtubules, mitochondria and few neurofilaments (Peters and Vaughn, 1967). Different types of glial cells have been documented in the neonatal optic nerve, such as mitotic cells, glioblasts (small glioblasts, large glioblasts and large glial precursors), astrocytes (immature, young and mature), oligodendrocytes (young, active and mature) and microglia (Skoff et al., 1976a, Vaughn, 1969). An electron microscopic quantitative study reported a relative proportion of different glial cell types at different ages in which P0 RON has approximately 70% astrocytes, 25% large glial precursor cells and 5% small glioblasts (Vaughn, 1969). Astrocytes are characterised by their stellate shape, homogeneous nuclei and dense cytoplasm, which contain glial filaments, ER filled with flocculent dense material, and oval opaque bodies (Vaughn, 1969). They send their processes to contact blood vessels, pia and nodes of Ranvier by their end feet, in which the latter is enveloped by either a single process or finger-like projection branching from a large process (Butt et al., 1994c). Oligodendrocytes start to appear at approximately P5, preceding the onset of myelination by one or two days (Skoff et al., 1976a, Vaughn, 1969). However, as the majority of glial cells in P0 RON are

astrocytes, the ultrastructural morphology and glial injury scoring were studied for all cells of P0 RON.

The effects of ischaemia on optic nerve morphology have been described at the ultrastructural level (Alix et al., 2012, Back et al., 2007, Waxman et al., 1992, Alix and Fern, 2009, Salter and Fern, 2005, Salter and Fern, 2008a, Wilke et al., 2004, Thomas et al., 2004). It has been reported that ischaemia mediates extensive injury to astrocytes of RON (Thomas et al., 2004, Wilke et al., 2004). Living astrocytes following ischaemic episodes exhibited swollen intracellular organelles (mitochondria and ER) (Thomas et al., 2004, Wilke et al., 2004, Waxman et al., 1992, Back et al., 2007). Moreover, clasmatodendrosis, which is the detachment of the glial process from its somata, is common in astrocytes following ischaemia (Thomas et al., 2004, Wilke et al., 2004, Salter and Fern, 2008a). Vacuoles started to appear in the glial cytoplasm after ischaemic exposure (Back et al., 2007, Wilke et al., 2004, Salter and Fern, 2008a). All of these morphological features of OGD-induced glial cell injury can be organised into a cell injury score (Constantinou and Fern, 2009). The scoring system for glial injury established by Constantinou and Fern was followed in this study.

7.6.2 Results:

7.6.2.1 Scoring System:

To study the degree of glial injury following 90 minutes of aCSF, OGD and OGD in combination with different antagonists, a blind analysis of cell injury was used following a previously used scoring system (Constantinou and Fern, 2009). The scoring system has four classifications beginning with score (0), in which the glial cells look healthy and have no signs of ischaemic injury, such as the appearance of vacuoles, perinuclear gap, swollen cytoplasmic organelles, and cell membrane destruction (Figure 7-27). Score (1) is characterised by the appearance of small vacuoles in the cytoplasm of glial cells while the cytoplasmic organelles are still healthy (Figure 7-28). Score (2) is characterised by the appearance of swollen intracellular organelles, especially mitochondria (Figure 7-29 A-B) and/or large vacuoles (Figure 7-29 C-D). Cell membrane destruction is a feature of score (3), in which either a part of the cell membrane is destroyed by a large vacuole (Figure 7-30 A) or all of the cell membrane is destroyed, as in the case of frank necrosis (Figure 7-30 B).

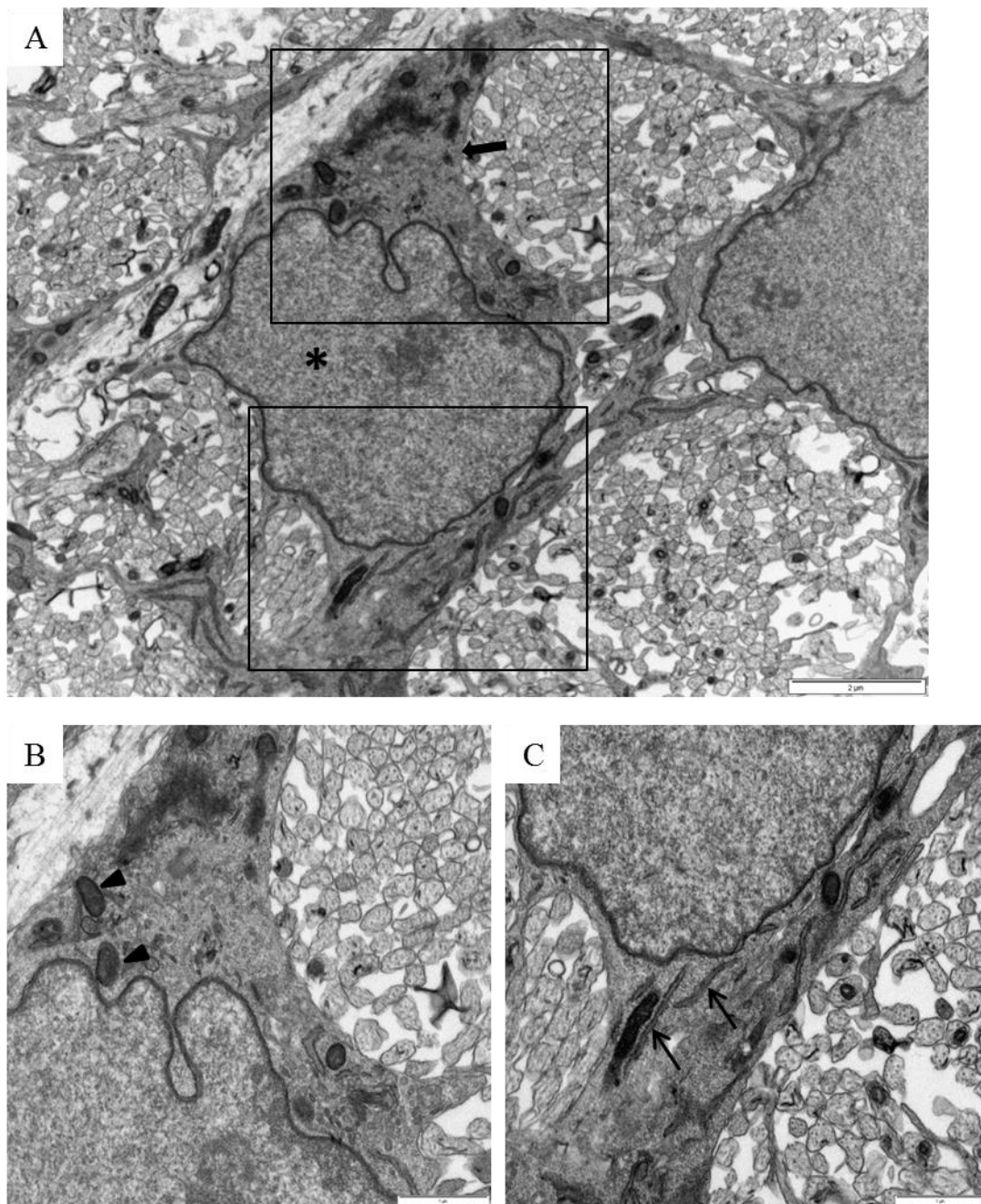


Figure 7-27: Score 0 glial ischaemic injury.

Electron micrograph showing the ultrastructural features of a glial cell in P0 RON. This glial cell represents score 0 because it shows healthy mitochondria (arrowheads), ER (arrows), nucleus (asterisk), and cell membrane (bold arrow), with no significant features of ischaemic injury. The upper part of the glia (the upper box) is shown in a higher magnification in (B), while the lower box is in (C).

Scale bar = 2 μm in (A) and 1 μm in both (B) and (C).

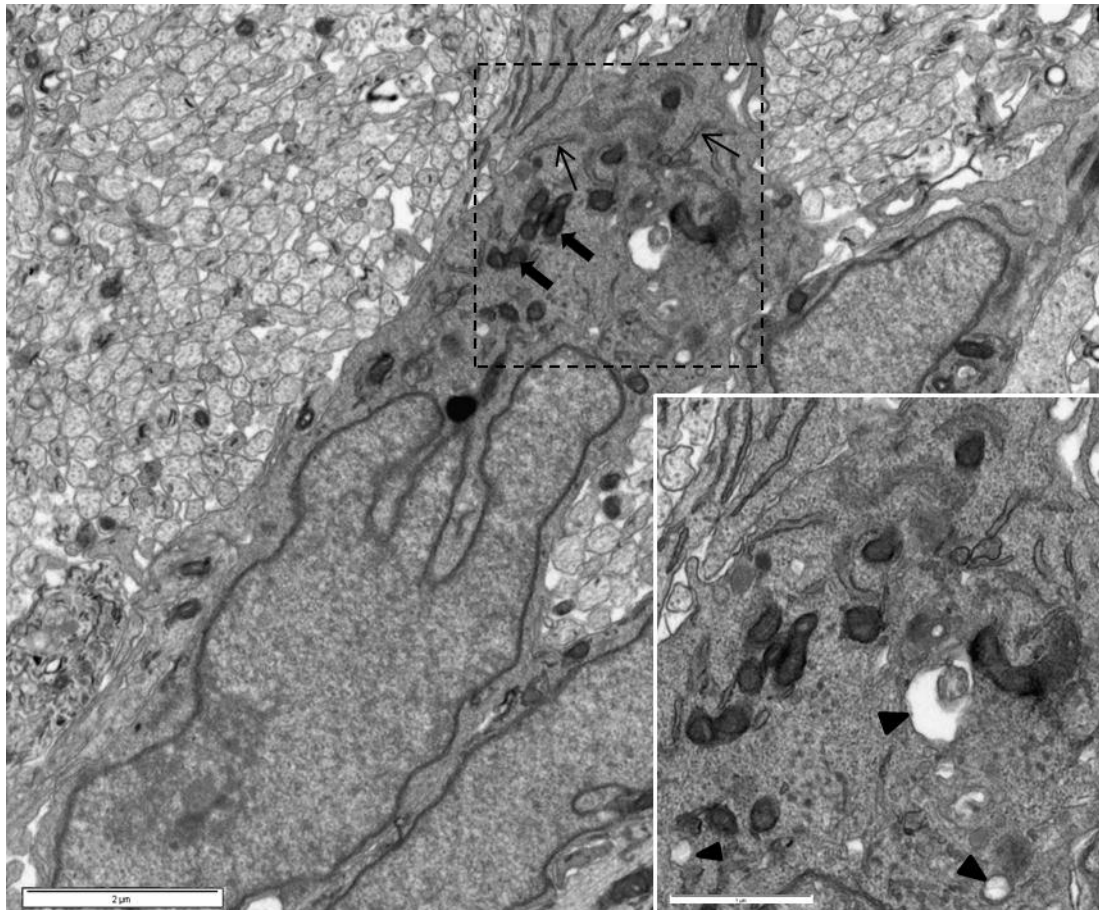


Figure 7-28: Score 1 glial ischaemic injury.

Electromicrograph showing the ultrastructural features of a glial cell in P0 RON. Boxed area is shown at higher power in the inset. This glial cell represents score 1 in glial injury as it has some small vacuoles (arrowheads) inside the cytoplasm, while the intracellular organelles, such as mitochondria (bold arrows) and ER (arrows), are still healthy.

Scale bar = 2 μm and 1 μm in the inset.

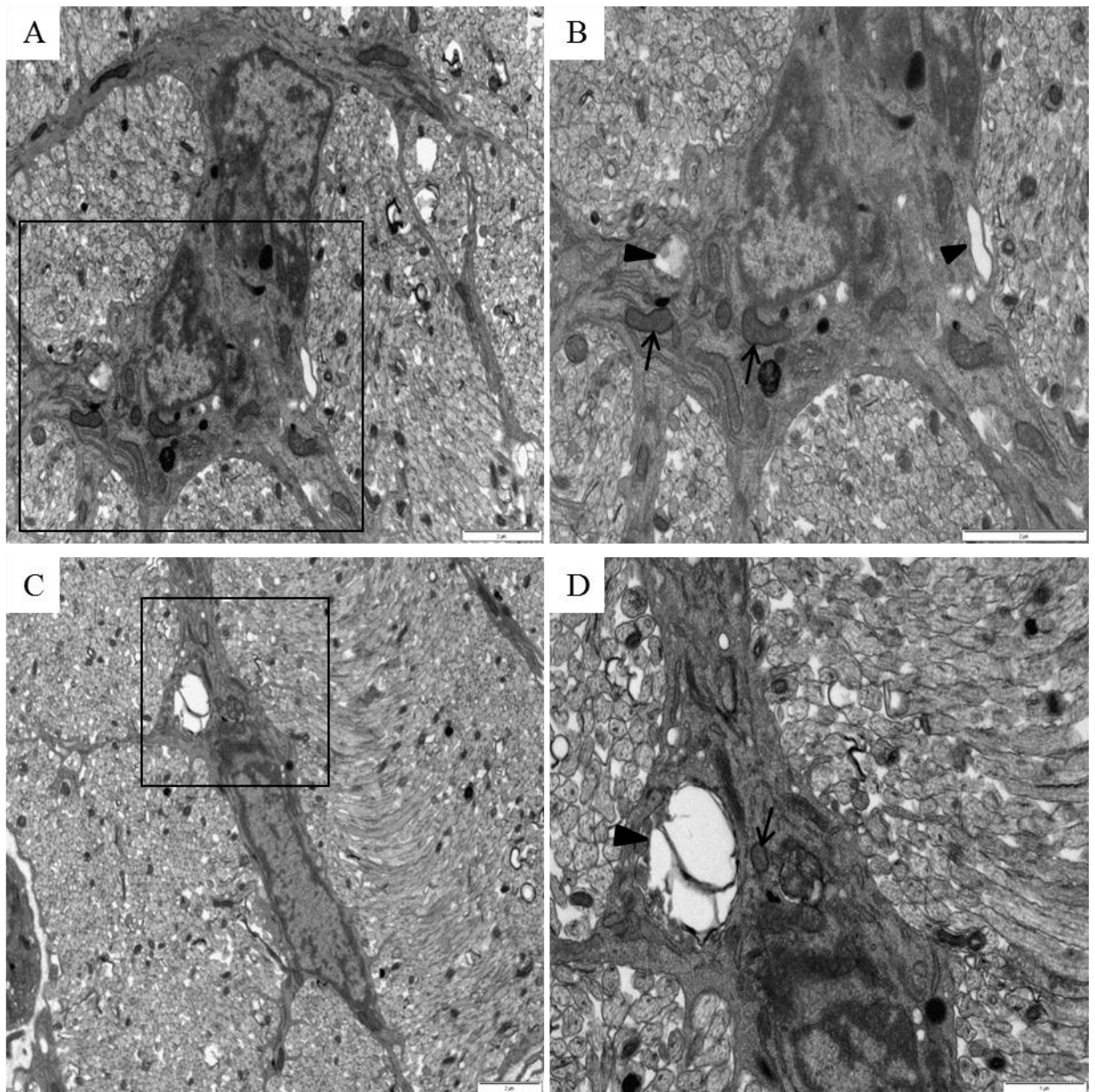


Figure 7-29: Score 2 glial ischaemic injury.

Electromicrograph showing the ultrastructural features of glial cells in P0 RON. The boxes in (A) and (C) are shown in a higher magnification in (B) and (D), respectively. (A) Shows small vacuoles (arrowheads) inside the glia cytoplasm, which are associated with swollen mitochondria (arrows). (C) Shows a large vacuole (arrowhead) inside the glial cytoplasm, regardless of the intracellular organelles in which the mitochondria (arrows) in this glia appear normal.

Scale bar = 2 μm in all images except in (D) = 1 μm .

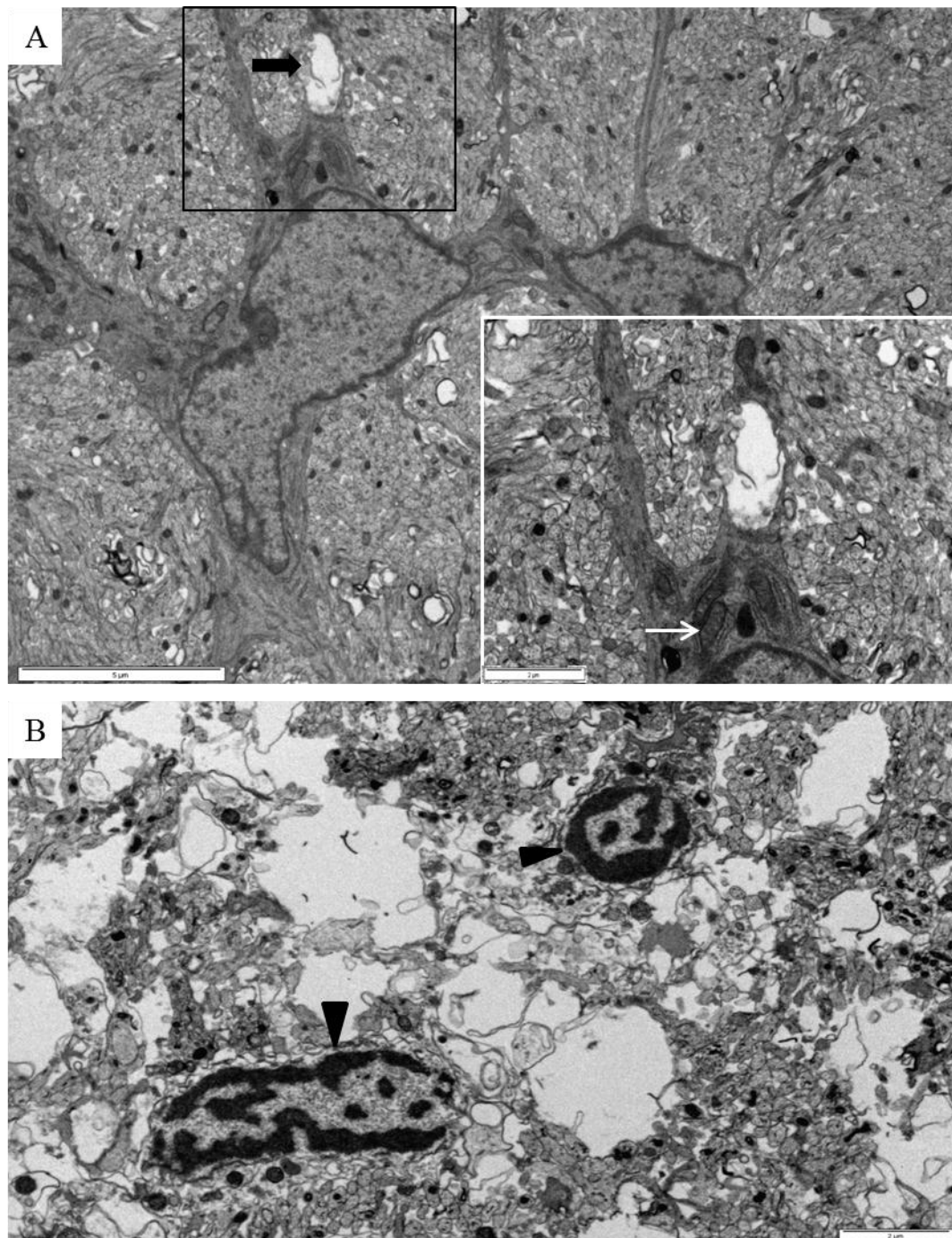


Figure 7-30: Score 3 glial ischaemic injury.

Electron micrograph showing the ultrastructural features of glial cells in P0 RON. Boxed area in (A) is shown at a higher power in the inset. (A) Shows a large vacuole (bold arrow) in the cytoplasm of a glial process, which destroys the cell membrane. The mitochondria are swollen (white arrow). (B) Shows a frank necrosis in which only the nucleus (arrowheads) can be identified.

Scale bar = 5 μm in (A) and 2 μm in both the high power inset and (B).

7.6.2.2 Controls:

Optic nerves perfused in aCSF for 100 minutes showed low levels of glial injury with a mean injury score of 0.35 ± 0.08 (Figure 7-39). Glial injury following the aCSF control was found to be limited to score 0 and 1, in which approximately 64.7% of the total glial injury is below score 0 and only 35.9% is in score 1 (Figure 7-38). A representative glial cell from the aCSF control which showed score 0 is shown in Figure 7-31A with an intact cell membrane, no signs of pathology, and normal intracellular organelles, such as mitochondria, ER and Golgi apparatus. On the other hand, score 1 glial cells from an aCSF control showed small vacuoles beginning to appear in their cytoplasm, while the intracellular organelles were still normal (Figure 7-31B).

In contrast to the aCSF control, switching to OGD solution for 90 minutes resulted in intensive injury to glial cells with a mean injury score of 2.4 ± 0.11 which is significantly different from that of aCSF control (Figure 7-39). In the OGD experiments, the majority of cells had a score of either 2 or 3 (41% and 51.28%, respectively) with only a small percentage seen of scores 0 or 1 (2.56% and 5.13%, respectively) (Figure 7-38). A representative glial cell from an OGD experiment showing score 0 is shown in Figure 7-32A with normal intracellular organelles, intact cell membrane and no significant pathology, while a score 1 glia has small cytoplasmic vacuoles but normal intracellular organelles (Figure 7-32B). More serious injuries were seen in score 3 glia, in which intracellular organelles were affected, such as swollen mitochondria and dilated mitochondria (Figure 7-33A). Score 4 glia showed multiple large vacuoles, which destroyed part of the glial cell membrane (Figure 7-33B).

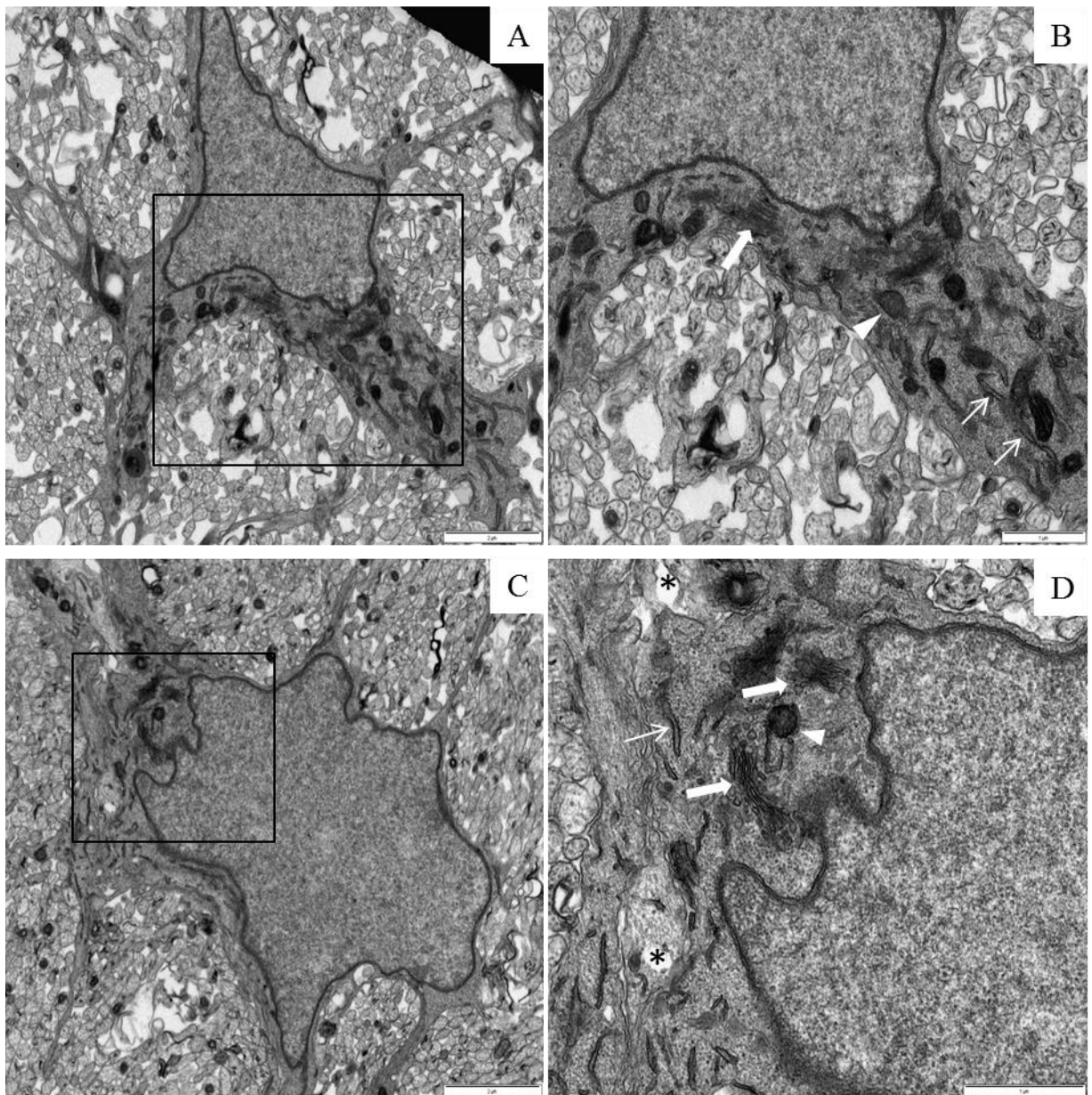


Figure 7-31: Micrograph of a P0 RON glial cells following an aCSF control experiment.

(A) Score 0: ultrastructural features of a glial cell which has normal mitochondria (arrowheads), ER (arrows) and Golgi apparatus (bold arrows). No significant pathology detected. The box in (A) is shown in a higher magnification in (B).

(B) Score 1: ultrastructural features of a glial cell which shows some small vacuoles (asterisk) in its cytoplasm. Otherwise, the intracellular organelles, such as mitochondria (arrowheads), ER (arrows) and Golgi apparatus (bold arrows), are normal. The box in (C) is shown in a higher magnification in (D).

Scale bar = 2 μm in (A) and (C) and 1 μm in (B) and (D).

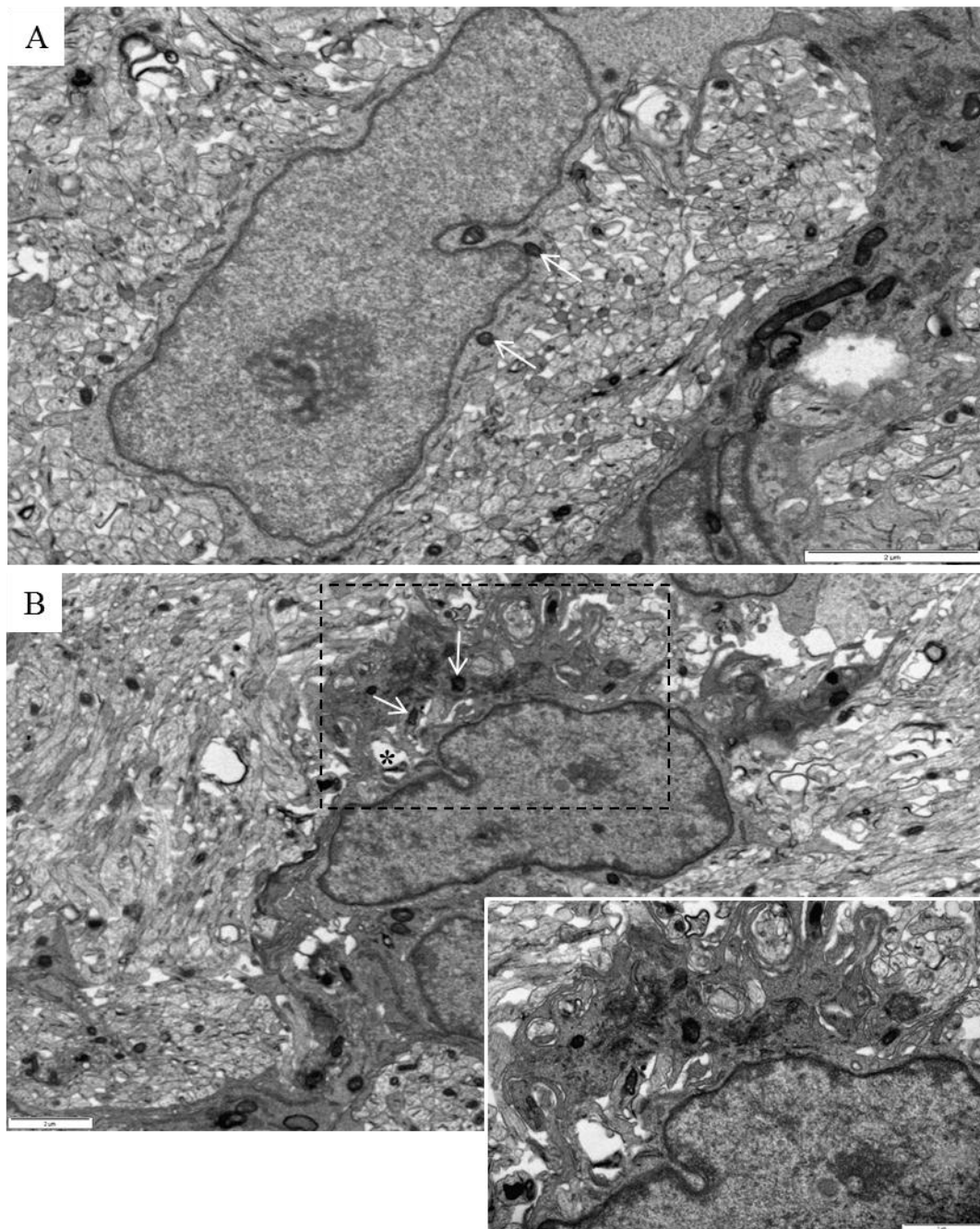


Figure 7-32: Micrograph of a P0 RON glial cell following an OGD experiment.

(A) Score 0: ultrastructural features of a glial cell which has normal mitochondria (arrows) and no significant pathology detected.

(B) Score 1: ultrastructural features of a glial cell which shows some small vacuoles (asterisk) in its cytoplasm. Otherwise, the intracellular organelles appear normal, such as mitochondria (arrows). Boxed area in (B) is shown at a higher power in the inset.

Scale bar = 2 μm except in the inset = 1 μm .

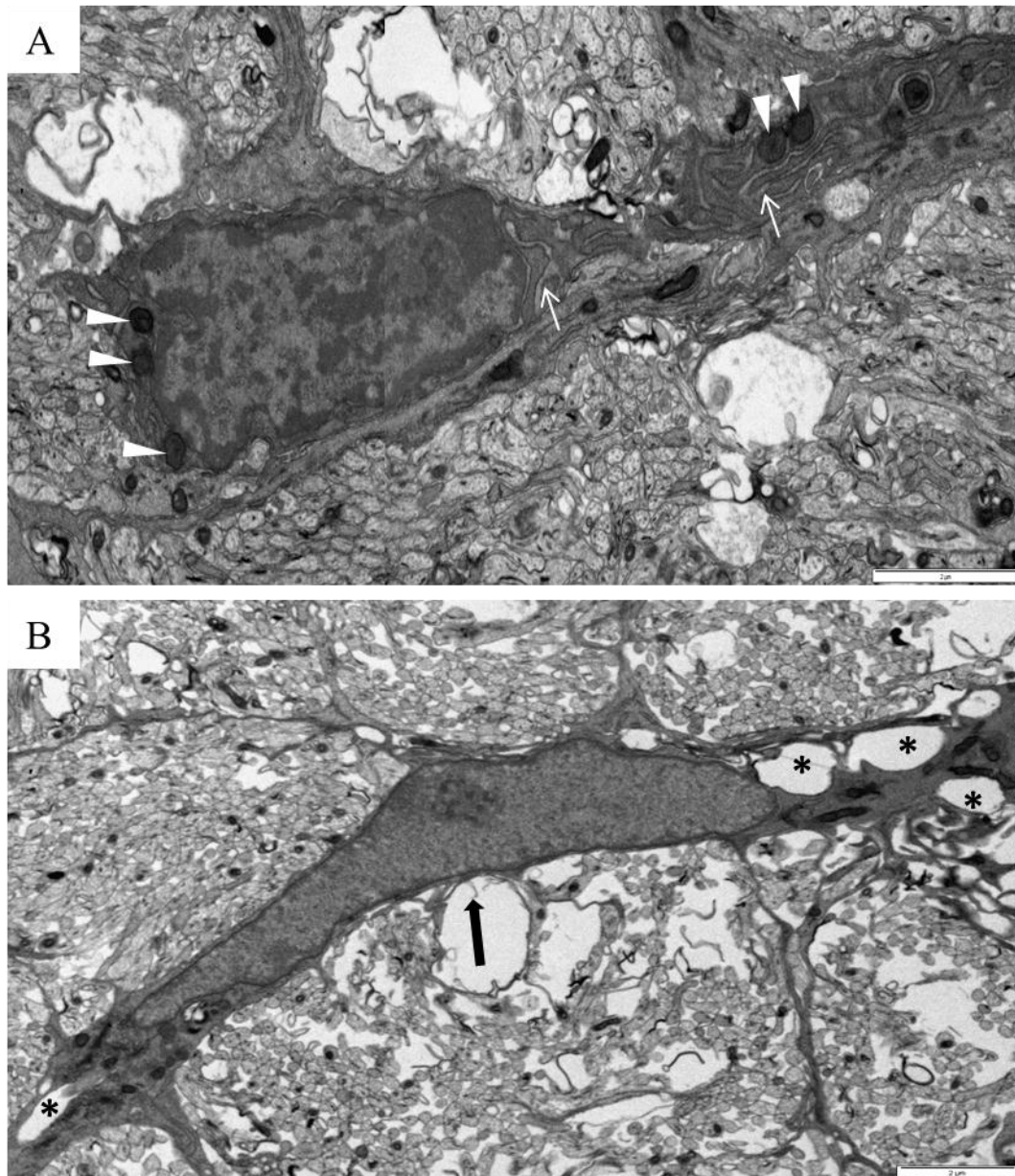


Figure 7-33: Micrograph of P0 RON glial cells following an OGD experiment.

(A) Score 2: ultrastructural features of a glial cell which has swollen mitochondria (arrowheads) and dilated ER (arrows).

(B) Score 3: ultrastructural features of a glial cell with multiple large vacuoles (asterisks) and damaged cell membrane (bold arrow).

Scale bar = 2 μm.

7.6.2.3 Antagonists' Application:

P0 RON glial cells were exposed to 10 minutes of aCSF with different receptor antagonists, such as picrotoxin, strychnine, mecamylamine or a combination of propranolol and phentolamine, followed by 90 minutes of OGD + antagonist. Studying the ultrastructural morphology of glial cells following OGD and antagonists revealed different levels of glial injuries. Both picrotoxin and strychnine significantly reduced the mean injury score when combined with OGD, as compared to the OGD alone (0.7 ± 0.15 and 1.0 ± 0.16 as compared to 2.4 ± 0.11 ; $p < 0.05$) (Figure 7-39). Following OGD + picrotoxin or OGD + strychnine experiments, it was found that the majority of glial cell injuries were in scores 0, 1 and 2, rather than score 3. More precisely, OGD + picrotoxin produced glial injury of approximately 55.26% for score 0, 23.68% for score 1, 15.78% for score 2 and only 5.26% for score 3; OGD + strychnine produced around 37.5% for score 0, 27.5% for score 1, 25% for score 2 and only 10% for score 3 (Figure 7-38). A representative micrograph showed different glial injury scores for OGD + strychnine and OGD + picrotoxin, as shown in Figure 7-34 and Figure 7-35, respectively.

On the other hand, OGD + mecamylamine or a combination of propranolol and phentolamine were found to produce a high degree of glial injury, represented by a high mean of injury scores which is not significantly different from that produced by OGD (2.1 ± 0.13 and 2.0 ± 0.09 as compared to 2.4 ± 0.11 , respectively; $p > 0.05$); see Figure 7-39. The majority of glial injuries were found in scores 2 and 3 following OGD with these two antagonists. Mecamylamine application during OGD produced glial injury of approximately 8.33% for score 0, 8.33% for score 1, 45.83% for score 2 and 37.5% for score 3, while a combination of propranolol and phentolamine produced approximately 2.17% for score 0, 8.69% for score 1, 67.39% for score 2 and 21.74%

for score 3 (Figure 7-38). A representative micrograph showed different glial injury scores for OGD + mecamylamine and OGD + propranolol and phentolamine, as shown in Figure 7-36 and Figure 7-37, respectively.

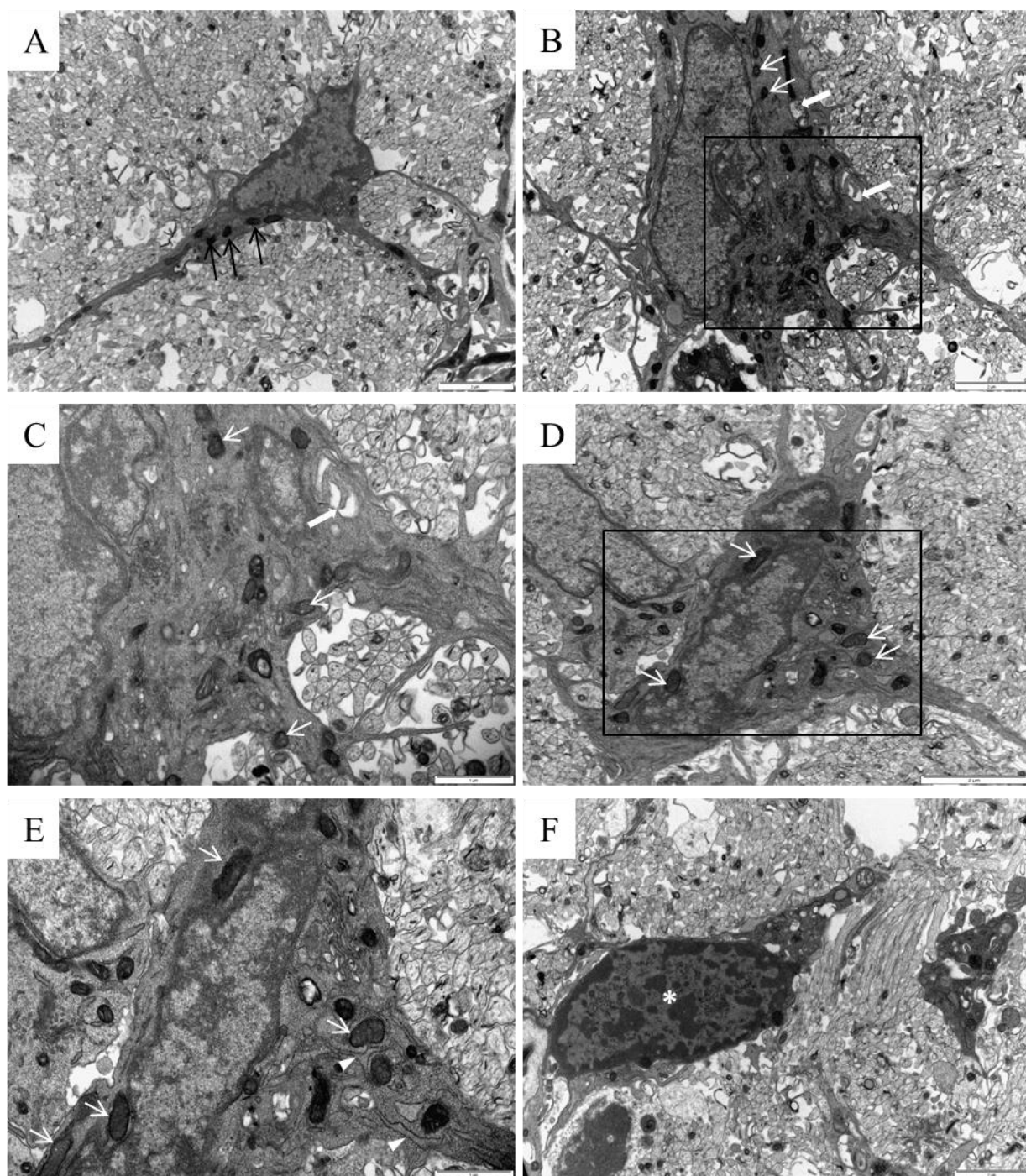


Figure 7-34: Micrograph of P0 RON glial cells following OGD + strychnine.

(A) Score 0: ultrastructural morphology of a glial cell with normal mitochondria (arrows) and no significant pathology detected.

(B, C) Score 1: ultrastructural morphology of a glial cell with multiple small vacuoles (bold arrows) and normal mitochondria (arrows). The box in (B) is shown in a higher magnification in (C).

(D, E) Score 2: ultrastructural morphology of a glial cell with abnormal intracellular organelles, such as swollen mitochondria (arrows) and dilated ER (arrowheads). The box in (D) is shown in a higher magnification in (E).

(F) Score 3: Frank necrosis where only the nucleus (asterisks) can be identified.

Scale bar = 2 μm except in (C) and (E) = 1 μm .

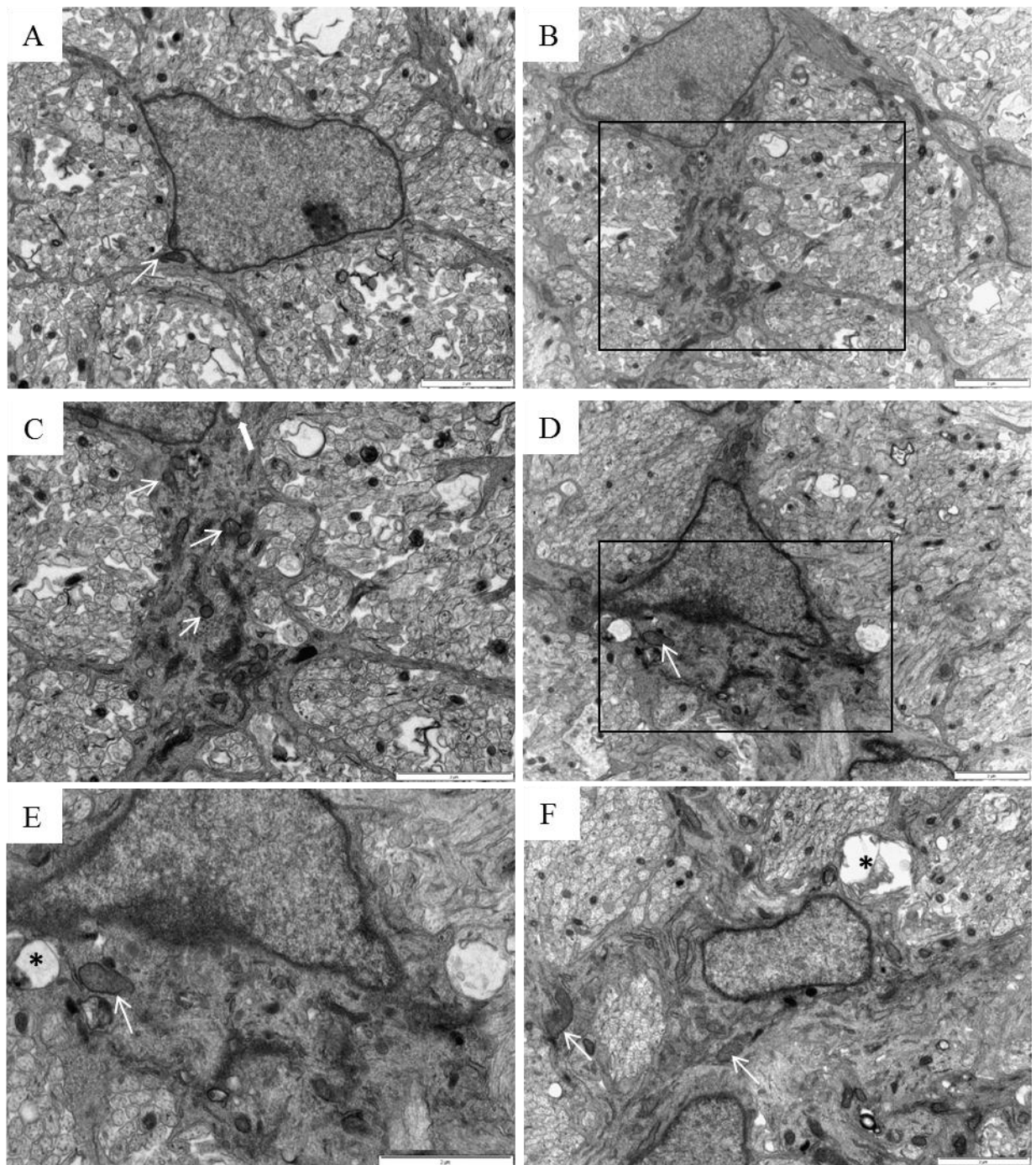


Figure 7-35: Micrograph of P0 RON glial cells following OGD + picrotoxin.

(A) Score 0: ultrastructural morphology of a glial cell with normal mitochondria (arrow) and no significant pathology detected.

(B, C) Score 1: ultrastructural morphology of a glial cell with a small vacuole (bold arrow) and normal mitochondria (arrows). The box in (B) is shown in a higher magnification in (C).

(D, E) Score 2: ultrastructural morphology of a glial cell which has an intracellular vacuole (asterisk) and swollen mitochondria (arrow). The box in (D) is shown in a higher magnification in (E).

(F) Score 3: ultrastructural morphology of a glial cell with some swollen mitochondria (arrows) and a large vacuole (asterisk), which destroyed the cell membrane in the process.

Scale bar = 2 μm .

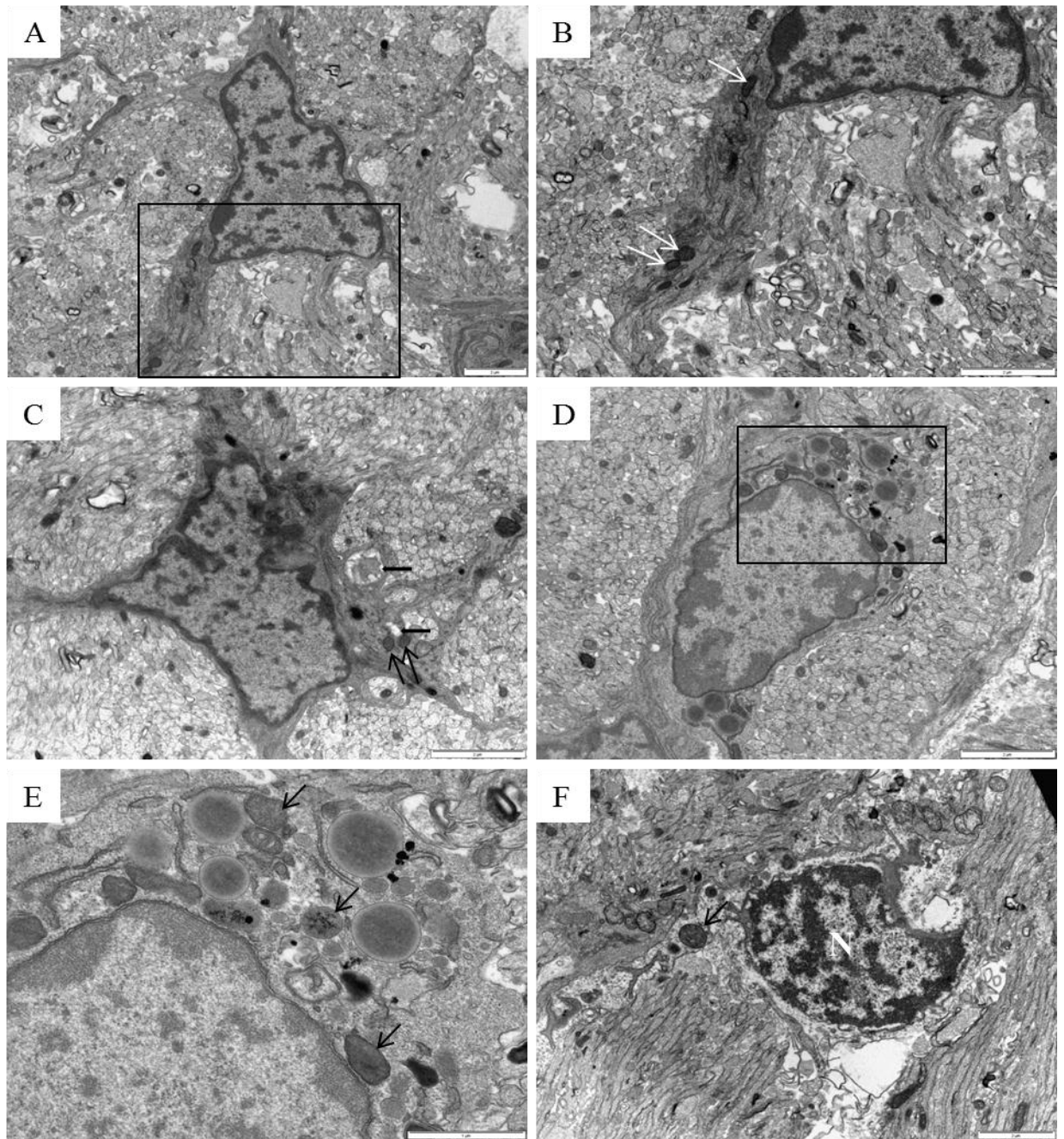


Figure 7-36: Micrograph of P0 RON glial cells following OGD + mecamylamine.

(A, B) Score 0: ultrastructural morphology of a glial cell with normal mitochondria (arrows) and no significant pathology detected. The box in (A) is shown in a higher magnification in (B).

(C) Score 1: ultrastructural morphology of a glial cell with small vacuoles (bold arrows) and normal mitochondria (arrows).

(D, E) Score 2: ultrastructural morphology of a glial cell with swollen mitochondria (arrows). The box in (D) is shown in a higher magnification in (E).

(F) Score 3: Frank necrosis where only the nucleus (N) can be identified.

Scale bar = 2 μm except in (E) = 1 μm .

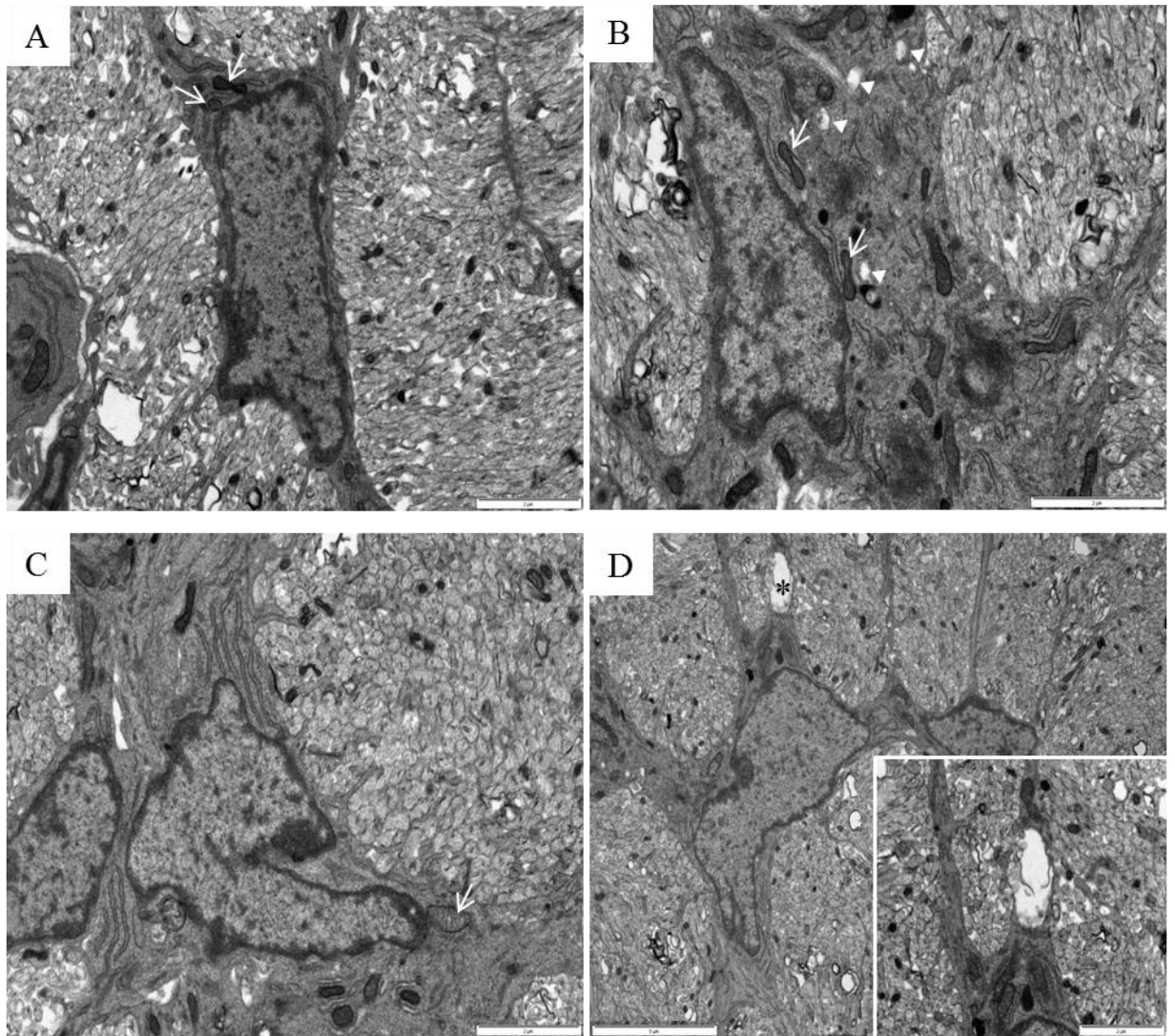


Figure 7-37: Micrograph of P0 RON glial cells following OGD + propranolol and phentolamine.

(A) Score 0: ultrastructural morphology of a glial cell with normal mitochondria (arrows) and no significant pathology detected.

(B) Score 1: ultrastructural morphology of a glial cell with small vacuoles (arrowheads) and normal mitochondria (arrows).

(C) Score 2: ultrastructural morphology of a glial cell with swollen mitochondria (arrows).

(D) Score 3: ultrastructural morphology of a glial cell with a large vacuole (asterisk), which destroyed the cell membrane in the glial process. Boxed area in (D) is shown at a higher magnification in the inset.

Scale bar = 2 μm except in (D) = 5 μm .

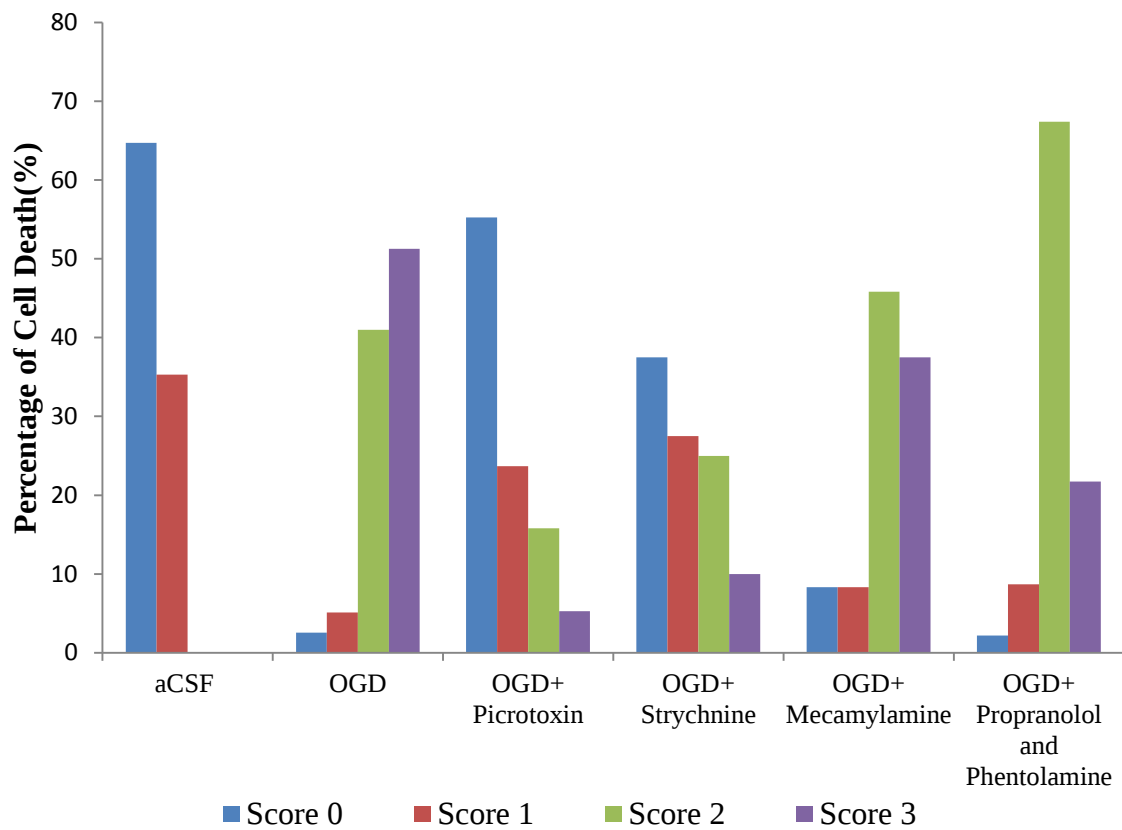


Figure 7-38: EM scoring for glial injury in aCSF and OGD controls, and OGD in the presence of various antagonists.

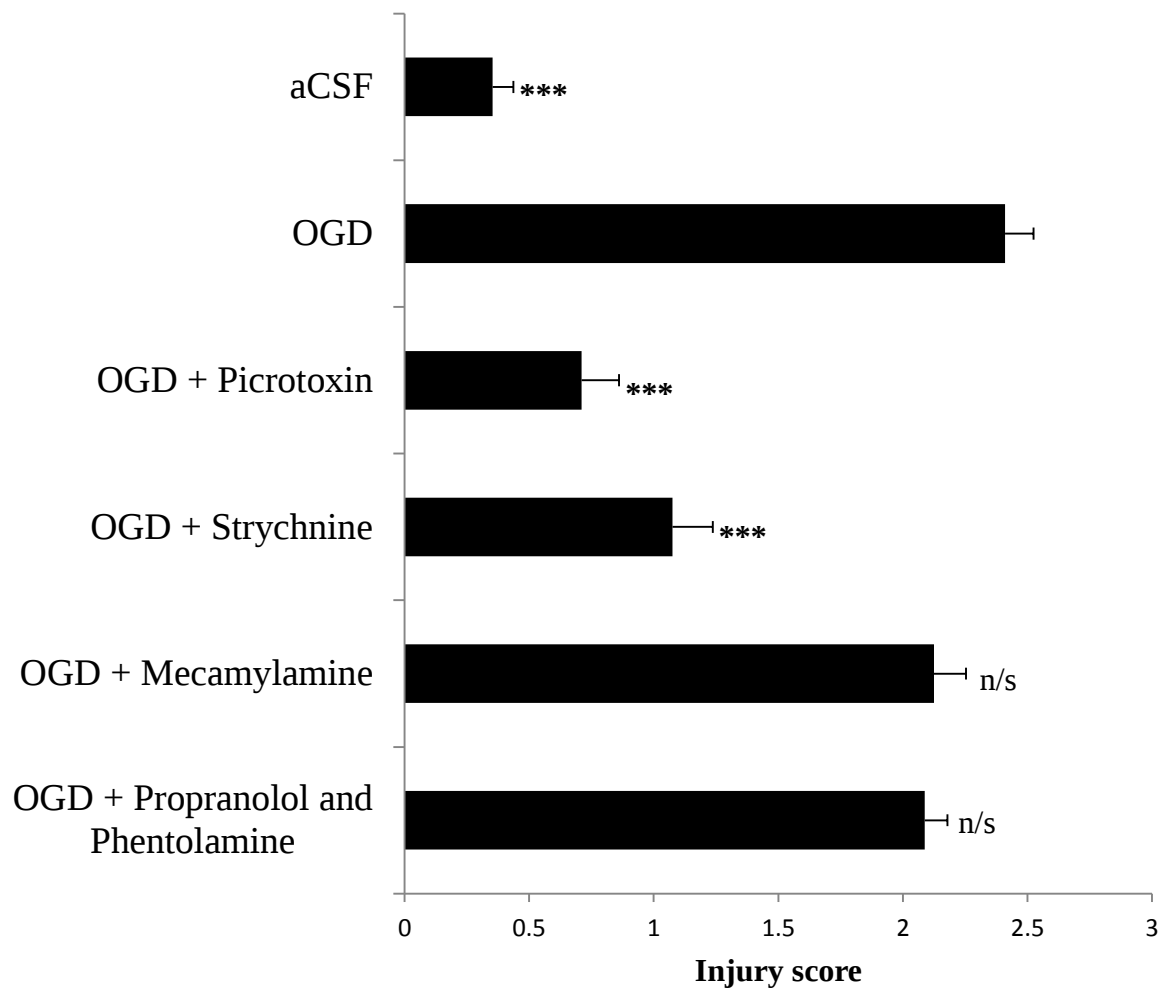


Figure 7-39: Mean injury scores from the various protocols.

*** represents a statistical significance as compared to OGD ($p < 0.05$). n/s represents a non-statistical significance between OGD and OGD + mecamylamine and between OGD and OGD + propranolol and phentolamine cell death.

7.6.3 Discussion:

In the current study, the ultrastructural morphology of randomly collected glial cells was used to determine an injury score for glial cells in the nRON. P0 RON perfused in aCSF solution produced a low level of glial injury, typified by the low mean glial injury score, while switching to OGD solution induced extensive cell injury with a higher injury score. This is not surprising as it has shown that P0 RON astrocytes are sensitive to ischaemic injury (Fern, 1998). It has been reported that ischaemia triggers elevated levels of nicotine (Yamamuro et al., 1996) and norepinephrine (Bhardwaj et al., 1990, Globus et al., 1989, Perego et al., 1992) in the extracellular space. Long exposure to nicotine and norepinephrine is found to irreversibly block P10 RON action potential conduction, although the site of action of this effect might be either axonal or glial. Moreover, it has been shown that nRON astrocytes are more sensitive to long exposure to neurotransmitters (nicotine + norepinephrine) than oligodendrocytes in P10 RON (Constantinou and Fern, 2009). Ultrastructural studies of P10 RON revealed that long exposure to nicotine + norepinephrine induced glial injury, mimicking the features of glial ischaemic injury (Constantinou and Fern, 2009). However, the current study revealed that blocking neither nicotinic receptors nor adrenergic receptors protected the P0 RON from ischaemic glial injury. This could be due to different receptor development and function between P0 and P10 RON glial cells. On the other hand, it is known that ischaemia evokes secretion of GABA (Ravindran et al., 1994, Shimada et al., 1993) and glycine (Luccini et al., 2010, Oja and Saransaari, 2009) to the extracellular space. Glial ultrastructure morphology revealed that blocking GABA or glycine receptors significantly reduced the level of P0 RON glial injury induced by ischaemia as compared to the OGD alone. This is consistent with the fact that GABA and glycine are

excitatory neurotransmitters during the early postnatal period and that blocking these receptors can be protective.

7.7 Localization of GABA_A and GLY Receptors in RON:

7.7.1 Introduction:

The morphology of optic nerve glial cells has been described under the confocal microscope (Butt et al., 1994a, Butt et al., 1994b, Butt and Ransom, 1993). Three types of Lucifer Yellow (LY) filled astrocytes have been detected in RON under confocal microscopes, depending on their morphological appearance: transversely, longitudinally and randomly oriented astrocytes (Butt and Ransom, 1993). Longitudinal astrocytes send their processes in a plane parallel to the optic nerve axons, while transverse astrocyte processes are perpendicular to the optic nerve axons (Butt et al., 1994a). Randomly oriented astrocytes are stellate cells in which their processes are radially branched from the cell body (Butt et al., 1994a). Transversely and randomly oriented astrocytes exist in RON at birth, while the longitudinal appear at P2 (Butt and Ransom, 1993). Each type has different maturation stages and process development, with the latter dependent on the measurement of the number and length of processes (Butt and Ransom, 1993). Random astrocytes became the prominent type in the adult RON, which reached approximately 61% of dye filled astrocytes followed by 30% transverse astrocytes and only 9% of longitudinal astrocytes (Butt et al., 1994a). Moreover, three-dimensional lysinated rhodamine dextran (LRD) filled astrocytes (transverse, longitudinal and random astrocytes) have been studied in the mouse optic nerve by laser scanning confocal microscopy (Butt et al., 1994b). This study revealed that all three morphological types of optic nerve astrocytes extend their processes to contact subpial and perivascular glia limitans, or exist free in the nerve.

Immunohistochemistry staining with NF showed RON axons as bundles running parallel to each other under confocal microscope (Ren et al., 2000). GFAP and NF are widely used as markers for astrocytes and axons, respectively (Arranz et al., 2008, Butt and Kirvell, 1996, Alix et al., 2012, Kalsi et al., 2004) and have been employed in the current study.

The expression of GABA has been studied in different parts of the CNS (Angulo et al., 2008). It was found that GABA transmitter expression is more localised to macroglia but not axons in RON; e.g., at E20, GABA was localised to macroglial somata and GFAP expressed by glial processes, with some overlap at the nerve periphery, while in P7 and beyond, GABA is expressed by oligodendrocytes and fibrous astrocytes (Lake, 1992). In P0–P7 RON culture, it was shown that GABA receptor is expressed by oligodendrocytes, type-2 (fibrous) astrocytes and O-2A progenitor cells, but is not expressed by type-1 (protoplasmic) astrocytes (Barres et al., 1990b). Moreover, immunostaining and high pressure liquid chromatography (HPLC) reported the expression of GABA and GAD by GFAP + astrocytes in cultured and intact whole RON, which attenuated after the third postnatal week (Ochi et al., 1993). This is consistent with the immune-electron microscopy of intact RON, which revealed the transient presence of GABA_A receptor in glial cells and pre-myelinated axons during the postnatal period and a reduced presence in the adult (Sakatani et al., 1992).

Several studies have focused on the distribution of glycine receptors in CNS. GlyR immunoreactivity and strychnine autoradiography have been used to study glycine receptor distribution at the light level (Lynch, 2004, White et al., 1990, Vandepol and Gorcs, 1988) and at the ultrastructural level by immuno-electron microscope (Triller et al., 1985). These studies revealed a high expression of glycine receptors in the spinal cord and medulla, less expression in the midbrain, hypothalamus

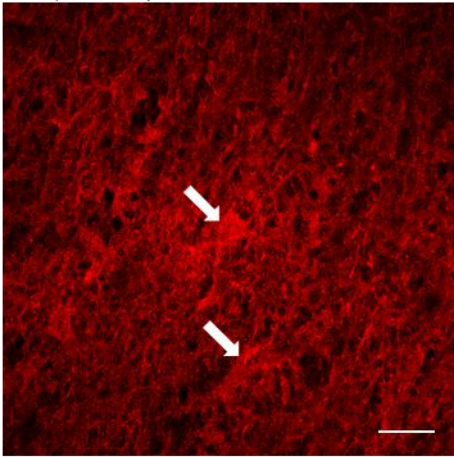
and thalamus, and no detectable glycine receptor in the higher brain (Rajendra et al., 1997, Lynch, 2004). It was found that glycine subunits were heavily expressed all over the white matter areas in all developmental stages (Belachew et al., 1998, Laube et al., 2002). Glycine receptor subunits are detected in glial cells of the rat spinal cord (Kirchhoff et al., 1996, Verkhratsky and Steinhauser, 2000). Different glycine and GABA subunits are detected by PCR in P0, P10 and adult RON (Domingues et al., 2010). In the current study, different ages (P0, P10 and adult) of RON were used to study the localisation of GABA_A and glycine receptors in RON glial cells and axons.

7.7.2 Results and Discussion:

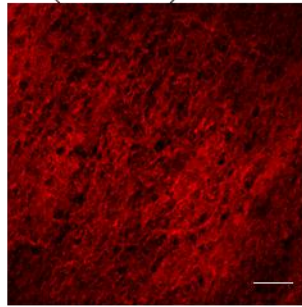
7.7.2.1 Localisation of GABA_ARs in RON:

GABA_AR was detected in adult, P10 and P0 RON by immunostaining. In adult and P10 RON, double labelling for GFAP revealed a colocalisation of astrocytes with GABA_AR, while some areas were only stained GABA receptor, as shown in Figure 7-40 and Figure 7-41. An intense staining and co-expression of astrocytes with GABA_AR is seen in P0 RON (Figure 7-42). This is consistent with the GABA expression in RON astrocytes of different ages (Ochi et al., 1993, Lake, 1992). GABA_AR (+) / GFAP (-) staining was seen in different ages of RON, suggesting GABA_AR expression in axons or oligodendrocytes. Double staining for NF revealed expression of GABA_AR on axons in P0 RON (Figure 7-45), but not in P10 (Figure 7-44) or adult RON (Figure 7-43). These results suggest the expression of GABA_A receptors on astrocytes of different RON ages and in axons only in P0 RON. This is consistent with the RON immune-electron microscopy, which revealed the transient expression of GABA_AR in glial cells and pre-myelinated axons during the postnatal period that attenuated with age (Sakatani et al., 1992). However, there is still a population of GABA_AR (+) cells, especially in P10 and adult RON, which does not stain with astrocyte or axon markers. These cells are shown to be aligned in rows between axons (Figure 7-43 and Figure 7-44), and this interfascicular appearance is a characteristic feature for RON oligodendrocytes (Butt and Kirvell, 1996, Kalsi et al., 2004). These cells are not seen in P0 RON (Figure 7-45), as oligodendrocytes are absent (Vaughn, 1969). For these reasons, we presumed that these cells are oligodendrocytes that express GABA_A receptors in P10 and adult RON. This is consistent with GABA expression by RON oligodendrocyte after P7 (Lake, 1992).

A (GABA)



a1 (GABA+)



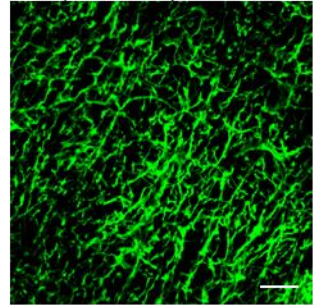
a2 (GFAP-)



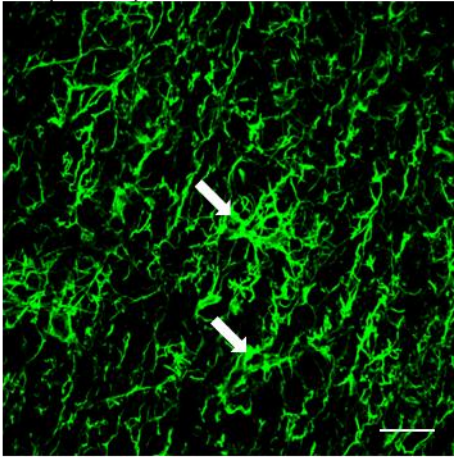
b1 (GABA-)



b2 (GFAP+)



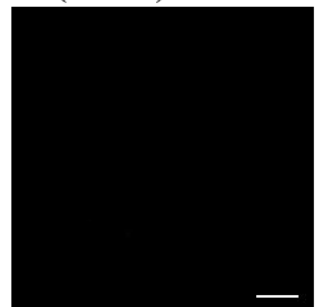
B (GFAP)



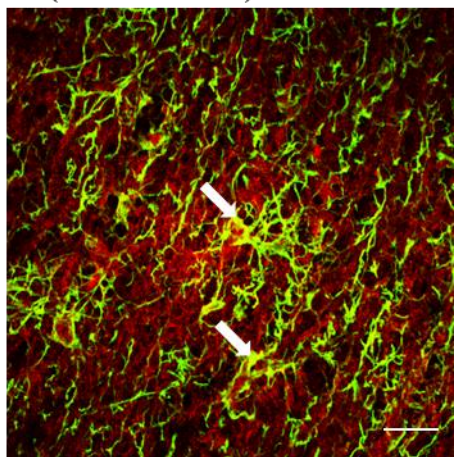
c1 (GABA-)



c2 (GFAP-)



C (GABA/ GFAP)



C1 (GABA/ GFAP)

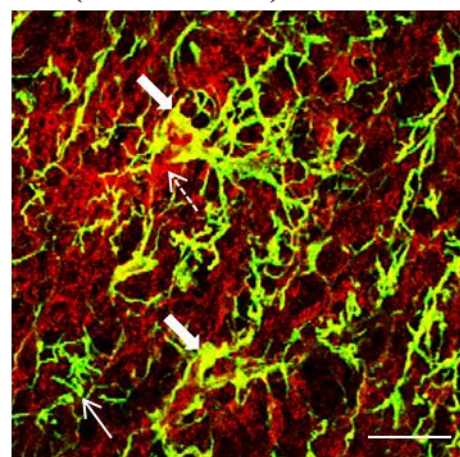


Figure 7-40: Adult RON staining for GABA_AR and GFAP.

A: Adult RON staining for GABA_A receptor (GABA/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GABA_A (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: Adult RON staining for astrocytes (GFAP/green).

b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse GFAP (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: Adult RON double staining for GABA_A receptors (GABA/red) and astrocytes (GFAP/green). The box in C is shown in a higher magnification in C1. Notice the GABA_AR and GFAP co-localisation (bold arrow). However, there are many areas that only stained for GABA_AR (dotted arrow) or GFAP (arrow).

c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 µm.

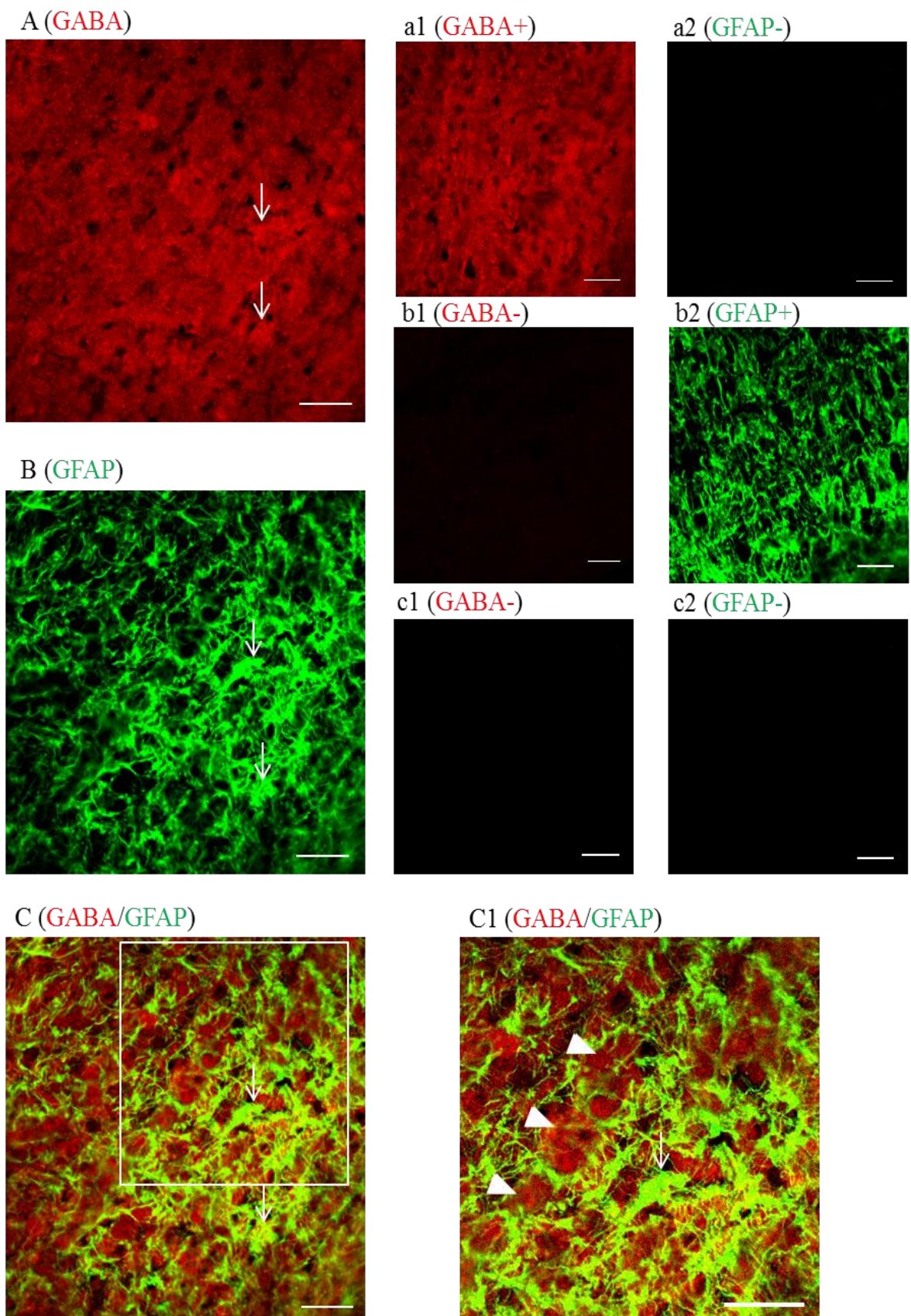


Figure 7-41: P10 RON staining for GABA_AR and GFAP.

A: P10 RON staining for GABA_A receptors (GABA/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GABA_A (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: P10 RON staining for astrocytes (GFAP/green).

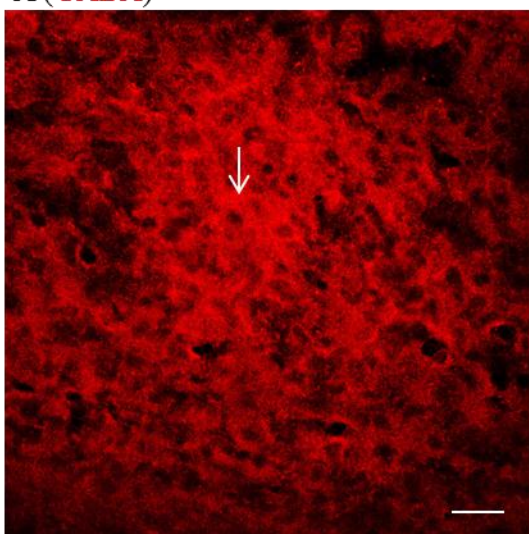
b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse GFAP (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: P10 RON double staining for GABA_A receptors (GABA/red) and astrocytes (GFAP/green). The box in C is shown in a higher magnification in C1. Notice the GABA_A and GFAP co-localisation (arrows). However, there are many areas that only stained for GABA_AR (arrowheads).

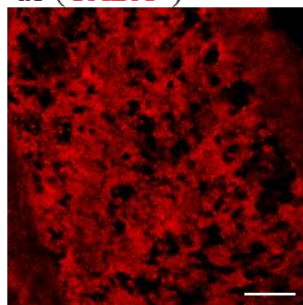
c1+ c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 µm.

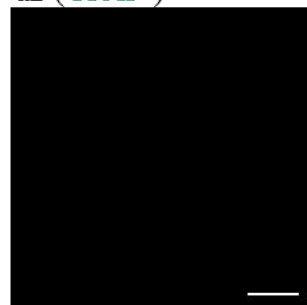
A (GABA)



a1 (GABA+)



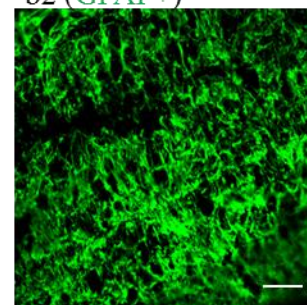
a2 (GFAP-)



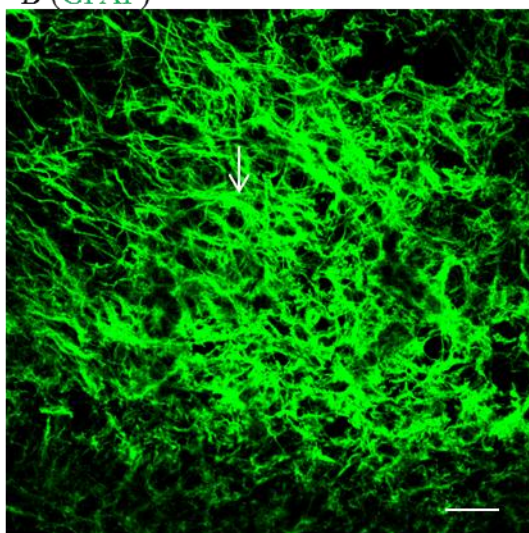
b1 (GABA-)



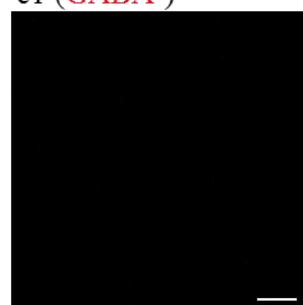
b2 (GFAP+)



B (GFAP)



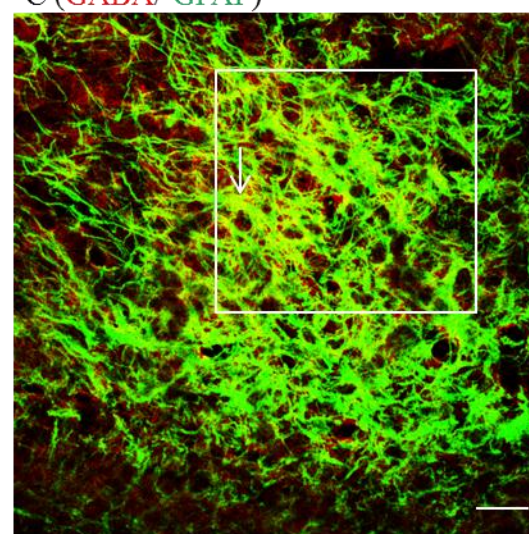
c1 (GABA-)



c2 (GFAP-)



C (GABA/ GFAP)



C1 (GABA/ GFAP)

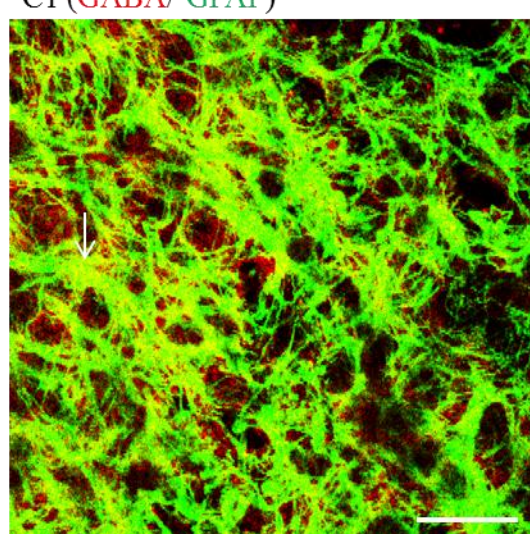


Figure 7-42: P0 RON staining for GABA_AR and GFAP.

A: P0 RON staining for GABA_A receptors (GABA/red).

a1 + a1: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GABA_A (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: P0 RON staining for astrocytes (GFAP/green).

b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse GFAP (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: P0 RON double staining for GABA_A receptors (GABA/red) and astrocytes (GFAP/green). The box in C is shown in a higher magnification in C1. Note that almost all of GABA_AR staining is found in astrocytes (arrows).

c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μ m.

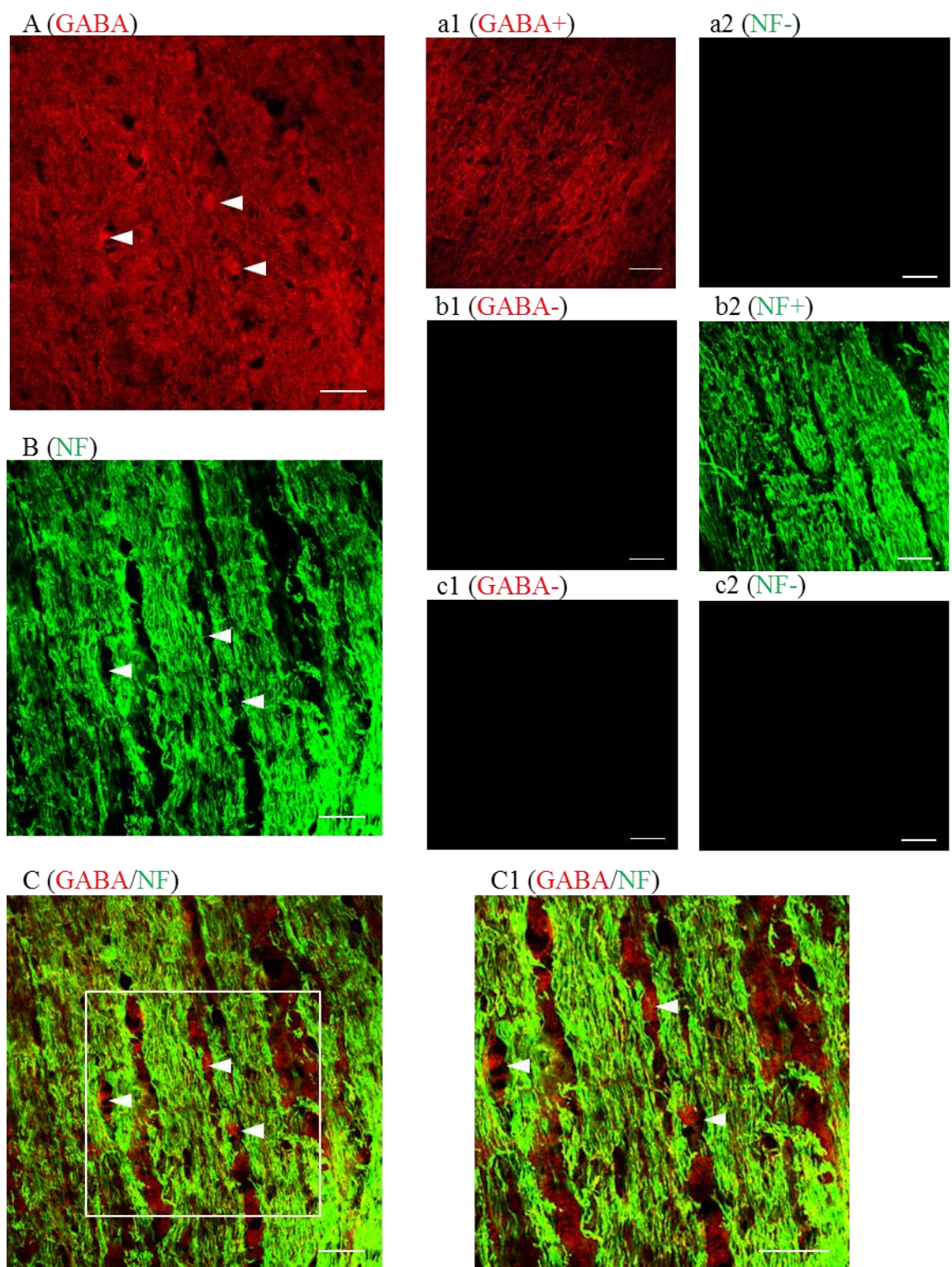


Figure 7-43: Adult RON staining for GABA_AR and NF.

A: Adult RON staining for GABA_A receptors (GABA/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GABA_A (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: Adult RON staining for axons (NF/green).

b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse NF (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: Adult RON double staining for GABA_A receptors (GABA/red) and axons (NF/green). The box in C is shown in a higher magnification in C1.

c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μ m.

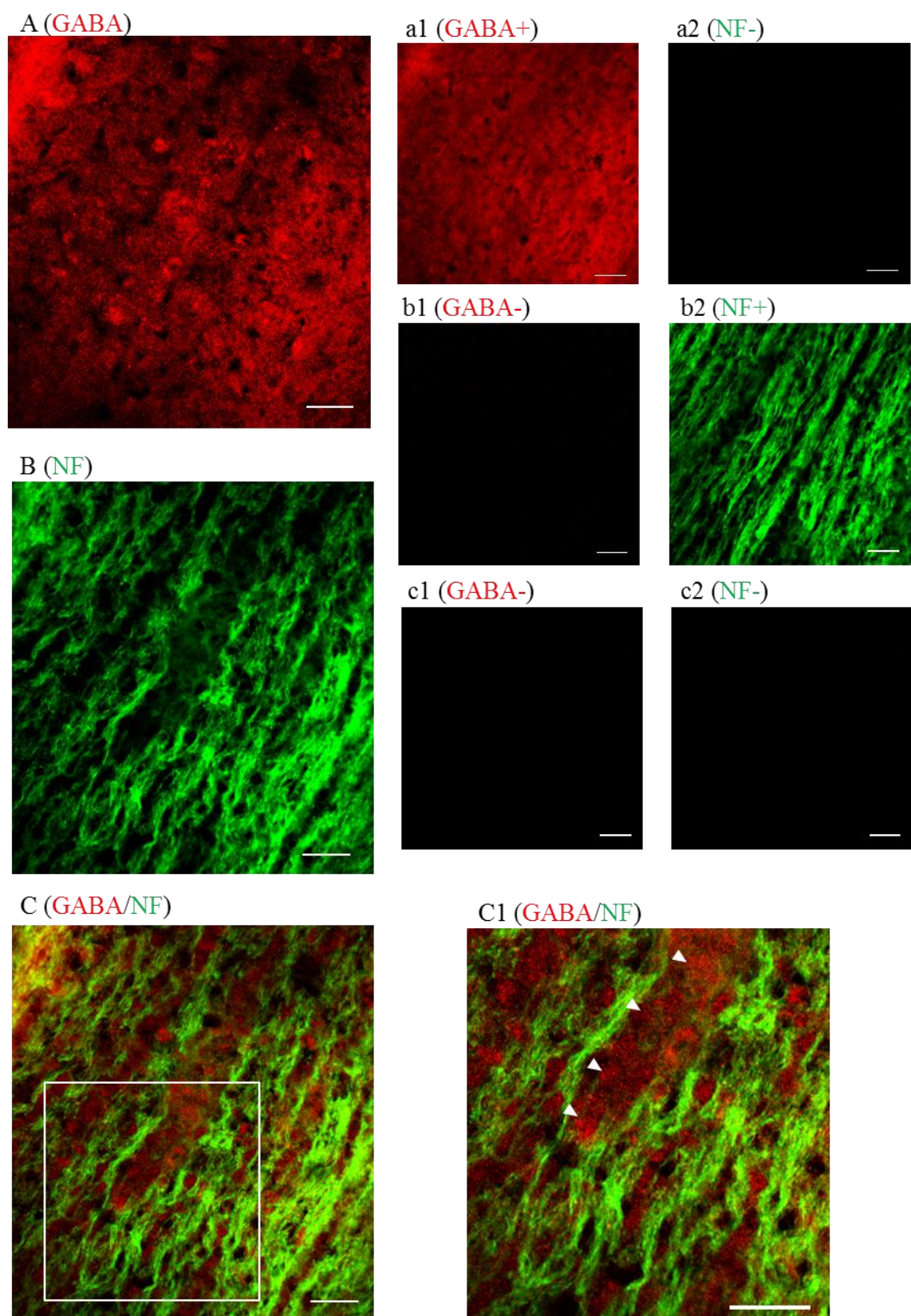


Figure 7-44: P10 RON staining for GABA_AR and NF.

A: P10 RON staining for GABA_A receptors (GABA/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GABA_A (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: P10 RON staining for axons (NF/green).

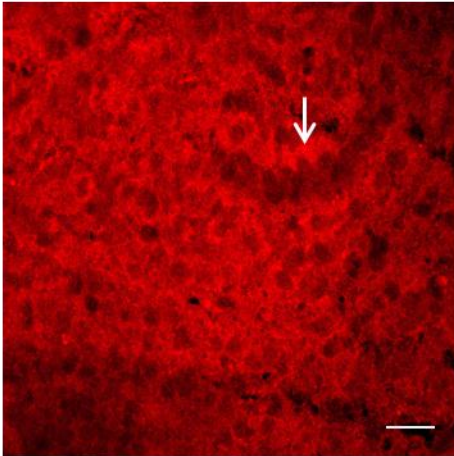
b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse NF (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: P10 RON double staining for GABA_A receptors (GABA/red) and axons (NF/green). The box in C is shown in a higher magnification in C1. Note that GABA_AR stained cells are aligned in rows between axons (arrowheads).

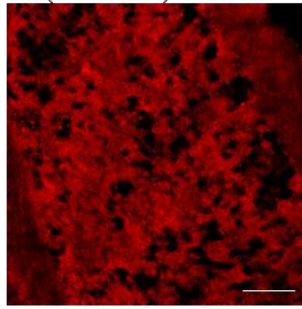
c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μ m.

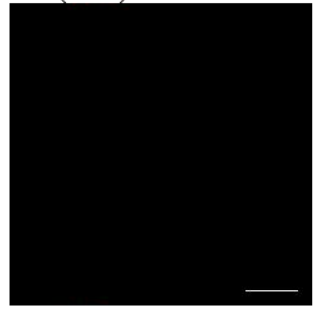
A (GABA)



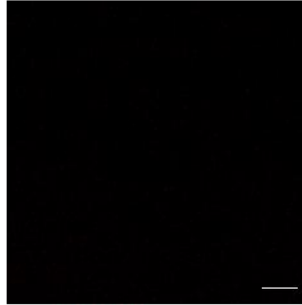
a1 (GABA+)



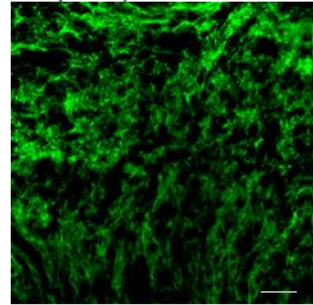
a2 (NF-)



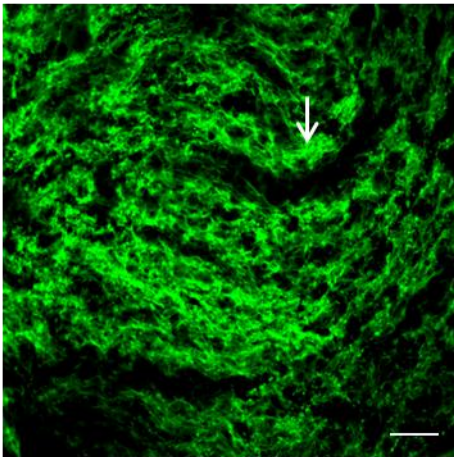
b1 (GABA-)



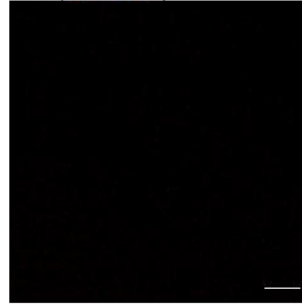
b2 (NF+)



B (NF)



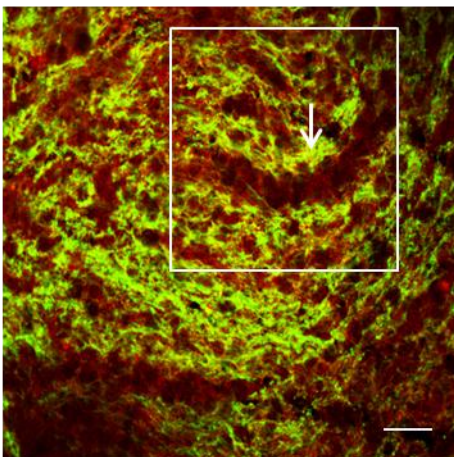
c1 (GABA-)



c2 (NF-)



C (GABA/NF)



C1 (GABA/NF)

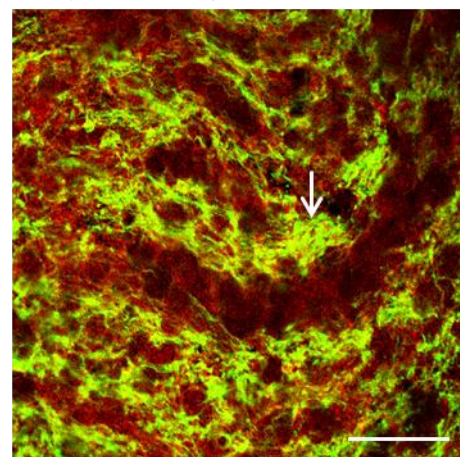


Figure 7-45: P0 RON staining for GABA_AR and NF.

A: P0 RON staining for GABA_A receptors (GABA/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GABA_A (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: P0 RON staining for axons (NF/green).

b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse NF (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: P0 RON double staining for GABA_A receptors (GABA/red) and axons (NF/green). The box in C is shown in a higher magnification in C1. The majority of GABA_AR staining is found in axons (arrow).

c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μm.

7.7.2.2 Localisation of GLYRs in RON:

Glycine receptor (GLYR) staining was detected in adult, P10 and P0 RON. Double staining with GFAP revealed co-localisation of astrocytes with glycine receptor staining in adult RON (Figure 7-46), P10 (Figure 7-47) and P0 (Figure 7-48). However, there were some areas which were GLYR(+) / GFAP(-), suggesting glycine receptor expression in other areas as axons and oligodendrocytes. Double staining with NF showed co-localisation of axons with glycine receptor staining in P0 (Figure 7-51) and P10 (Figure 7-50), but not in adult RON (Figure 7-49). Glycine staining in adult RON is seen in cells arranged in rows between NF+ axons (Figure 7-49). We presume that these cells could be astrocytes or oligodendrocytes, as this interfascicular appearance is a characteristic feature for RON oligodendrocytes (Butt and Kirvell, 1996, Kalsi et al., 2004). These results suggest that glycine receptors are expressed by astrocytes and axons of P0 and P10, while they are present in astrocytes and oligodendrocytes of adult RON.

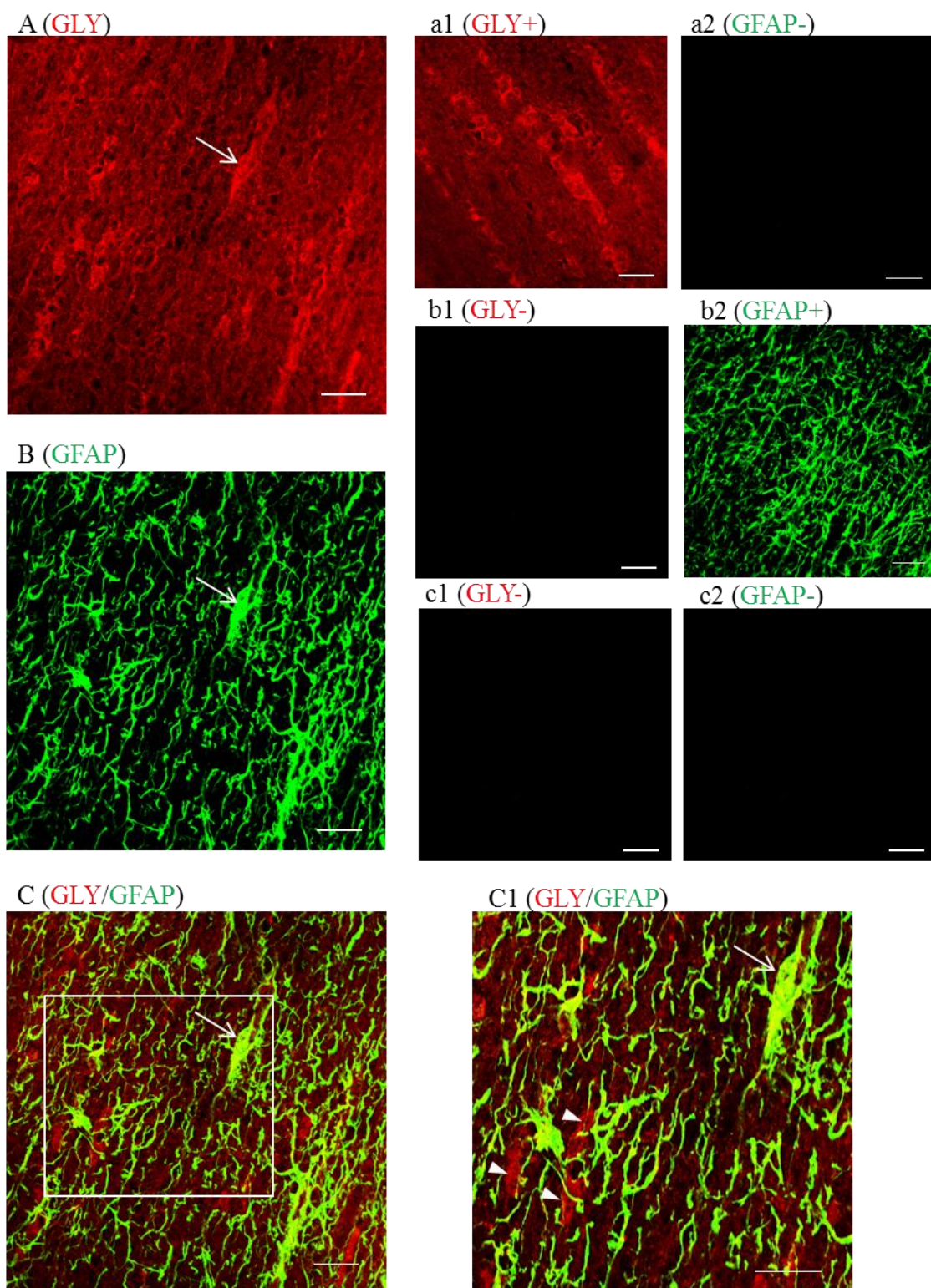


Figure 7-46: Adult RON staining for glycine receptor and GFAP.

A: Adult RON staining for glycine receptors (GLY/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GLY (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: Adult RON staining for astrocytes (GFAP/green).

b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse GLY (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: Adult RON double staining for glycine receptors (GLY/red) and astrocytes (GFAP/green). The box in C is shown in a higher magnification in C1. Notice the glycine receptor and GFAP co-localisation (arrows). However, there are some areas that are only stained for glycine (arrowheads).

c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μ m.

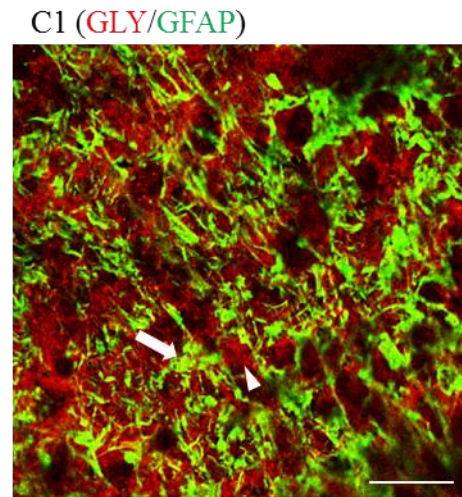
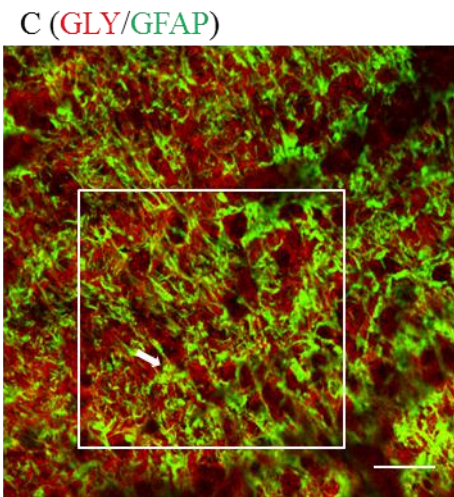
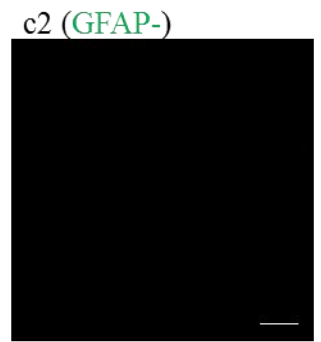
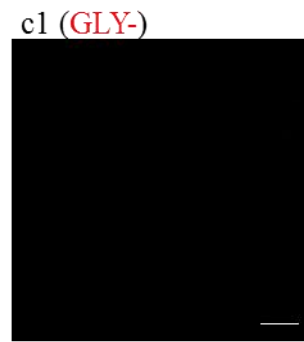
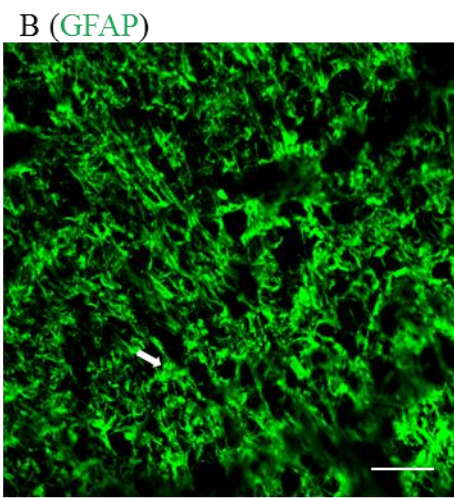
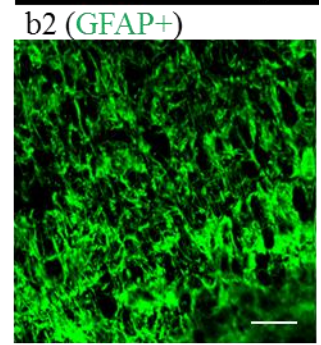
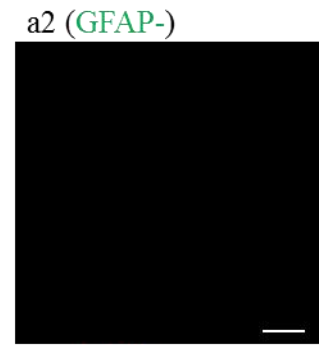
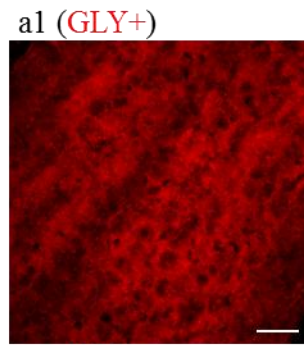
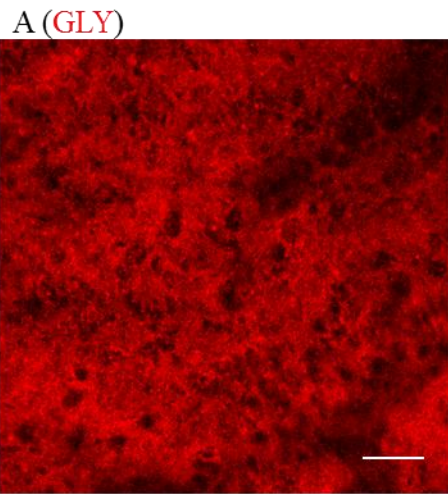


Figure 7-47: P10 RON staining for glycine receptor and GFAP.

A: P10 RON staining for glycine receptors (GLY/red). Glycine staining is diffused all over the optic nerve.

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GLY (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: P10 RON staining for astrocytes (GFAP/green).

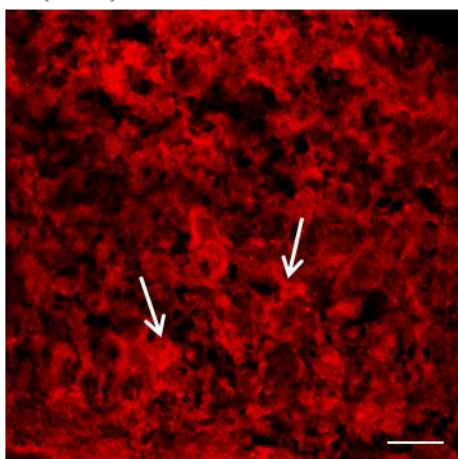
b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse GLY (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: P10 RON double staining for glycine receptors (GLY/red) and astrocytes (GFAP/green). The box in C is shown in a higher magnification in C1. Note the co-localisation between astrocytes and glycine staining (bold arrows). However, there are areas stained only with glycine receptor (arrowhead).

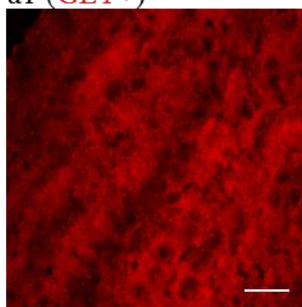
c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μ m.

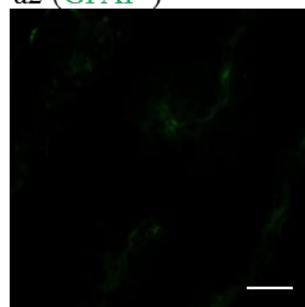
A (GLY)



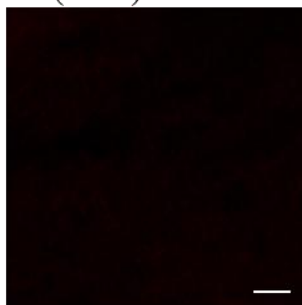
a1 (GLY+)



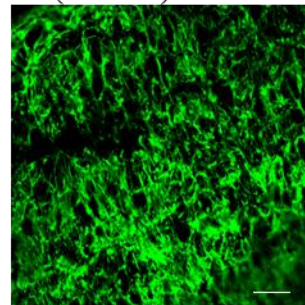
a2 (GFAP-)



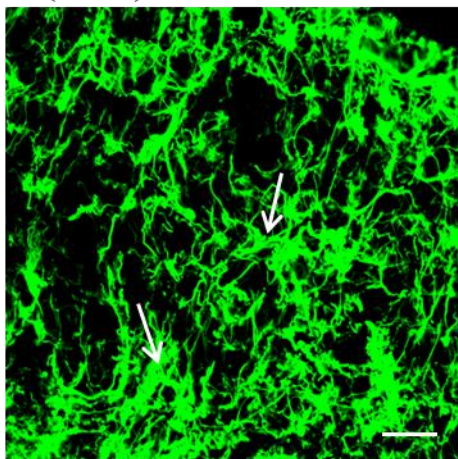
b1 (GLY-)



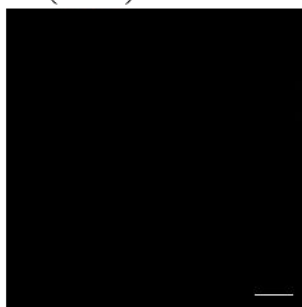
b2 (GFAP+)



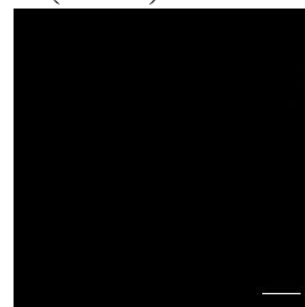
B (GFAP)



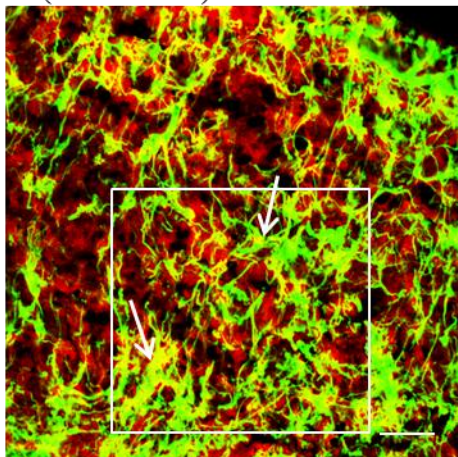
c1 (GLY-)



c2 (GFAP-)



C (GLY/GFAP)



C1 (GLY/GFAP)

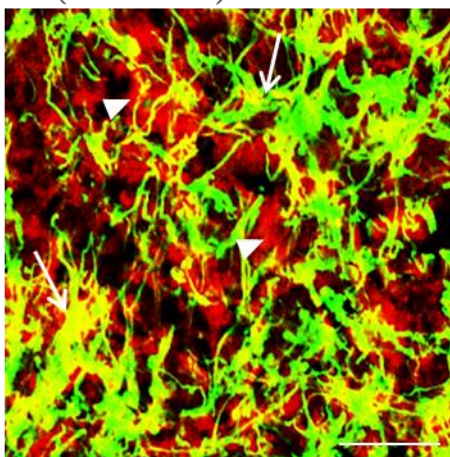


Figure 7-48: P0 RON staining for glycine receptor and GFAP.

A: P0 RON staining for glycine receptors (GLY/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GLY (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: P0 RON staining for astrocytes (GFAP/green).

b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse GLY (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: P0 RON double staining for glycine receptors (GLY/red) and astrocytes (GFAP/green). The box in C is shown in a higher magnification in C1. Note the co-localisation between astrocytes and glycine staining (arrows). However, there are areas stained only with glycine receptor (arrowheads).

c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μ m.

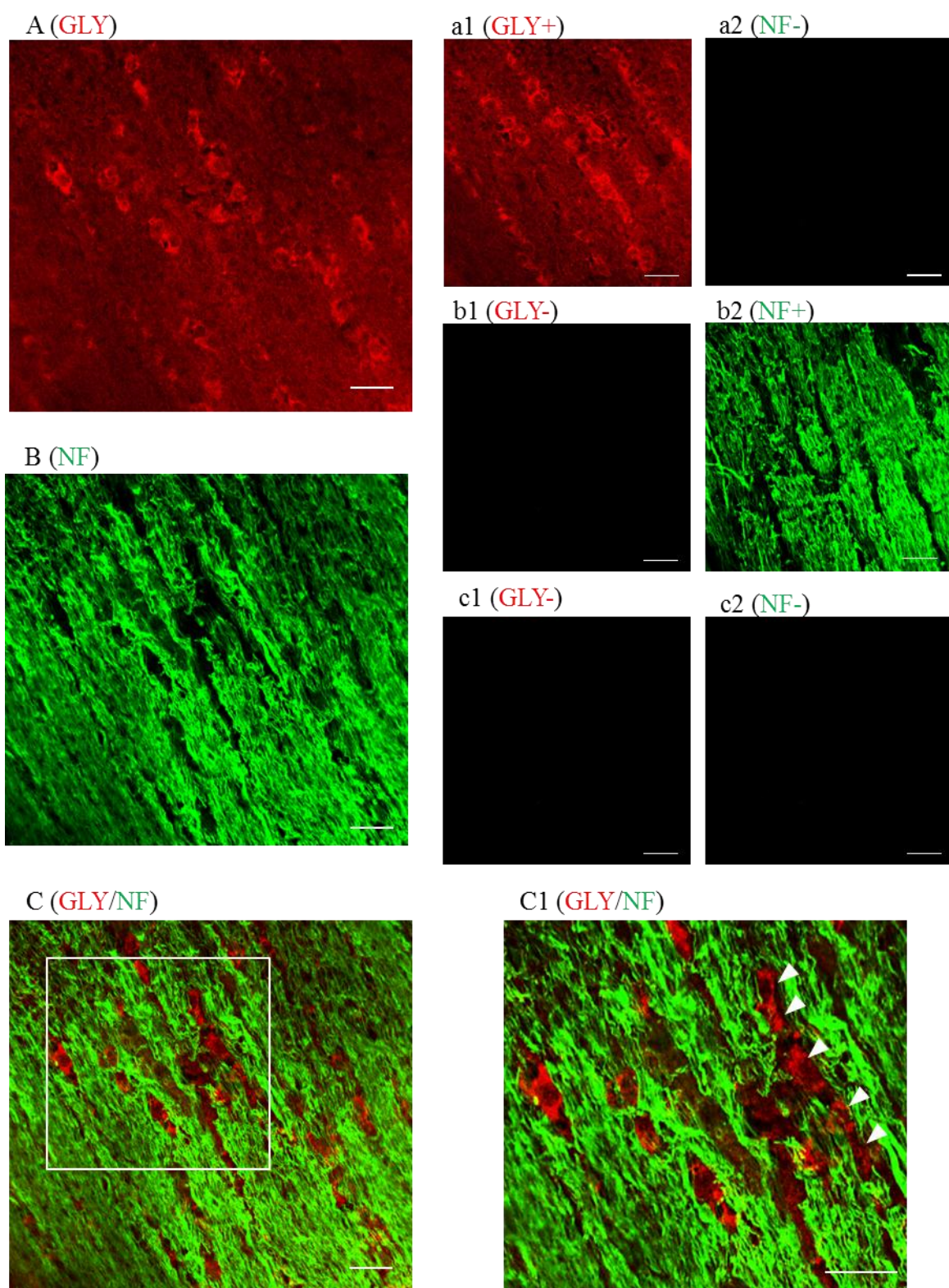


Figure 7-49: Adult RON staining for glycine receptor and NF.

A: Adult RON staining for glycine receptors (GLY/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GLY (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: Adult RON staining for axons (NF/green).

b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse GLY (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: Adult RON double staining for glycine receptors (GLY/red) and axons (NF/green). The box in C is shown in a higher magnification in C1. Note that glycine receptor (+) cells are aligned in a row (arrowheads).

c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μ m.

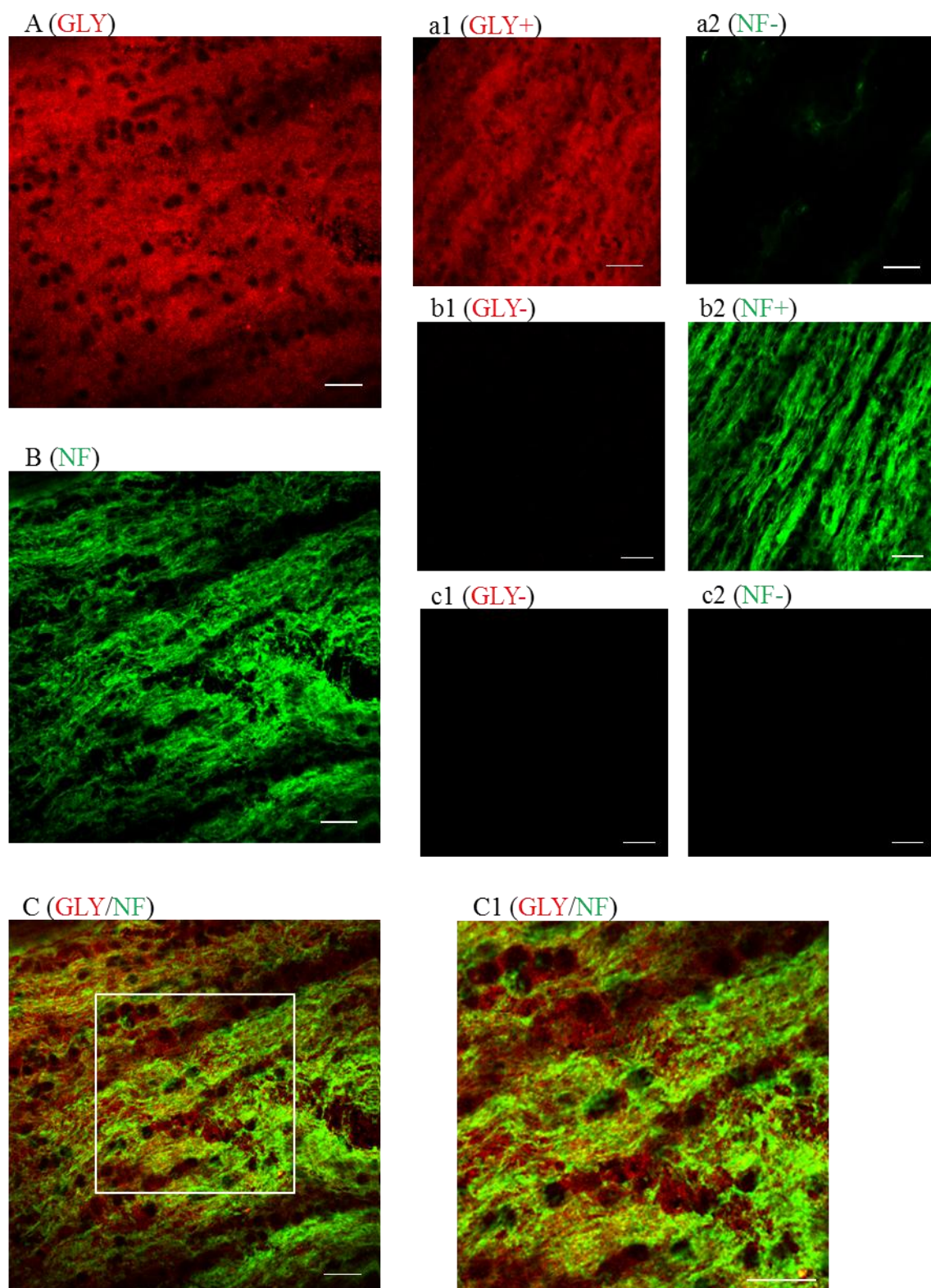


Figure 7-50: P10 RON staining for glycine receptor and NF.

A: P10 RON staining for glycine receptors (GLY/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GLY (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: P10 RON staining for axons (NF/green).

b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse GLY (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: P10 RON double staining for glycine receptors (GLY/red) and axons (NF/green). The box in C is shown in a higher magnification in C1.

c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μ m.

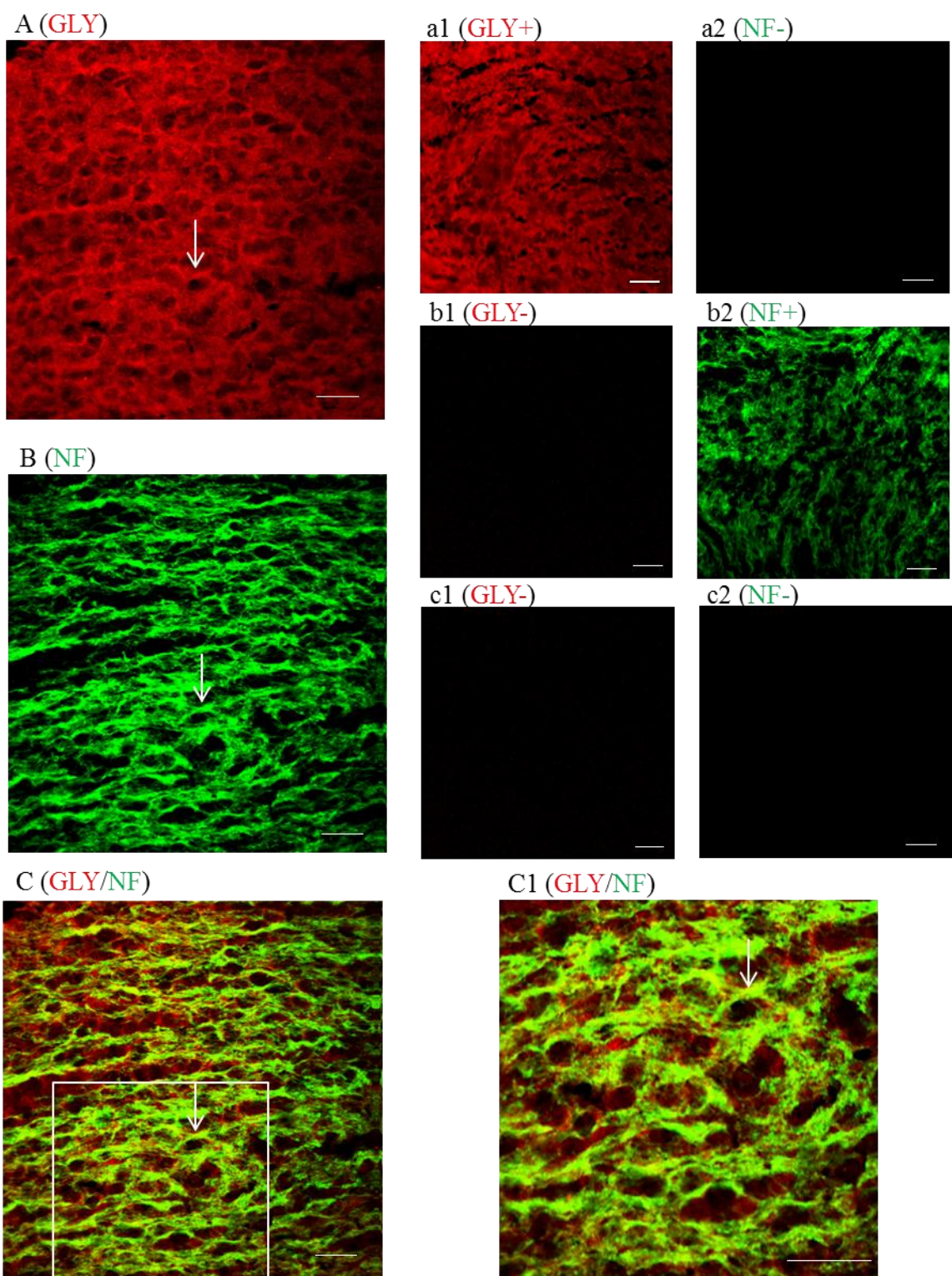


Figure 7-51: P0 RON staining for glycine receptor and NF.

A: P0 RON staining for glycine receptors (GLY/red).

a1 + a2: Negative controls for A. a1 and a2 stained with only one primary antibody: anti-rabbit GLY (red), and two different secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

B: P0 RON staining for axons (NF/green).

b1 + b2: Negative controls for B. b1 and b2 stained with only one primary antibody: anti-mouse GLY (green), and two secondary antibodies: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

C + C1: P0 RON double staining for glycine receptors (GLY/red) and axons (NF/green). The box in C is shown in a higher magnification in C1. Almost all the axons are stained with glycine (arrows).

c1 + c2: Negative controls where there is no primary antibody added, while both secondary antibodies are applied: anti-rabbit Alexa-568 fluorophore (red) and anti-mouse Alexa-488 fluorophore (green).

Scale bar = 2 μ m.

7.8 Conclusions:

7.8.1 Summary of Results:

As previously discussed, it has been found that the extracellular level of neurotransmitters increased during ischaemia. Ischaemia affects both neurons and glial cells. Both types of cells are known to express a wide range of neurotransmitter receptors. In this part of the thesis, I focused on the effects of GABA, glycine, nicotine and norepinephrine on ischaemic injury of developing central white matter astrocytes.

The mechanism of ischaemic injury in P0 RON astrocytes is Ca^{2+} -dependent (Fern, 1998). In the current study, measuring the intracellular Ca^{2+} level (340/380 ratio) and dye intensity (360 intensity) of Fura-2 loaded astrocytes was the method used to assess astrocyte viability. Ninety minutes of OGD was sufficient to significantly increase the percentage of cell death as compared to aCSF control (see Figure 7-52). Perfusion with 100 μM GABA and 100 μM glycine may have produced receptor desensitisation, as it reduced the percentage of cell death in both aCSF and OGD conditions. Blocking each receptor separately with 100 μM picrotoxin or 2 μM strychnine protected astrocytes from ischaemia-induced cell death. Higher concentrations of these antagonists did not produce any additional protection. Strychnine was shown to reduce the percentage of cell death to a very low level, which could not be produced by picrotoxin. However, the reduction in cell death produced by strychnine is not significantly different from that produced by picrotoxin. This could be related to the powerful depolarisation effect of glycine in nRON, which is found to be seven times greater than that achieved by muscimol application (Simmonds, 1983). Moreover, a combination of both antagonists does not add any additional protection to astrocytes as compared to each separately. These two antagonists not only reduced the

percentage of cell death, but also affected the distribution of cell death (see Figure 7-53). The initial cell death was postponed by approximately 30 minutes by picrotoxin, 20 minutes by strychnine, and 10 minutes in combination, compared to that seen in OGD alone. Immunohistochemistry revealed that these antagonists were acting on GABA_A and glycine receptors, which were located on both astrocytes and axons in P0 RON. Studying the ischaemic injury of P0 RON glial cells at the ultrastructural level revealed the protective effect of picrotoxin and strychnine, which is consistent with the Ca²⁺ imaging results. On the other hand, activation of nAChRs or adrenoceptors by nicotine or norepinephrine perfusion had no significant effect on the aCSF or OGD end result. Blocking these two receptors with 10 µM or 30 µM mecamylamine or 100 nM α-Btx, respectively, or with a combination of propranolol and phentolamine resulted in no protective action during OGD. Studying the ischaemic injury of P0 RON glial cells at the ultrastructural level revealed that mecamylamine or a combination of propranolol and phentolamine failed to protect glial cells from ischaemia which is consistent with the Ca²⁺ imaging results.

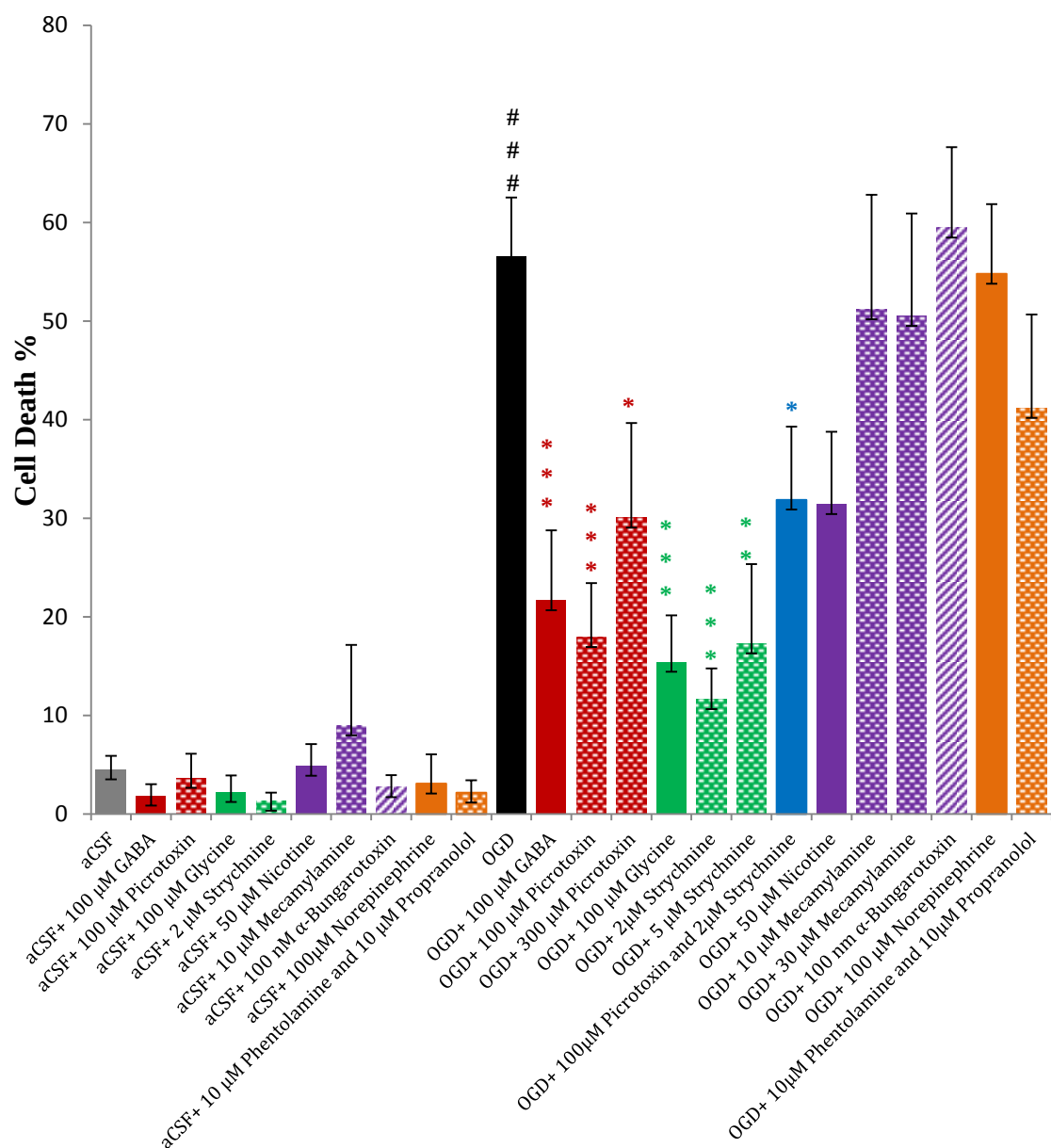


Figure 7-52: Histogram summarising the incidence of cell death in all agonists and antagonists and compared to controls (aCSF control and OGD control). *** represents a statistical significance as compared to OGD and ### represents a statistical significance as compared to aCSF ($p < 0.05$).

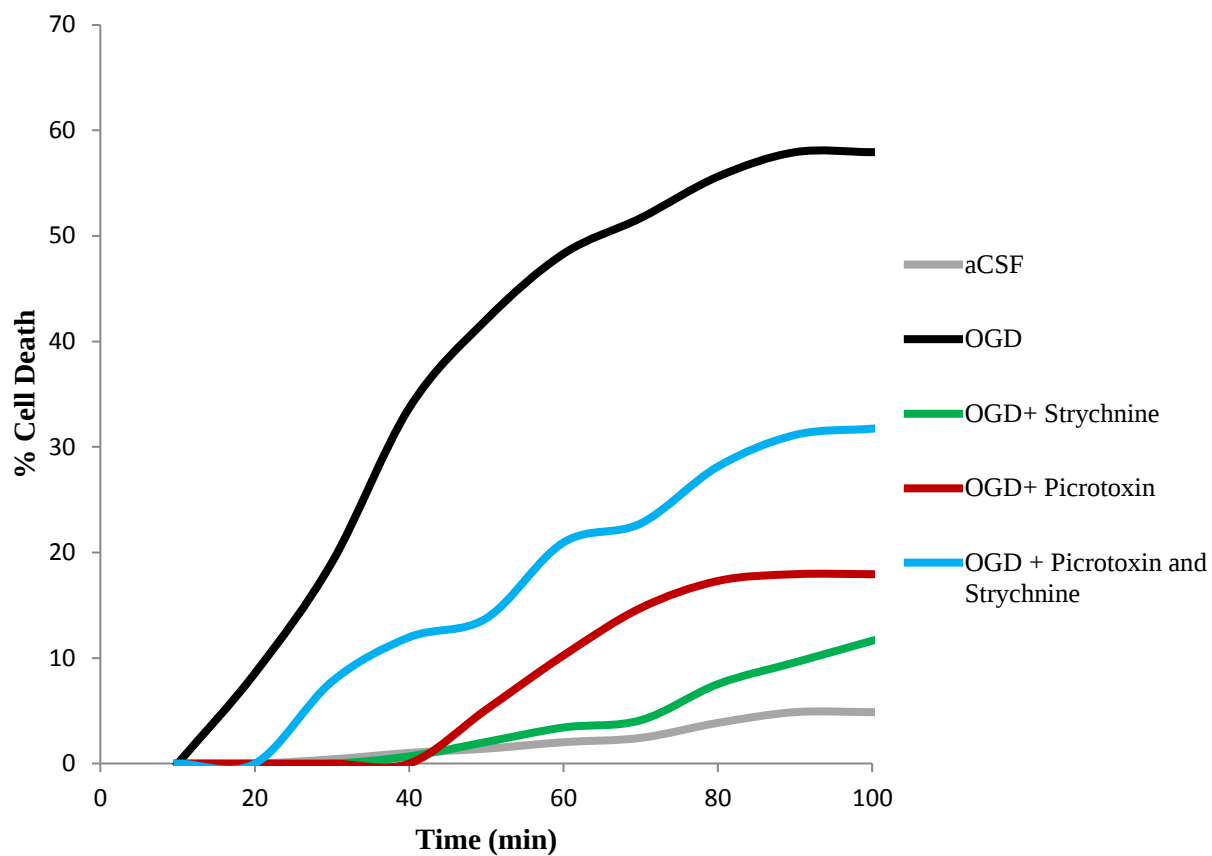


Figure 7-53: A comparison between the cell death during controls (aCSF and OGD controls) and OGD + 100 μ M picrotoxin and/or 2 μ M strychnine.

7.8.2 Discussion and Future Work:

The significance of WM during brain ischaemia has recently become an important field of study. Ischaemia is known to affect both WM and GM. WM occupies approximately 10% of the rodent brain volume, while it reaches 50% in the human brain, giving WM a vital role clinically (Baltan, 2009). Several studies have revealed complex mechanisms of ischaemic WM injury at different ages, locations and cell type (Alix and Fern, 2009, Fern, 1998, Fern, 2001, Fern et al., 1998, Fern and Moller, 2000, Fern and Ransom, 1997, Fern et al., 1995a, Salter and Fern, 2008a, Shannon et al., 2007, Stys, 1998, Stys, 2005, Thomas et al., 2004, Wilke et al., 2004, Alix et al., 2012, Salter and Fern, 2005). WM has no neuronal cell bodies (other than a few exceptions), and instead contains axons and glial cells. Glial cells are responsible for preserving a healthy environment, and any defect in glial function can lead to a lethal outcome. Focusing on astrocytes, our knowledge of the function of these cells has been transformed from being 'brain glue' to cells resembling neurons in responding to transmitters and propagating the signals (Haydon, 2001). These unexpected astrocyte functions have attracted much research interest in recent years. Astrocytes also play important roles in neurotransmitter homeostasis, pH regulation, K^+ buffering, energy metabolism, neurite outgrowth, synaptic remodelling and BBB formation (Barres, 2008, Dienel and Hertz, 2005, Rossi et al., 2007, Wang and Bordey, 2008, Chen and Swanson, 2003). WM astrocytes are replete with neurotransmitter receptors, such as glutamate, GABA, glycine, nAChRs and adrenergic receptors, as discussed previously in detail. Different subtypes of these receptors are expressed on astrocytes during development. PCR shows different receptor subunits of GABA, glycine, nAChRs and adrenergic receptors at different ages in RON glial cells (P0, P10 and adult) (Domingues et al., 2010). However, information about the presence of functional

receptors and the exact location of these receptors is lacking in P0 WM. In the current study, the localisation of GABA and glycine receptors has been studied in P0, P10 and adult RON. I found that nicotine and norepinephrine had no action on P0 astrocytes. The questions raised here are: Are there any functional nAChRs or adrenergic receptors at this age (P0) or not? If so, where are they localised? When do astrocytes start to have functional nAChRs and adrenergic receptors? One promising technique to measure changes in membrane potential is using voltage sensitive dyes (Brauner et al., 1984, Bradley et al., 2009, Peterka et al., 2011, Glover et al., 2008, Chemla and Chavane, 2010). This technique can be used to study the effect of neurotransmitters and their antagonists on glial cell membrane potential. This technique is easy to perform compared with glass microelectrodes, and the dyes are highly soluble in water and have a low phototoxicity (Bradley et al., 2009, Brauner et al., 1984). Membrane depolarisation increases the dye fluorescence intensity due to dye accumulation inside the cytoplasm, while membrane hyperpolarisation decreases the fluorescence intensity in the cytosol as the dye is sequestered in the cell membrane (Brailoiu et al., 2009). It is worth trying this technique to study the depolarisation/hyperpolarisation effect of different neurotransmitters and their blockers, thereby searching for the presence of functional receptors.

In conclusion, the work reported in this part of the thesis has presented new data extending our knowledge of the contribution of some neurotransmitters in ischaemic events of developing central white matter, furthering our appreciation of how blocking some of these can be pharmacologically manipulated to achieve favourable therapeutic outcomes.

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