Ischaemia and neurotransmitters in mature and immature white matter

A thesis submitted to the University of Leicester for the degree of Doctor of Philosophy in the Faculty of Medicine and Biological Sciences.

By

Tahani R. Mohamed Huria M.D., MSc

Department of Cell Physiology and Pharmacology,
Faculty of Medicine and Biological Sciences,
University of Leicester

Abstract

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Tahani Huria

Optic nerves are an appropriate and widely employed model used to study the function and the pathophysiology of central white matter. This thesis investigates ischaemic injury mechanisms in mature and immature white matter, using isolated adult and neonatal Wistar rat and balb-c mouse optic nerve. A central theme to this work is that both myelinated and non-myelinated central white matter injury is a partially glutamate-dependent process. Electrophysiology was used to record the compound action potential (CAP) under normal and pathological conditions in both myelinated and pre-myelinated (post-natal day 2: P2) optic nerves. Following a period of oxygen and glucose deprivation (OGD), both white matters were susceptible to excitotoxicity; mediated by over-activation of N-methyl D-aspartate type glutamate receptors (NMDA-Rs). The previously described higher tolerance of mature mouse optic nerve to OGD was eliminated by exogenous stimulation of NMDA-Rs via direct perfusion with agonists during OGD. My data reconcile earlier contradictions in the literature regarding the significance of NMDA-Rs for ischaemic injury in white matter in the two animals. A second major finding; ischaemic injury in P2 RONs was completely prevented by the NMDARs antagonist MK-801. Interestingly, both MK-801 and a second antagonist, memantine, were toxic to P2 RONs when perfused under control conditions. The presence of NMDA-Rs on pre-myelinated axons was confirmed by immuno-staining.

Neurotransmitters other than glutamate, such as GABA and glycine may also play a role in ischaemic injury of P2, with GABA and glycine receptor block being particularly protective of the CAP against damage. Electron-micrographs of pre-myelinated optic nerve axons and glia confirmed the data collected by electrophysiological recording of the CAP. These findings show that ischaemic damage of immature white matter is mediated largely by NMDA-Rs and that other neurotransmitter receptors also contribute to injury.

For my daughter and my family

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Chapter I

Introduction

In this thesis the mechanism of ischaemic injury observed following oxygen and glucose deprivation in both myelinated and non-myelinated white matter was investigated. Ischaemic injury to central white matter is responsible for several diseases in the brain, such as stroke in adult and periventricular leukomalacia in neonates. Several studies have been done to study the mechanism of white matter injury in adult, but little attention paid to study the mechanism of injury of white matter in neonates. Here a Wistar optic nerve model was used to study the ischaemic injury in both myelinated and non-myelinated white matter, and the role of glutamate and other neurotransmitters in mediating these conditions were investigated using electrophysiology to record the compound action potential and electron-microscopy for morphological changes. In this introduction I shall briefly describe the structure and function of white matter, generation of action potential and the pathophysiology of ischaemia in adults and neonates. In addition, a brief description of the optic nerve, used as the main experimental model in this study, will be given.

White matter

Both the brain and the spinal cord consist of white and grey matter (Wen and Chklovskii, 2005). The grey matter of the brain is composed of neuron cell bodies (often called nuclei or ganglia), axons, glial cells and capillaries (Wakana *et al.,* 2004; Stoffel and Bosio, 1997). It appears as an outer convoluted rim overlying the white matter such as the cortex, and also exists as islands within the white matter such as the deep nuclei (Wakana *et al.,* 2004). It lacks any significant amount of myelin and lipid component responsible for the white appearance of white matter and so appears to be pinkish-tan in colour (Wakana *et al.,* 2004; Siegel *et al.,* 1994). White matter consists mainly of axons, glial cells and blood vessels (Butt, 2005; Baltan, 2009). All central axons of a diameter less than 0.2 µm are non-myelinated, while those with a greater diameter tend to be myelinated (Butt *et al.,* 1998). The myelin membranes of the largest axons are part of Schwann cells in the peripheral nervous system (PNS) and oligodendrocytes in the central nervous system (CNS) (Demerens *et al.,* 1996). The lipid content of the myelin sheath gives the tissue a white appearance; therefore, areas of the nervous system that contain myelinated axon pathways are considered to be white matter (Waxman and Sims, 1984).

The electron microscope reveals the typical morphological features of myelinated axons (Hirano and Llena, 1995). The axoplasm contains neurofilaments (10 nm in diameter, common in mature axons), microtubules (24 nm in diameter, common in immature axons), and long filamentous structures that run along the axis of the axons (Hirano and Llena, 1995). Microtubules participate in axoplasmic transport, and both microtubules and neurofilaments help the axon in stabilising its shape. Smooth endoplasmic reticulum and mitochondria are abundant in axons, because the ATP consumption of an axon is high, and mitochondria contain enzymes necessary to transform glucose into high energy compound (Hirano and Llena, 1995). In 1984 Hildebrand and Waxman found that the axons of the developing rat optic nerve have vesiculotubular clusters that appear to be attached to the membrane, and these may be involved in axolemmal remodelling. At the time of myelination, the oligodendrocyte process deposits compact myelin around the axons (Huppi *et al.,* 1998).

In cross-section by electron microscope, myelin is viewed like a sequences of alternating dark and less-dark lines (protein layers) separated by non-stained zones (the lipid hydrocarbon chains) (Butt *et al.,* 1998). The dark layer is a dense layer attached to the inner leaflets of the plasma membrane and is called the inner tongue process; while the less-dark layer facing the extracellular surface, to which it is not attached, is called the external tongue process, and this is continuous with the oligodendrocyte cell body (Martini, 2005) *(see Fig 1).* The myelin membrane is composed of proteins and lipids: the most abundant proteins are proteolipid protein (PLP) and myelin basic protein (MBP), while myelin-associated glycoprotein (MAG)

is considered as a minor constituent (Stoffel and Bosio, 1997). The most important lipids are cholesterol, complex phospholipids and glycosphingolipids, comprising about 80 % of the dry weight of both CNS and PNS myelin which form the lipid bilayer (Stoffel and Bosio, 1997). Specifically, the myelin bilayer contains cholesterol (ethanolamine plasminogen); and the glycolipids (galactocerebroside (GalC), galactosulfaide (GalDG) and ganglioside GM1) are minor components (Siegel *et al,* 1999). The glycolipids are the dominant class of lipids in the myelin bilayer (Stoffel and Bosio, 1997). The lipid components, the glycolipids and cholesterol, give the myelin membrane very unique properties such as high electrical resistance. Both lipid and protein components are crucial for the long-term stability of the myelin sheath (Stoffel and Bosio, 1997).

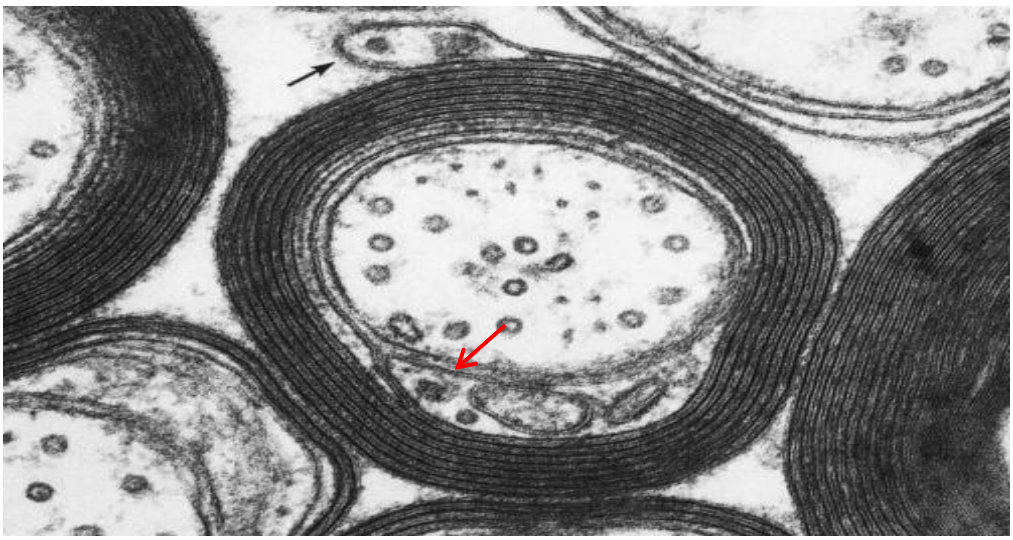


Figure 1- Cross-section of myelin by electron microscope.

A cross-section of myelin shows that the dark layer is a dense layer attached to the inner leaflets of the plasma membrane, which is called the inner tongue process (red arrow). The less-dark layer facing the extracellular surface, to which it is not attached, is called the external tongue process (black arrow). The figure is from Siegel *et al.* (1999).

There is a close relationship between oligodendrocytes and axons. In 1928 Rio-Hortega identified that it is the oligodendrocytes that are responsible for myelin sheath formation (Butt, 2005); and subsequent authors revealed different oligodendrocytes myelinate axons of different diameters, so bigger axons have a thicker myelin sheath (Waxman and Sims, 1984; Berry *et al.,* 1995; Butt *et al.,* 1998). Axons release growth factors that control the number of populating oligodendrocytes (Barres and Raff, 1994). Oligodendrocytes are neuro-ectodermal in origin and have cell soma ranging from 10-20 µm in diameter; they are globular and dense with an irregular cell margin (Waxman and Sims, 1984), and few cell processes can be seen (Ludwin, 1997) (*see Fig 2)*. The cytoplasm contains many organelles: endoplasmic reticulum, Golgi apparatus, mitochondria, free ribosomes, multi-vesicular bodies and coated vessels. Oligodendrocytes generally have an ovoid nucleus with clumps of dense heterochromatin (Barres and Raff, 1994). There are typical morphological features that distinguish oligodendrocytes from other glial cells, such as the absence of glial filaments and the presence of 24 nm microtubules at the margins of the cells, as well as the presence of lamellar dense bodies (Siegel *et al.,* 1994).

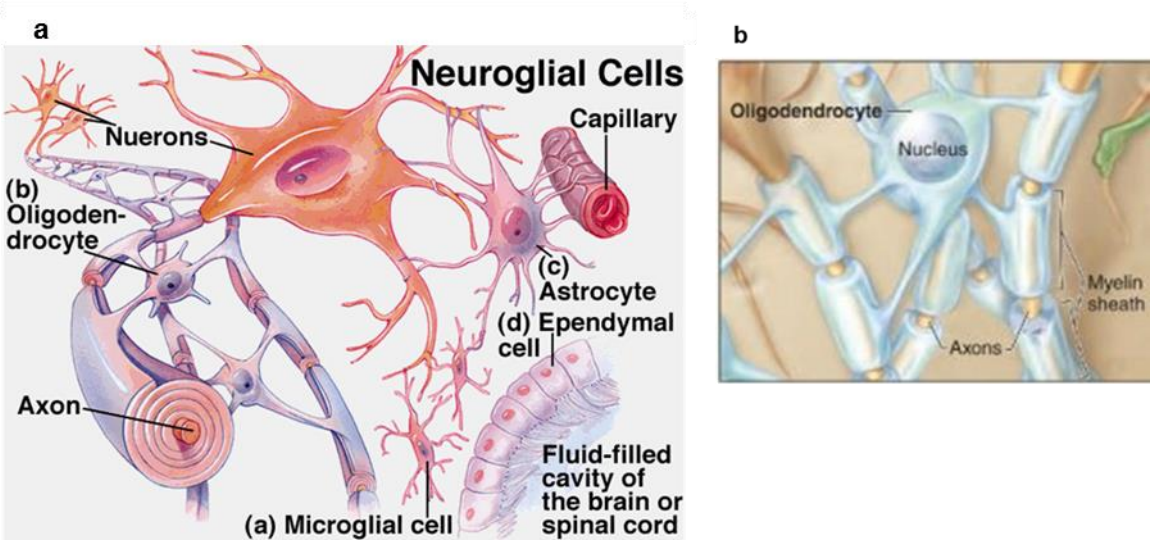


Figure 2- Oligodendrocytes and astrocytes.

An oligodendrocyte makes a compact myelin sheath around the axon while astrocytes interact with the blood vessels and synapses. Fig (a) is taken from (http://course1.winona.edu/sberg/ILLUST/neuroglialCellFunctions.gif), and fig (b) is taken from (http://www.cram.com/cards/chapter-12-nervous-system-nervous-tissue-2356543).

There are three types of oligodendrocytes in the brain; interfasicular oligodendrocytes are restricted to white matter; their size during myelination is 20 µm becoming 10-15 µm in adulthood with a large nucleus (Barres and Raff, 1994). The second type is satellite oligodendrocytes, which are perineural and are of 10 µm in size; they are restricted to grey matter and may serve to regulate the microenvironment around neurons (Barres and Raff, 1994). The third type is the intermediate oligodendrocytes, which have a small nucleus (Ludwin, 1997). Butt *et al.* (1989) also classified oligodendrocytes according to the size or the thickness of the myelin sheath: type I are small cells of 15-30 micro-diameter axons with thin myelin; type II and type III are intermediate; and the last type is composed of large cells of axons with thick myelin. Mori and Leblond (1970) distinguished three types of oligodendrocytes: light, medium, and dark. The dark types have the densest cytoplasm, and the light types are the most actively dividing cells; they become progressively darker as they mature.

Oligodendrocytes contain proteins that can be used to generate specific antibodies as a molecular marker such as MBP, proteolipid protein, MAG, and GalC, which are applied routinely for anatomical analysis *in vivo* and *in vitro (* Jahn *et al.,* 2009). Oligodendrocytes originate from migratory and mitotic precursors and mature progressively into post-mitotic myelin-producing cells (Bauman and Pham-Dinh, 2001). They begin first as oligodendrocyte precursor cells that originate from the neuroepithelial cells of the ventricular zones at very early stages during embryonic life, then transform into oligodendrocyte progenitor cells which migrate throughout the CNS and settle along the fibre tracts of future white matter (Bauman and

Pham-Dinh, 2001). These cells subsequently transform into pre-oligodendrocytes, which are multi-processed cells that preserve the property of cell division and gain the marker O4 (Sommer and Schachner, 1981). The pre-oligodendrocytes become immature oligodendrocytes, categorised in the rat by the presence of the marker GalC; they then become non-myelinating mature oligodendrocytes, and finally are transformed into myelinating mature oligodendrocytes (Bauman and Pham-Dinh, 2001), *(see Fig 3).*

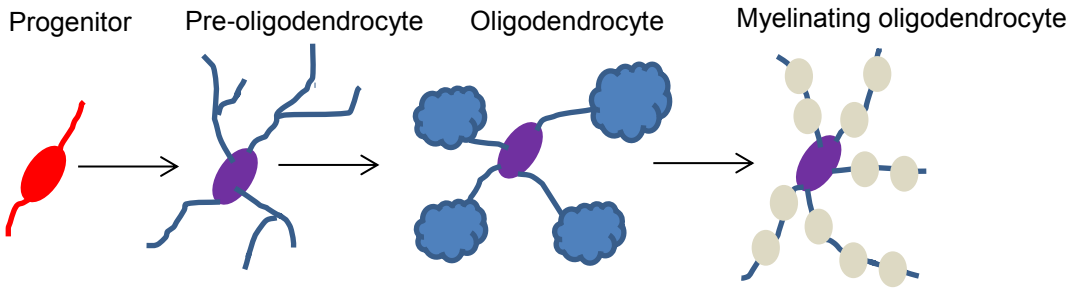


Figure 3- The developmental stages of oligodendrocytes.

The process begins with immature progenitor cells and concludes with fully myelinated oligodendrocytes (adapted from Back, 2006).

Astrocytes are ectodermal in origin and are of two types: protoplasmic astrocytes with clear cytoplasm, which are restricted to grey matter; and fibrous astrocytes, which populate the white matter and are characterised by an ovoid nucleus with homogenous chromatin and a large number of glial filaments arranged in tight bundles (Miller and Raff, 1984). Astrocytes control and limit the diffusion of neurotransmitters into extracellular space (Thomas *et al.,* 2011). The other function includes the uptake of K^+ released from the node of Ranvier following an action potential (Ransom and Orkand, 1996). It has been recognised that astrocytes react with other substances released by axons such as glutamate and ATP (Kriegler and Chiu, 1993). ATP released from axons increases the synthesis and release of the leukaemia inhibitory factor (LIF) in astrocytes, which in turn promotes myelination (Ishibashi *et al.,* 2006). It has been shown that glutamate and ATP released from the axons result in rises in intracellular Ca^{2+} in white matter glia (Hamilton *et al.,* 2009). Both glutamate and ATP evoke Ca^{2+} signals in NG-2 glia *in situ*, acting on glutamate and purine receptors (Hamilton *et al.,* 2009). White matter astrocytes also release neurotransmitters to propagate intercellular Ca^{2+} signals to neighbouring glia (Hamilton *et al.,* 2008). Cytoplasmic markers of astrocytes include glial fibrillary acidic protein (GFAP) (Pfeiffer *et al.,* 1992).

In addition to oligodendrocytes and astrocytes, a third type of macroglial cell has been identified in CNS white matter: the NG-2 cell (Butt *et al.,* 2002). NG-2 cells express the NG-2 chondroitin sulphate and are primarily considered as adult oligodendrocyte precursor cells in the mature CNS, as well as oligodendrocyte precursor cells in the developing CNS (Chang *et al.,* 2000; Nishiyama *et al.,* 2002). Mature NG-2 glia contacts the nodes of Ranvier, making them perfectly suitable to respond to changes in axon integrity (Butt *et al.,* 1999). Mature NG-2 cells are morphologically similar to astrocytes but they lack astrocyte markers, raising the question of whether or not they are a separate population (Butt *et al.,* 2002). The latter named NG-2 glia as synantocytes to differentiate them from oligodendrocytes forming oligodendrocyte precursor cells (Butt *et al.,* 2002). Finally, microglia of mesodermal origin populate both the white matter and the grey matter of the CNS in a resting state and are supposed to become very mobile, active macrophages during disease (Butt *et al.,* 2002). Microglia invades the CNS at the time of vascularisation via the pia mater, the walls of blood vessels (Butt *et al.,* 2002).

Steps of myelination

The mechanism of myelination is not completely understood, however there is some evidence regarding the sequential steps involved (Bauman and Pham-Dinh, 2001):

1) Migration of oligodendrocytes to axons that are to be myelinated;

2) Connection of the oligodendrocyte process to the axon;

3) Twisting of the process around the axon, with a pre-arranged number of myelin sheaths and the recognition of the space not to be myelinated, e.g. the node of Ranvier.

Myelination starts caudorostrally in the brain and rostrocaudally in the spinal cord (Bauman and Pham-Dinh, 2001). Myelination can be detected using electron microscopy between P6 and P8 (Foster *et al.,* 1982; Hildebrand and Waxman, 1984); myelin associated glycoprotein has been successfully stained at P7 by using immunofluorescence (Rasband *et al.,* 1999). As development continues the length of single layer sheaths increases, compact myelin formed by sheaths that are composed of at least five layers can be first seen between P10 and P12 (Hildebrand and Waxman, 1984). In the mouse, myelination starts in the spinal cord at birth and extends to almost all areas of the brain by 45-60 days postnatal time. In contrast, myelination in humans starts in the second half of foetal life in the spinal cord and peaks during the first year of the postnatal period; it continues until 20 years of age in some of the cortical fibres (Back *et al.,* 2001; Miller *et al.,* 2012).

Optic Nerve

The optic nerve is a unique part of the CNS and is used for research because of a lack of intrinsic neurons and neuronal cell bodies forming one of the simplest parts of the CNS (Raff *et al.,* 1987). The eye is derived from the neural tube, which is neuro-ectoderm in origin (Orgul and Cioffi, 1996). The earliest stage of prenatal development is embryogenesis, which finishes at the end of gestational week 3. At this time; the optic groove appears on either side of the midline at the expanded cranial end of the still-open neural folds (Orgul and Cioffi, 1996). Organogenesis is the second period of prenatal development that follows the embryogenesis; it begins at gestational week 4 and continues until week 8. Week 4 is characterised by the formation of the optic vesicle from optic sulci on either side of the forebrain (Orgul and Cioffi, 1996). The vesicles continue to grow, then are invaginated and form the optic cup, which is connected to the diencephalon by the constricted and hollow tube called the optic stalk (Raff, 1989). The embryonic fissure is formed as a result of the invagination of the vesicles; in humans this process starts at 22 days of development (Stiles & Jernigan, 2010). The forming stalk ultimately becomes the rudiments of the optic nerve, which is then ensheathed in all three meningeal layers (dura, arachnoid and pia mater) (Raff, 1989). The inner layers of the retina begin to differentiate during embryonic days 14-16 (E14-16) and the ganglion cells are made leading to the formation of tiny axons (0.2-0.3 μm) which extend and invade the optic stalk forming groups of different size (Kuwabara, 1975). The axons of the optic nerve are approximately 0.2 μm in diameter at birth and separated by an abundant extracellular space with a small number of glial cells; the optic nerve diameter

increases from about 1.6 mm inside the eye, to 3.5 mm in the orbit, to 4.5mm in the cranial space (Foster *et al.,* 1982).

The optic nerve has 2 types of macroglial cells: astrocytes and oligodendrocytes (Raff *et al.,* 1987). Astrocytes appear on embryonic day 16 (E16), and oligodendrocytes appear on the day 21 (E21) (Miller *et al.,* 1985). Astrocytes develop from astrocyte precursor cells, whereas oligodendrocytes develop from NG-2 cells that are bi-potential in cell cultures (O-2A cells) (Raff*,* 1989). Many studies have indicated that O-2A progenitor cells are motile (Barbarese *et al.,* 1983). Temple and Raff (1986) and Wolswijk *et al.* (1990) studied the neonatal optic nerve through time-lapse photography and proposed that O-2A progenitor cells are the only progenitor cells that migrate until they differentiate into oligodendrocytes. This hypothesis is consistent with another study which found that O-2A progenitor cells are motile and migrate along the optic nerve from the optic chiasm to the eye during development (Small *et al.,* 1987).

Previous studies using E17 rats showed that O-2A progenitor cells are found in small numbers at the chiasm end but not at the eye end of the optic nerve; though by the second postnatal week they are distributed along the optic nerve (Small *et al.,* 1987). With regard to the two main glial cell types present in the optic nerve, astrocytes develop first, followed by oligodendrocytes (Miller *et al.,* 1985; Raff, 1989; Butt and Ransom, 1993). Oligodendrocytes form the myelin sheaths; while the astrocytes extend processes into the node of Ranvier, nourish the adjacent axons and stabilise the local extracellular ion concentration (Raff *et al.,* 1987). Astrocytes induce the endothelial cells to form tight vessels (Janzer & Raff, 1987) and secrete

growth factors which are responsible for the proliferation and differentiation of O-2A progenitor cells (Noble & Murray, 1984; *Raff et al.,* 1985). Interestingly, astrocytes in the lamina cribosa area of the optic nerve stop O-2A progenitor cells from entering the retina to prevent differentiation and myelination from occurring there. Such myelination would damage the retina by making it opaque and therefore impairing vision (Small *et al.,* 1987). The lamina cribosa is a non-myelinated region where the nerve pierces the sclera and acts as a barrier to prevent the migration of O-2A progenitor cells into the retina (Berliner, 1931).

In the PNS the myelinating Schwann cells can repair injury following differentiation and re-myelination, whereas in the CNS the O-2A progenitor cells found in the adult brain might not be able to migrate and differentiate in the demyelinated areas. Reactive gliosis associated with demyelinated lesions may prevent the O-2A progenitor cells from entering lesions (Raff *et la.,* 1987). More work is needed to answer the question of why this cell does not re-myelinate following injury.

Siegel *et al.* (1999) observed that larger numbers of mitochondria are located in the non-myelinated segments of axons than in the myelinated segments. In the optic nerve, the non-myelinated pre-laminar and laminar regions exhibit higher mitochondrial enzyme activity when compared to the post-laminar myelinated regions; the mitochondrial function is essentially producing the energy ATP which is essential for the survival of the cells (Bristow *et al.,* 2002). The metabolic requirements are different in individual cells even at the intracellular level (Bristow *et al.,* 2002), where the outer segments of the photoreceptor cells of the retina are packed with

mitochondria and have a high degree of cytochrome-c-oxidase activity compared to the inner segments, due in part because the outer segments require a large amount of energy to maintain membrane potential (Wong-Riley, 2010; Runge *et al.,* 1986,). Myelinated nerve fibres conduct by salutatory conduction, while non-myelinated nerve fibres require more energy to repolarise their plasma membranes (Bristow *et al.,* 2002). Therefore, there are large numbers of mitochondria and high enzyme activity in the non-myelinated regions of the lamina cribosa. Electron-microscopic studies have confirmed that the non-myelinated fibres of the human lamina cribosa have more mitochondria than the myelinated fibres (Hollander *et al.,* 1995). In contrast, Bristow *et al.* (2002) found that most of the nerve fibres in rabbits are myelinated, and mitochondrial accumulation in the cribrosa and beyond was not seen.

Action potential

The function of the axon is to deliver information in the form of electrical activity from one neuronal cell to a number of end targets (Goldberg, 2003). This electrical activity is known as the action potential (Goldberg, 2003). There are two major ions that play a role in action potentials: Na^+ and K^+ (Bers, 2001). However, other ions Ca^{2+} and Cl^- may help contribute to the resting membrane potential (Bers, 2001; Siegel *et al.,* 1994). At rest, Na^+ and Cl^- ions are concentrated extracellular while K^+ is concentrated intracellular (Bers, 2001; Siegel *et al.,* 1994).

The equilibrium potential for an ion (E_{ion}) is described by the Nernst equation which calculates, for defined intra and extracellular ion concentrations, the theoretical membrane potential if the membrane was 100% perfectly selective for that ion (Bers, 2001; Siegel *et al.,* 1994).

Nernst Equation (Bers, 2001):

$$E_{ion} = RT/ZF \ In \ [ion]_o/[ion]_i$$

E_{ion} - Equilibrium potential for ions (mv).

R- Gas constant (8.3 joule.K^{-1}.mol^{-1}).

T- Temperature in Kelvin.

F- Faraday's constant (96500 coulombs. mol-^{1}).

Z- Valency of ion (e.g. +1 for monovalent cations).

E_{ion} is a theoretical value where there is no net movement of charge. For K^+ ions the large concentration gradient allows ions to efflux, however increasing K^+ efflux increases intracellular negative charge. The point where these are opposing movements are equal is termed the equilibrium potential for K^+ (E_k) (Bers, 2001). Most cells in the resting state reside close to E_k as this is the predominant permeating ion at rest; however, they do not have a resting membrane potential at E_k due to the fact that the membrane is not 100% selectively permeable to a single ion. Small inward Na^+ and Ca^{2+} fluxes via spontaneous channel opening, carriers and other transporters cause a slight depolarisation and so the resting membrane potential is generally around - 70mV rather than close to E_k (~ - 90 mV) (Bers, 2001). Increase in permeability to other ions, particularly Na^+ and Ca^{2+} cause a depolarisation of the membrane potential. Charges in ionic permeability are the basis of electrical signalling in neuronal signalling (Bers, 2001; Siegel *et al.,* 1994).

Phases of action potential

(Barnett and Larkman, 2007; Bers, 2001; Hodgkin and Huxley, 1952).

1- Threshold phase

It precedes the action potential, in which the inward Na^+ current exceeds the outward K^+ current leads to opening of more voltage-gated Na^+-channels. The initial depolarization reaches a threshold of ~ 10-20 mV above the resting potential.

2- Rising phase

Increase in Na^+ permeability due to opening of more voltage-gated Na^+-channels pushing the membrane potential towards E_{Na}.

3- Overshoot phase

The membrane potential is above zero.

4- Peak

High level of depolarization occurs during this phase that precedes the falling phase.

5- Falling phase

Opening of voltage-gated K^+-channels and inactivation of Na^+-channels lead to membrane repolarization by increased permeability of K^+ and decreased Na^+ respectively, so pushing membrane potential towards E_K *(see Fig 4A).*

6- Undershoot phase

The membrane potential is hyperpolarized because the membrane potential is lower than the resting potential due to the large K^+ flux. The myelinated optic nerve action potential lacks the undershoot phase, the down-stroke being due largely to the inactivation of Na^+-channels (Gordon *et al.,* 1988).

7- Refractory period

In this Period Na^+-channels recover from inactivation.

More positive signals in the cytoplasm will depolarize the membrane, and more negative signals in the cytoplasm will hyperpolarize the membrane (Siegel *et al.,* 1994). An action potential may last 0.4 msec at any one area and propagate at a speed of 100 m/sec (Siegel *et al.,* 1994).

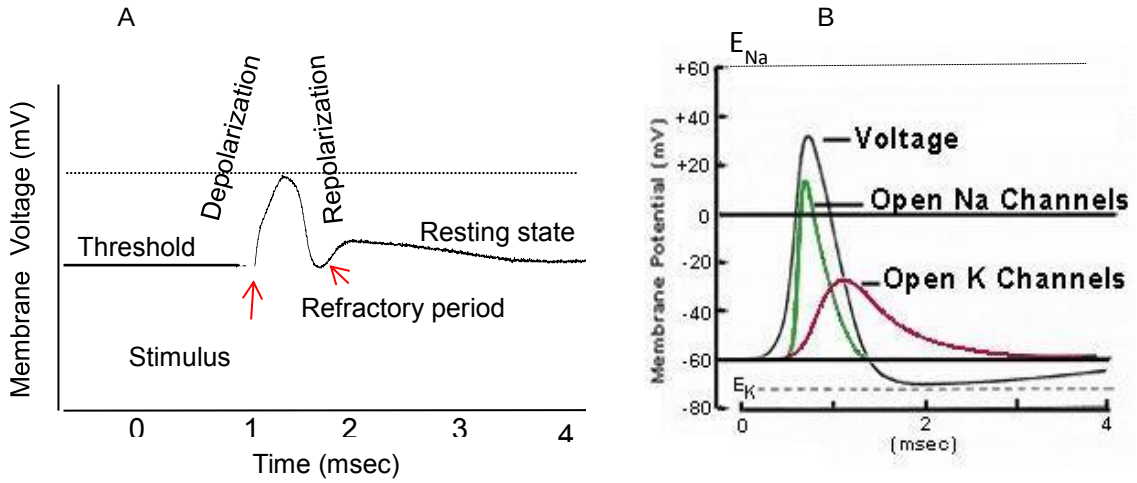


Figure 4A- Action potential

A: Multi phases of extracellular action potential start from the threshold and end with the refractory period. **B**: Changes in membrane permeability for Na+ and K+ during action potential. Both Na+ and K^+ are driven toward the equilibrium potential of + (60-65mV) and – (80-85mV) respectively, (Siegel *et al.*, 1994; Bers, 2001).

Action potential generation in developing and developed axons

As an axon can be non-myelinated or myelinated, the action potential travels down the axon by two quite different methods: continuous action potential conduction for non-myelinated axons, and saltatory conduction for myelinated axons (Debanne *et al.,* 2011). In both myelinated and non-myelinated axons, action potentials are initiated at the initial segment of the axon (AIS) and are generated by a threshold stimulus received from the dendrites and the cell body (*see Fig 4B)* (Debanne *et al.,* 2011). In non-myelinated axons, the action potential is generated as a wave all the way along the axon (Giuliodori and DiCarlo, 2004). Once initiated, the action potential spreads and triggers the adjacent voltage-gated channels that cause action potential propagation to occur (Giuliodori and DiCarlo, 2004). Action potential occurs as a rapid alternative depolarization and repolarization of a small portion of the axon membrane (Debanne *et al.,* 2011). When Na^+-channels open induce axonal membrane depolarization and Na^+ ions diffuse into the axon, then the axon repolarize when K^+-channels open and K^+ exit the axon (Agur and Dalley, 2008; Darke *et al.,* 2009). A series of action potentials is required to propagate a signal along the whole axon (Agur and Dalley, 2008; Darke *et al.,* 2009). Each single action potential is triggered by spread of the passive current generated by the previous action potential (Tank, 2008; Darke *et al.,* 2009). The action potential in myelinated axons jumps between the nodes of Ranvier, where voltage-gated Na^+-channels are concentrated; hence, when the nerve

impulses travel down myelinated axons they appear to jump from one node to the next called saltatory conduction (Lai and Jan, 2006). Action potential at each node is triggered by a threshold stimulus created by passive current from the previous node (Barrett *et al.,* 2009). Action potential is faster in myelinated axons than non-myelinated axons because of high density of Na^+-channels at Node of Ranvier. When the previous node repolarizes lead to opening of Na^+-channels in the next stimulated node and influx of Na^+ ions that cause depolarization in this area(Agur and Dalley, 2008; Darke *et al.,* 2009). Once the action potential reaches the nerve terminal, it causes depolarisation and the opening of voltage-gated Ca^{2+}-channels, allowing the entry of Ca^{2+} into the cell and producing an increase in the concentration of intracellular Ca^{2+} (Lai and Jan, 2006). This causes the mobilisation of the neurotransmitter-containing vesicles and the fusion of these vesicles with the presynaptic membrane, releasing the neurotransmitters by exocytosis, which involves sequences of protein interactions (Bender and Trussell, 2009). Two proteins in the vesicle walls, synaptotagmin and synaptobrevin, act together with the complementary proteins in the axon terminal (e.g. snap-25 and syntaxin) to localise the vesicles in the appropriate sites for neurotransmitter release (Bender and Trussell, 2009). Once the neurotransmitter is released into the synaptic cleft, it binds to post-synaptic receptors, for example ionotropic receptors which are direct ligand-gated receptors (Foust *et al.,* 2010; Palmer *et al.,* 2010; Bender and Trussell, 2009). This interaction between these receptors and the neurotransmitters causes the ion-channels to open or close and subsequently changes the membrane potential or the post-synaptic cell function depending on the channel type (Foust *et al.,* 2010;

Palmer *et al.,* 2010; Bender and Trussell, 2009). Other post-synaptic receptors are metabotropic receptors (G-protein and second messenger coupled). The second messenger system includes adenylyl cyclase, guanylyl cyclase and phospholipase-C, which produce cyclic-adenine monophosphate (cAMP), cyclic guanosine-monophosphate (cGMP), and both diacylglycerol (DAG) and inositol-triphosphate (IP3) respectively (Debanne *et al.,* 2011).

Various studies have used voltage-sensitive dyes and other approaches to determine the spike initiation zone of action potential (Foust *et al.,* 2010; Palmer *et al.,* 2010; Bender and Trussell, 2009). Na^+-spikes usually occur in the axon in the soma, in a region called the AIS (Mathy *et al.,* 2009; Williams and Stuart, 1999). In myelinated axons, action potential is located between 15 µm-40 µm from the soma (Atherton *et al.,* 2008; Khaliq and Raman, 2006). In contrast, in non-myelinated axons the action potential is initiated 20 µm-40 µm from the axon hillock (Boudkkazi *et al.,* 2007; Yu *et al.,* 2008). The spike threshold was identified by Noble and Stein (1966) and defined as the voltage where the whole collected inward membrane current exceeds the outward membrane current. Previously, it had been suggested that the voltage threshold was 10-20 mv lower in the AIS (more hyperpolarised) than in the cell body (Coombs *et al.,* 1957). Action potential propagation along non-myelinated axons depends on the passive spread of the current of the active region of the membrane to depolarise the next segment (Coombs *et al.,* 1957). A rapid influx of Na^+ ions occurs near the edge of the action potential, which leads to the depolarisation of a new segment of membrane toward the threshold. Subsequently, current flows out due to the opening of K^+-channels; accordingly, the membrane potential

returns to the resting value (Debanne *et al.,* 2011). The inactivation of voltage Na^+-channels and hyperpolarising K^+-channels prevents the axonal membrane that has been excited from being directly re-excitable (Hodgkin, 1954; Debanne *et al.,* 2011). As a result, the action potential cannot propagate backward and therefore the conduction is unidirectional (Debanne *et al.,* 2011). Once the action potential leaves the activated area, the Na^+-channels become re-primed, K^+ conductance decreases, and the membrane becomes susceptible to re-excitation (Colquhoun and Ritchie, 1972).

During the early stages of development prior to axon myelination, action potential conduction is mediated by a low density of Na^+-channels (~ 2/μm^2) spread along the axon (Waxman *et al.,* 1989; Shatz, 1996); the velocity of the action potential conduction increases dramatically during development from nearly 0.2m/sec at birth to nearly 30m/sec in the adult (Foster *et al.,* 1982). This increase in the velocity of the action potential conduction is correlated with the increase in the axonal diameter and myelination. The most powerful structural factors that control the conduction velocity of the mammalian axons are the diameter of the axon and the presence of myelin (Waxman, 1980). The smallest axons in the CNS are nearly 0.1 microns in diameter (Waxman, 1980); and the majority of axons are > 0.3 microns are myelinated in the CNS (Waxman *et al.,* 1989). conduction velocity in non-myelinated axons depends mainly on the availability of Na^+-channels; a low concentration of the external Na^+, or the blocking of Na^+-channels by Na^+-channel blockers such as TTX, leads to a large decline in the conduction velocity and an eventual block (Katz, 1947; Colquhoun and Ritchie, 1972). Higher densities of Na^+-channels produce an increase in the action potential amplitude and

rise-time; consequently the adjacent axonal regions are excited faster, and as a result the velocity of the conduction is increased (Colquhoun and Ritchie, 1972; Del Castillo and Moore, 1959; Hodgkin, 1954). Conduction velocity depends toughly on the rise time of the action potential which in turn depends on the density of Na^+-channels responsible for the onset of the action potential (Waxman, 1975).

The second major parameter controlling conduction velocity is membrane capacitance (Hodgkin, 1954). If the capacitance of the membrane is small, the time required to reach the threshold is short, and hence the conduction velocity is high (Hodgkin, 1954). Myelinated axons have a high membrane resistance and low membrane capacitance. The third parameters is temperature, Na^+-channel kinetics are affected by temperature; low temperature makes Na^+-channels open and close more slowly, thus reducing the conduction velocity (Chapman, 1967; Franz and Iggo, 1968; Hodgkin and Katz, 1949). However, in myelinated axons the conduction velocity is also dependent on neuro-glia interactions that mediate myelination (Arbuthnot *et al.,* 1980; Constantinou and Fern, 2009; Fields, 2008). For example, the depolarisation of a single oligodendrocyte was found to increase the conduction velocity of the axons, which it myelinated by nearly 10% (Binczak *et al.,* 2001). Action potential conduction velocity is generally slow in non-myelinated axons (approximately 0.25 m/s– 0.38 m/s) in comparison to myelinated axons (Andersen *et al.,* 2000; Waxman et al., 1989), in which it is very fast and is principally dependent on the axonal diameter; therefore when the axon diameter exceeds 1-2 µm, the conduction velocity will be rapid (Rushton, 1951).

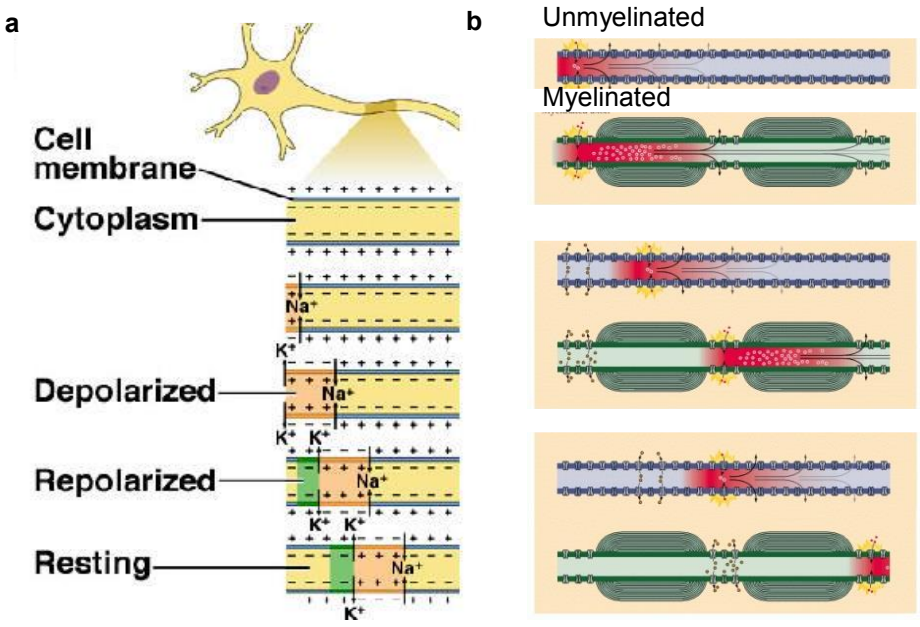


Figure 4 B- Schematic representation of action potential.

a: The difference in electrical potential across the axonal membrane in un-stimulated neuron is called resting potential When the nerve is stimulated lead to opening of Na^+-channels and increases the permeability of the membrane to Na^+ intracellular that lead to reduce the negativity of intracellular charges and cause the nerve to depolarise. Opening of K^+-channels lead to flux of K^+ outside the nerve and repolarize the membrane called repolarization. Membrane potential following repolarization might become more negative than the resting potential and then called hyperpolarization, (http://www.ncbi.nlm.nih.gov/books/NBK10921/figure/A204/?report=objectonly).

b: Comparison of speed of action potential conduction in unmyelinated and myelinated axons, (http://faculty.southwest.tn.edu/rburkett/A&P1%20Muscle%20Physiology.htm).

The tripartite synapses:

The junction between pre and post synaptic neurons modulated by a synaptic astrocyte is known as the tripartite *(see Fig 4C)* (Zhang and Haydon, 2005). Astrocytes are associated with synapses structurally and functionally. Several neurotransmitters are released from pre-synaptic axons lead to activation of glutamate receptors which lead to increase in intracellular Ca^{2+} in astrocytes or inhibition of GABA receptors on the post synaptic neurons (Eroglu and Barres, 2010). Astrocytes processes take up the glutamate from the synaptic cleft and metabolize it into glutamine then return it to the neurons to prevent over excitation of the receptors (Zhang and Haydon, 2005). Astrocytes also secrete growth factors and provide energy via glycogen. Microglia secretes cytokines and scavenges cellular debris. The most important function of glia at the synapse is to maintain ion homeostasis by regulating the concentration of extracellular K^+ and PH (Eroglu and Barres, 2010).

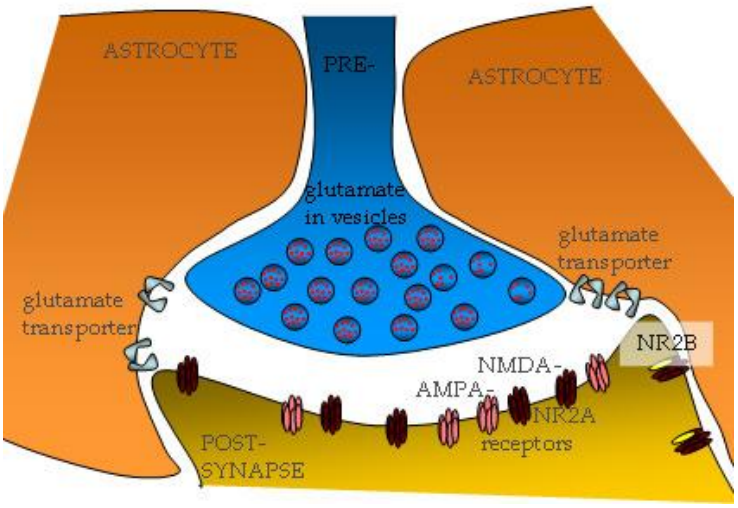


Figure 4C- Tripartite synapses.

The astrocyte processes (orange) ensheath the perisynaptic area. Astrocyte processes contain transporters take up glutamate from the synaptic cleft and metabolise it into glutamine and transferred it to axonal neurons. Pre-synaptic neuron (blue) contains the synaptic vesicles with the neurotransmitter. Post-synaptic neuron (yellow) contains glutamate receptors (NMDA & AMPA) and other neurotransmitter receptors (http://www.neuroresearch.de/popup_01_index.html).

Channels in myelinated and non-myelinated axons

A high concentration of voltage-gated Na^+-channels is expressed on the AIS, therefore, Na^+ current density is 34-fold greater in the AIS than in the soma (Hu *et al.,* 2009). Three isoforms of Na^+-channels are present in the AIS, which drive the ascending phase of action potential: Nav1.1, Nav1.2 and Nav1.6 (Debanne *et al.,* 2011). Nav1.1 is found in the AIS of both GABAergic neurons and retinal ganglion cells (Ogiwara *et al.,* 2007; Van Wart *et al.,* 2007). Nav1.2 present in the AIS of non-myelinated axons, while Nav1.6 is in the AIS of myelinated axons. Nav 1.2 is expressed first during development, and then is replaced gradually by Nav 1.6 along with myelination (Boiko *et al.,* 2001; Boiko *et al.,* 2003). Na^+-channels show a very low threshold at the distal part of the AIS, which could explain the action potential initiation primarily mediated by Nav1.6 and the back propagation principally maintained by Nav1.2 (Dulla and Huguenard, 2009; Hu *et al.,* 2009).

Among non-myelinated axons, Nav1.2 channels are homogenously distributed along the axons and support the conduction of action potentials; however, Nav1.6 is excluded from the non-myelinated region and clustered exclusively at the nodes of Ranvier (Debanne *et al.,* 2011). There are three Na^+ currents produced by Na^+-channels: inactivating transient Na^+ current (I_{NaT}), persistent Na^+ current (I_{NaP}), and resurgent Na^+ current (I_{NaR}); the latter current is activated upon repolarisation (Raman and Bean, 1997). Both I_{NaP} and I_{NaR} play an important role in controlling neuronal excitability and repetitive firing (Magistretti and Alonso, 2002). However, I_{NaP} is generated in the proximal axon (Astman *et al.,* 2006; Stuart and Sakmann, 1995), whereas

I_{NaR} is generated in the AIS and is present all along the axon (Kim *et al.,* 2010).

In addition to the above channels, in both myelinated and non-myelinated axons there is a specific voltage-gated-K^+ (Kv) conductance also expressed in the AIS (Debanne *et al.,* 2011). Several isoforms of K^+-channels are present: Kv1-Kv7; the current carried by these channels is 10 times greater in the distal area of the AIS than that measured in the soma (Kole *et al.,* 2007). However, both Kv1.1 and Kv1.2 are most frequently associated with the initial segment of both the excitatory and inhibitory cortical and hippocampal neurons (Inda *et al.,* 2006; Lorincz and Nusser, 2008). The Kv2.2 channel usually promotes inter-spike hyperpolarisation in the AIS of the medial nucleus of the trapezoid body(Johnston *et al.,* 2008), while the Kv7 channels are essential in controlling the resting membrane potential and action potential threshold in the AIS of many neurons (Pan *et al.,* 2001; Shah *et al.,* 2008; Yue and Yaari, 2006). There are five voltage-gated K^+-channel subunits expressed in non-myelinated axons (Debanne *et al.,* 2011). Kues and Wunder (1992) identified Kv1.3 channels in the parallel axons of cerebellar granule cells. The mossy fibre axons of the hippocampus express Kv3.3 and Kv3.4 channels (Chang *et al.,* 2007). Kv7 channels determine the excitability of pyramidal CA1 cells (Vervaeke *et al.,* 2006).

Recently, the appearance of P/Q- and L-type voltage-gated VGCC clusters at putative node sites has been identified as an early event in the formation of the nodes of Ranvier and myelination in central white matter (Alix *et al.,* 2012). Alix *et al.* (2012) found that myelinated and non-myelinated axons of white matter express VGCCs; these channels are expressed in the

axolemma of developing axons and act as the main source of Ca^{2+} influx under ischaemic conditions (Alix *et al.,* 2012; Alix *et al.,* 2008). During ischaemia, pre-myelinated axons of small size (< 0.4µm in diameter) are protected by a glutamate receptor blockade, whereas pre-myelinated axons of diameter > 0.4µm are protected by a combination of VGCC and glutamate receptor blockades (Alix *et al.,* 2012).

Channels in the node of Ranvier and axon terminals

As mentioned previously, action potential conduction is salutatory in myelinated axons due to the molecular organisation of the node of Ranvier. Two different domains of the axolemma separate the node of Ranvier from the internode (Abraham *et al.,* 1987). These domains are the paranodal-axoglial junction and the juxtaparanodal regions, which are characterised by the manifestation of a high concentration of voltage gated Na^{+}-channels and specific protein complexes (ankyrin G, NrCAM and 186 kDa neurofascin) that are highly enriched at the node (Bennett and Lambert, 1999; Jenkins and Bennett, 2001). Here, the Nav1.6 isoform is found in mature central axons, switching from Nav1.2 during development (Caldwell *et al.,* 2000; Duflocq *et al.,* 2008; Lorincz and Nusser, 2010). The density of the Nav1.6 subunit in the node of Ranvier of myelinated axons is nearly double that observed in the AIS (~ 350 Na^{+}-channel/μm^{2}) (Lorincz and Nusser, 2010).

Persistent and transient Na^+ currents have been identified in the node of Ranvier of myelinated axons (Benoit and Dubois, 1987; Dubois and Bergman, 1975). Salutatory conduction at the node of Ranvier is protected by the juxtaparanodal expression of Kv1.1 and Kv1.2 channels (Debanne *et al.,* 2011). However, the axon terminals also express several types of channels that exhibit an important role in action potential conduction: e.g. Nav1.2; and several isoforms of Kv-channel (e.g. Kv1.1, Kv1.2, Kv3 & Kv7) (Debanne *et al.,* 2011). Kv1 and Kv3 have been identified in the terminals of many inhibitory and excitatory neurons; Kv3 channels keep action potentials brief, therefore limiting Ca^{2+} influx and subsequently the release probability (Goldberg *et al.,* 2005). In addition to Nav-channels and Kv-channels, Cav1.2 (L-type) Ca^{2+}-channels also dominate in the axon terminal (Tippens *et al.,* 2008). Both non-myelinated axons and axon terminals contain hyperpolarisation-activated cyclic nucleotide-gated cationic channels (HCN) and the G-protein-gated inwardly rectifying potassium channels (GIRK) in presynaptic terminals, which are activated by $GABA_B$ receptors where they are thought to control the duration of the action potential (Fernandez-Alacid *et al.,* 2009; Ladera *et al.,* 2008).

Both myelinated and non-myelinated axons express NMDA and non-NMDA type of glutamate receptors and other neurotransmitter receptors such as $GABA_A$ and nACh receptors (Debanne *et al.,* 2011). Both the AIS and nerve terminals express several types of receptors that play an important role in white matter physiology and pathology. The AIS expresses a high concentration of the α_2-subunit of $GABA_A$ receptors, which controls axonal excitability (Brunig *et al.,* 2002). While, $GABA_B$ receptors are widely

expressed on presynaptic excitatory and inhibitory terminals (Bettler *et al.,* 2004; Trigo *et al.,* 2008). In addition to GABA receptors, there are ionotropic and metabotropic glutamate receptors which dominate in the nerve terminals and regulate synaptic transmission (Pinheiro and Mulle, 2008). Moreover, purine receptors are expressed on the axon terminals, where they modulate transmission release. Three families of purine receptors have been identified: P1Rs (G-protein coupled) that are activated by adenosine and are subdivided into A_1, $A_{2A,}$ A_{2B} and A_3 Rs; P2XRs (ligand-gated) which are activated by nucleotides and are subdivided into $P2X_{1-7}$; and P2Y (G-protein coupled) that are activated by nucleotides and are subdivided into $P2Y_{1-14}$ (Burnstock, 2008). Therefore, ATP and its degradation products ADP and adenosine are considered as important signalling molecules in the brain (Burnstock, 2007).

The neurotransmitter glutamate

The amino acid L-glutamate is the major excitatory neurotransmitter in the brain, and it mediates its actions by glutamate receptors (Kew and Kemp, 2005). More than 80% of the synapses of the brain use glutamate as their principle neurotransmitter. There are two pools of glutamate in the brain which characterise its function (Rodriguez *et al.,* 2013): synaptic glutamate (sGLU) and extra-synaptic glutamate (eGLU). sGLU is stored in the synaptic vesicles and released into the synaptic cleft as a Ca^{2+}-dependent neurotransmitter in response to the initiation of an action potential (Barbour and Hausser, 1997). Once the concentration of sGLU reaches 6–7 mM in the synaptic cleft, the receptors located around the synaptic cleft are stimulated (Moss and Bolam, 2008). Astrocytic terminals are present at many

glutamatergic terminals and express glutamate receptors and transporters; therefore, astrocytes control and limit the diffusion of glutamate into the extracellular space (Barbour and Hausser, 1997). This sGLU is taken back and metabolised into glutamine by glutamine synthetase in the astrocytes to prevent the sustained activation of synaptic receptors (Marcaggi and Attwell, 2004). Glutamine is an important molecule for the preservation of the normal activity of glutamatergic synapses, and is released into the extracellular space by astrocytes to be used by neurones; it is then converted back to glutamate and transferred into the glial cells (Marcaggi and Attwell, 2004).

This is known as the glutamate-glutamine cycle, which represents nearly 40% of glutamate turnover (Sofroniew and Vinters, 2010; Morales and Rodriguez, 2012).

Astrocytes elicit contrasting actions on glutamatergic synapses: they depress glutamate neurotransmission when acting on pre-synaptic metabotropic receptors, and activate glutamate neurotransmission when acting on post-synaptic NMDA receptors (Araque *et al.,* 1998). The eGLU is defined as a small percentage of sGLU, which diffuses from the synaptic cleft and intermixes with other glutamate molecules released from non-neuronal cells, stimulating the extra-synaptic receptors (Asztely *et al.,* 1997). The concentration of glutamate in the extracellular space is 1-3 µM (Hansson *et al.,* 2000); this concentration in the extracellular space is maintained at this level by the clearance of extra glutamate from the extracellular space via the surrounding cells at the synaptic cleft; this process is dominated by astrocytes in order to prevent excitotoxic injury to the neurones (Logan and Synder, 1971; Hansson *et al.,* 2000). The first cells which respond to a brain

injury within a few minutes are astrocytes, which exhibit swelling due to the changes in the intracellular Ca^{2+} concentration mediated by glutamate receptors, as well as an increase in the extracellular glutamate concentration and a consequent decrease in the extracellular space volume (Norenberg, 1998). Such reactions result in the release of glutamate into the extracellular space, which limits the diffusion of glutamate from the lesion site; the extra glutamate cannot be cleared from the extracellular space, which affects the neuronal function and ends in neurotoxicity and even cell death (Norenberg,1998). Therefore, astrocytic glutamate receptors play a very important role in both physiological and pathological conditions in the CNS (Norenberg, 1998).

Glutamate transporters

Astrocytes express glutamate transporters that maintain the physiological levels of glutamate concentration in the extracellular space. It is possible that this prevents over-excitation of the glutamate and neurotoxicity that can occur under a variety of pathological conditions in the brain (Danbolt, 2001; Robinson and Dowd, 1997). There are several types of glutamate transporters present on the glia cell membrane (Danbolt, 2001; Huang and Bergles, 2004). Glutamate transporter-1 (GLT-1), displays the highest percentage of expression in the brain and it is also named as excitatory amino acid transporter (EAAT2) (Danbolt, 2001). Glutamate aspartate transporter is the main transporter (GLAST) expressed by oligodendrocytes, it is also named as EAAT1 (Matute, 2011). Excitatory amino acid carrier-1 (EAAC1 or EAAT3) is the neuronal transporter that is present in a

subpopulation of adult oligodendrocyte progenitor cells (Domercq *et al.,* 1999). Glutamate transporters are present in the plasma membrane as well as the membrane of synaptic vesicles, and are particularly applicable to the regulation of glutamate uptake in wide-ranging CNS regions (Liu *et al.,* 2008). Vesicular glutamate transporters (VGLUTs) consist of three homologous proteins (VGLUT1-3), while VGLUTs 1 and 2 are mainly expressed in glutamatergic neurones. VGLUT1 and VGLUT3 are expressed in the hippocampus; and VGLUT2 is expressed in the cerebral cortex (layer IV) (Liguz-Lecznar and Skangiel-Kramska, 2007). There are significant differences between EAATs and VGLUTs (Liguz-Lecznar and Skangiel-Kramska, 2007). First, in contrast to EAATs, the accumulation of glutamate in synaptic vesicles is not a Na^+-electrochemical-dependent process; second, EAATs have a higher affinity for glutamate than VGLUTs; and third, EAATs recognise both glutamate and aspartate as substrates, whereas VGLUTs do not recognise aspartate (Amara and Kuhar, 1993; Kanner, 1993; Maycox *et al.,* 1990). The homeostasis of the extracellular glutamate concentration is a critical function controlled by neuronal and glial glutamate transporters. The expression or function of reduced glutamate transporters has been predicted to increase the extracellular glutamate concentration, followed by the excessive activation of glutamate receptors and excitotoxicity and even cell death (Mao, 2005).

Glutamate receptors (GluRs)

GluRs have been studied extensively and are considered to be one of the most important receptor groups (Chiu *et al.,* 1999), because they are implicated in a variety of neurologic brain disorders that include ischaemic brain damage, epilepsy, and neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Dingledine *et al.,* 1999). There are two types of GluRs: ionotropic (ligand-gated ion channels) and metabotropic (G-protein-coupled) receptors, based on their pharmacological and electrophysiological properties (Pin and Duvoisin, 1995). The activation of these receptors is responsible for the excitatory synaptic transmission and synaptic plasticity that underlie long-term potentiation (LTP) and long-term depression (LTD), which are involved in learning and memory (Traynelis *et al.,* 2010). These receptors regulate a broad spectrum of processes in the CNS, retina and PNS, and are postulated to play very important roles in numerous neurological diseases such as stroke, multiple sclerosis (MS), psychological diseases and periventricular leukomalacia (PVL) in the neonate (Dingledine *et al.,* 1999). At the present time, GluRs are considered a potential target for identifying therapeutic strategies for various CNS disorders in both adults and neonates (Traynelis *et al.,* 2010).

Types of GluRs:

1- Ionotropic-GluRs (iGluRs)

2- Metabotropic-GluRs (mGluRs)

1- iGluRs

There has been notable progress in the past decade toward understanding the structural basis of iGluRs such as agonist binding, partial agonist, antagonist and allosteric modulation of the receptor. All iGluRs are members of the superfamily of ligand-gated ion channels; they are non-selective in their permeability to cations such as K^+, Na^+, and sometimes Ca^{2+} (Traynelis *et al.,* 2010). They are multimeric and share a common basic structure, subdivided into N-methyl-D-aspartate receptors (NMDA-Rs) and non-N-methyl-D-aspartate receptors (non-NMDA-Rs) based on responses evoked by the selective agonists N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl1-4-isoazolepropionic acid (AMPA), and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) (Dingledine *et al.,* 1999; Traynelis *et al.,* 2010). Recently, new receptors have been identified and named ‘delta’ receptors; these are considered iGluRs and have an important role in some aspects of synaptic plasticity (Traynelis *et al.,* 2010; Chiu *et al.,* 1999). Kew and Kemp (2005) explained that, iGluRs have 4 trans-membrane domains (TMI-TMIV). The second domain (TMII) does not cross the membrane, but forms a re-entrant loop (Kew and Kemp, 2005). The second and third domains (TMII & TMIII) are linked by a long extracellular N-terminal, while the short intracellular C-terminal follows the third trans-membrane domain (TMIII) (Kew and Kemp, 2005) (*see Fig 5)*. These structures and sequences of iGluRs have a similarity to the bacterial periplasmic amino acid binding protein structure (Traynelis *et al.,* 2010).

1.1- NMDA-Rs

NMDA-R channel complexes have been extensively studied and characterised. NMDA-Rs are glutamate-gated cation channels whose activation contributes to depolarisation by causing a Na^{+} and Ca^{2+} influx (Cull-Candy *et al.,* 2001). The NMDA-R is a unique receptor: it is characterised by high permeability to Ca^{2+} and is blocked by Mg^{2+} in a voltage-dependent manner; it also requires two different agonists for its activation, glutamate and glycine (Cull-Candy *et al.,* 2001; Lynch and Guttmann, 2001). In addition to glutamate and glycine binding sites, the NMDA-R has a polyamine regulatory site that regulates receptor activation and separates recognition sites for Mg^{2+} and Zn^{2+} that inhibit ion influx through agonist binding (Flores-Soto *et al.,* 2012).

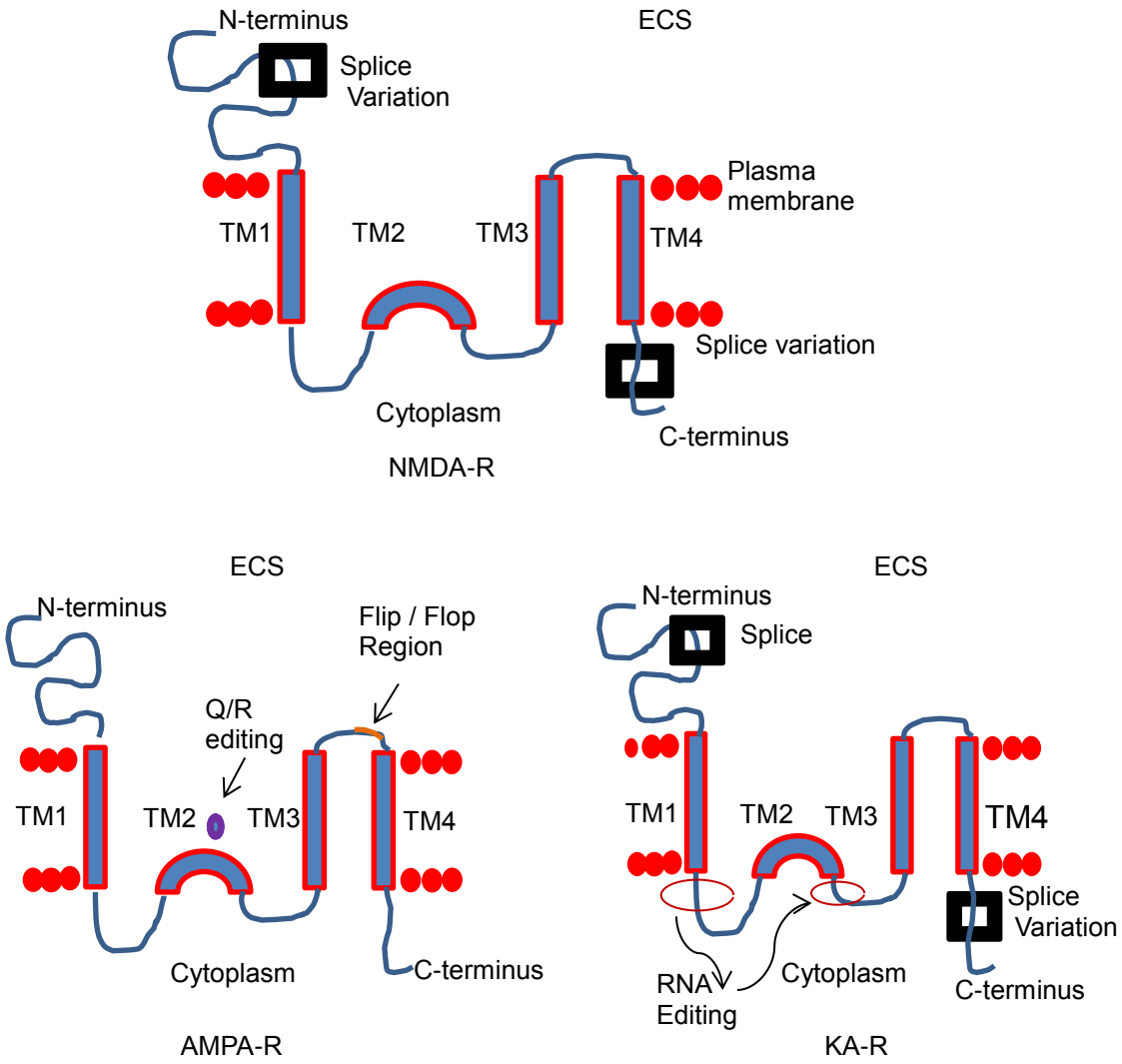


Figure 5- Pharmacological structure of NMDA-Rs and non-NMDA-Rs.

All iGluRs share the same basic structures where the second domain (TM2) does not cross the membrane, but forms a re-entrance loop that in turn forms the intracellular C-terminal and extracellular-N-terminal (adapted from Dingledine *et al.*, 1999).

NMDA-Rs are heteromeric complexes made up of three different subunits (NR1, NR2, NR3), with NR1 being an obligatory subunit and essential component for all NMDA-Rs (Cull-Candy *et al.,* 2001). There are eight different variants of NR1 subunits generated by alternative splicing from a single gene. Six separate genes encode four different NR2 subunits (NR2A, NR2B, NR2C and NR2D) and two NR3 subunits (Dingledine *et al.,* 1999). The NR1 subunit has a glycine binding site, and combines with NR2 subunits that have a glutamate binding site to produce functional NMDA-Rs (Schorge and Colquhoun, 2003). When (2NR1 and 2 NR2) subunits combined, form NR1-NR2 tetramers that produce functional form of NMDA-Rs with physiological and pharmacological properties in different parts of the brain (Traynelis *et al.,* 2010; Cull-Candy *et al.,* 2001).

The expression of functional recombinant NMDA-Rs in mammalian cells needs the co-expression of at least one NR1 and one NR2 subtype (Traynelis *et al.,* 2010; Cull-Candy *et al.,* 2001). The selection of the NR2 subunit confers additional physiological and pharmacological properties on the receptors; the NR1-NR2C channel appears to be more sensitive to Mg^{2+} blockade and has a high affinity for glycine binding, whereas the NR1-NR2A channel responds more to reducing agents than the others (Dingledine *et al.,* 1999; Traynelis *et al.,* 2010; Cull-Candy *et al.,* 2001). The expression of NMDA-Rs has been reported in astrocytes (Schipke *et al.,* 2001; Lalo *et al.,* 2006) and in oligodendroglia (Karadottir *et al.,* 2005; Salter and Fern, 2005; Micu *et al.,* 2006). Functional NMDA-R subunits are expressed in central white matter where they mediate ischaemic injury to the glia and may be

involved in cell-to-cell signalling (Domingues *et al.,* 2011). NMDA-R subunit expression differs throughout the central nervous system: the NR1 subunit is universal in the CNS, while NR2C subunits are expressed mainly in the cerebellum (Rigby *et al.,* 1996). The NR2D subunits are expressed early during development and are found in the diencephalon and midbrain in adults (Rigby *et al.,* 1996).

Al-Hallaq *et al.* (2002) used co-immnuopreciptation studies and suggested that the NR1, NR2 and NR3A subunits could co-exist in native single receptor complexes. Further study found that mice that are lacking the NR3A subunits have increased NMDA-mediated currents and spine density in cortical neurones (Das *et al.,* 1998). Accordingly, some native NMDA-Rs may be composed of NR1 in combination with one or more NR2 subunits, or NR1 in combination with both NR2 and NR3 subunits, for example (NR1/NR2A,NR1/NR2B, or NR1/NR2A/NR2B or NR1/NR2A/NR3A) (Kew and Kemp, 2005). Stys and Lipton (2007) suggested that NMDA-Rs have a very important role in mediating the Ca^{2+}-dependent injury of oligodendrocytes and the myelin sheath where the NR3 subunits seem to be involved. It has been established that the NR1-NR2A complex in particular has a higher affinity for competitive NMDA-R antagonists than agonists (Siegel *et al.,* 1994).

Another study using RNA isolated from neonatal optic nerves and brains showed that NR1 exon 5 alternative splicing is selectively regulated in the glia of white matter, and the NR3B gene experiences alternative splicing in both white matter and grey matter (Domingues *et al.,* 2011). The NR3B gene has four isoforms which, when expressed with NR1 and NR2 subunits, modify the glutamate-induced increase in Ca^{2+} concentration and the glutamate potency at NMDA-Rs in an NR2-depedent manner (Domingues *et al.,* 2011). Heterologous co-expression studies of NMDA-Rs using the rat neo-cortex have shown that NR1 encodes an essential subunit of NMDA-Rs, and there is a progressive alteration in subunit composition post-natally that contributes to NMDA-R variation during cortical development (Sheng *et al.,* 1994). The latter found that NR1 mRNA expression is low at birth, then increases from two weeks, and decreases with ageing. NR2B mRNA expression is high from P1 until adulthood, whereas NR2A mRNA expression is absent at birth and starts to appear from the next three weeks until adulthood (Sheng *et al.,* 1994). The latter also showed that NR2A and NR2B are co-immuno-precipitated, using antibodies to either subunit from P7 to adulthood; they proposed that NR2A and NR2B are segregated into three different subtypes of NMDA-R (Sheng *et al.,* 1994). Another study using RNA and immunoblot- analysis demonstrated that the NMDA-R subunits have a different expression during development (Watanabe *et al.,* 1992).

NMDA-Rs have several binding sites for compounds that activate or inhibit its function (Kemp and McKernan, 2002). These compounds are used in the development of therapeutic strategies for a multiplicity of brain diseases, and most are classified as iGluR agonists. NMDA-R agonists are di-carboxylic short chain amino acids such as glutamate, aspartate and NMDA (Siegel *et al.,* 1994). Glutamate is the most potent endogenous agonist of NMDA-Rs; its binding site is localised on the NR2 subunit (Laube *et al.,* 1997; Anson *et al.,* 1998). The second most potent and selective agonist is L-homocystate, followed by aspartate and then quinolinate respectively (Priestley and Kemp, 1994). NMDA is a less potent agonist but is more commonly used for NMDA receptors than glutamate since it exhibits more selectivity for NMDA-Rs. Homoquinolinate has a binding site localised on the NR2B subunit in the brain tissue (Brown *et al.,* 1998). The glycine binding site is localised on the NR1 subunit of the receptor, both glutamate and glycine binding sites of NMDA-Rs are located extracellular (Kuryatov *et al.,* 1994; Kew *et al.,* 2000). The activation of NMDA-Rs by glutamate and glycine is potentiated with increasing extracellular alkalinity (pH 6.8-8.4) (Traynelis and Cull-Candy, 1991). S-Hydroxy-ethyl-vinyl glycine is a potent and full agonist at the glycine site of NMDA-Rs, whereas L-alanine and D-cycloserine are partial agonists at the glycine site due to decreased intrinsic activity (Leeson *et al.,* 1994).

NMDA-Rs also have positive allosteric potentiators or modulators; the first that were described are the polyamines spermine and spermidine (Ransom and Stec, 1988). Polyamines are endogenous molecules that may exert important regulatory effects on NMDA-Rs under physiological and pathological conditions (Williams, 2009). They are acting at extracellular sites on the receptors to increase or decrease currents and Ca^{2+} flux through NMDA-Rs (Williams, 2009). Most of the modulators facilitate the function of NMDA-Rs by enhancing receptor affinity to glycine (Martina *et al.,* 2003). Aminoglycoside antibiotics, having more than three amino groups, potentiate NMDA-Rs via the polyamine site (Masuko *et al.,* 1999; Harvey and Skolnick 1999). Additional NMDA-R potentiators have been described that modulate the responses of the receptor by affecting the NR2A and NR2B subunits, including endogenous neurosteroids and pregnenolone-sulphate (Stawski *et al.,* 2010). NMDA-R has two separate binding sites, one for Mg^{2+} and one for Zn^{2+}; the possible explanation in that is the extracellular Mg^{2+} exerts a voltage-dependent blockade of NMDA-Rs, whereas the extracellular Zn^{2+} is a voltage-independent blockade (Siegel *et al.,* 1994).

A large variety of glutamate receptor antagonists has been discovered by extending the carbon chain of the corresponding agonists which have a high selectivity for the glutamate site (Kew and Kemp, 2005). Examples of these include (R)-2-amino-5-phosphonopentanoate (D-AP5) and (S)-alpha-amino-5-phosphono-methyl [1, 1´-biphenyl]-3-propanoic acid (SDZ EAB-515), although these antagonists have very poor penetration through the blood brain barrier (Kew and Kemp, 2005). There are a large number of antagonists that target different sites and subunits on NMDA-Rs (Kew and

Kemp, 2005). The first identified selective NMDA-R antagonists were ketamine, phencyclidine and dizocipline (MK-801), which are highly potent and active *in vivo* (Anis *et al.,* 1983; Wong *et al.,* 1986). 5-phosphonomethylquinoxantiend ions are selective antagonists at the NR1/NR2A subunits of NMDA-Rs, while the NR2B subunits of NMDA-Rs are selectively blocked by conantokin G (Con G) which is a17-amino-acid peptide isolated from the venom of the marine cone snail (Donevan and McCabe, 2000).

There are several classes of selective NR2B antagonists (Chenard *et al.,* 1995). The first antagonists identified were derived from ifenprodil (Williams, 1993) and the others are (1S, 2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propranolol (Troxprodil/CP101, 606) (Chenard *et al.,* 1995). These antagonists are very active and cross the blood brain barrier; however, they have non-selective action affecting adrenergic receptors and human Ether-à-go-go-Related Gene (hERG) ion channel (McCauley *et al.,* 2004). The unwanted side effect that results from binding many drugs to this channel is known as drug-induced acquired long QT syndrome (ALQTS), which can lead to cardiac arrhythmia and even sudden death due to the delayed repolarisation of cardiac ventricular action potential. Another disadvantage related to such drug binding is poor oral bioavailability and pharmacokinetics due to high first pass liver metabolism and clearance rates (Kew and Kemp, 2005).

Benzamidines are recently identified NR2B antagonists that show very good oral bioavailability and pharmacokinetics (Claiborne *et al.,* 2003). Also, some competitive antagonists such as kynurenic and quinoxaline-dicarboxylic acids block both the glycine site of glutamate receptors and AMPA-Rs; this is because of the structural similarities in the two receptors (Siegel *et al.,* 1994). Quinolones and indoles are antagonists that block the glycine site of NMDA-Rs and inhibit NMDA-R responses at high concentrations (Siegel *et al.,* 1994). Bristow *et al.(*1996) using *in vivo* study identified that kynurenic acid was the first glycine site antagonist, had a good oral bioavailability, and was bound tightly to the plasma membrane protein which facilitated its blood brain barrier penetration.

1.2- Non-NMDA-Rs

1.2.1- AMPA-Rs:

AMPA-Rs are a subfamily of glutamate-gated ion channels which play a very important role in neuronal development and synaptic plasticity, and which mediate fast excitatory neurotransmission in most of the synapses in the CNS (Hansen *et al.,* 2007). Molecular and biological studies have revealed that AMPA-Rs are tetrameric assemblies for four subunits (GluR1-GluR4 or GluRA-GluRD) (Collingridge *et al.,* 2009). Each subunit is present in several forms owing to RNA editing and alternative splicing (Dingledine *et al.,* 1999; Palmer *et al.,* 2005). The GluR2 subunit plays a key role in determining the functional properties of the AMPA-Rs (Hansen *et al.,* 2007). Receptors with the GluR2 subunit exhibit low Ca^{2+} permeability, whereas

those without show a high Ca^{2+} permeability (Hansen *et al.,* 2007). The GluR2 subunit has a unique effect on AMPA-Rs due to the presence of the single amino acid arginine at position 607, whereas the other subunits have glutamine at this position (Greger *et al.* 2002). At this site, called the Q/R site, arginine is replaced by gene-encoded glutamine through RNA editing rather than through DNA encoding (Koike *et al.,* 2000). RNA editing is developmentally regulated, and during foetal life there is a small percentage of GluR2 that is not edited (GluR2Q) but coexists with the edited form (GluR2R); while postnatally all GluR2 is present in the edited form (Sommer *et al.,* 1991). Once the glutamine is replaced with arginine at the Q/R site, the receptor becomes highly impermeable to Ca^{2+} (Sommer *et al.,* 1991). GluR2-lacking AMPA-Rs are predominantly expressed in white matter cells such as radial glia, pre-oligodendrocytes and sub-plate neurons during the first post-natal week, and then become highly expressed on cortical neurons during the second post-natal week (Talos *et al.,* 2006). The latter revealed that AMPA-R subunits are developmentally regulated in developing human white matter and grey matter (Talos *et al.,* 2006).

The concentration of glutamate required to activate AMPA-Rs is 15μM, which is less than the concentration of glutamate inside the synaptic cleft (Hansen *et al.,* 2007). AMPA-R agonists (glutamate and AMPA) are full agonists and induce a fast desensitisation response; and kainate is a partial agonist which induces little desensitisation at AMPA-Rs (Kew and Kemp, 2005; Arai *et al.,* 2000). AMPA-Rs are antagonised by competitive and non-competitive antagonists; competitive antagonists such as quinoxalinediones (e.g. CNQX) are potent and selective at AMPA-Rs (Drejer and Honore, 1988)

but have some affinity at the glycine site of NMDA-Rs (Birch *et al.,* 1988). Some of the selective and competitive antagonists at AMPA-Rs, such as PNQX, are of little clinical use because of limited water solubility and nephrotoxicity (Turski *et al.,* 1998). Non-competitive antagonists such as (GYKI 52466) are selective at AMPA-Rs (Solyom and Tarnawa, 2002). The 2, 3-benzodiazepines, including (GYKI 53784 / LY303070), exhibit slight selectivity between AMPA-R subtypes but are more selective at AMPA-Rs than kainate (KA-Rs) and NMDA-Rs (Bleakman *et al.,*1996; Ruel *et al.,* 2002).

1.2.2- KA-Rs

KA-Rs are the third type of iGluRs; they have GluR5-GluR7, KA1 and KA2 subunits that form homomeric and heteromeric channels (Alt *et al.,* 2004). Several molecular and biological studies have revealed that the GluR6 and GluR7 subunits can assemble with KA2 but not with GluR2-GluR4 (Alt *et al.,* 2004). GluR5, GluR6 and GluR7 form functional homomeric channels and show a low affinity for kainate binding with a dissociation constant in the range of 50-100 nM; whereas KA1 and KA2 subunits do not form functional homomeric channels and show a high affinity for kainate binding with a dissociation constant in the range of 5-15 nM (Pinheiro and Mulle, 2008; Sommer *et al.,* 1992; Schiffer *et al.,* 1997). GluR7 exhibits a very low affinity for both glutamate and kainate compared to GluR5 and GluR6 (Schiffer *et al.,* 1997; Duncan *et al.,* 2012). There is 75%-80% homology between GluR5, GluR6 and GluR7; and 68% homology between the KA1 and KA2 subunits, whereas the two subfamilies of KA-Rs exhibit only 45% homology (Pinheiro

and Mulle, 2008). The KA-R subunits display less than 40% homology with the AMPA-R subunits (Pinheiro and Mulle, 2006). The mRNA of KA-Rs exhibits alternative splicing and RNA editing like AMPA-Rs (Dingledine *et al.,* 1999; Pinheiro and Mulle, 2006; Bettler and Mulle, 1995).

KA-Rs are presynaptic, synaptic and postsynaptic receptors and are expressed throughout the CNS. For example, the GluR5 subunits are expressed predominantly in the interneurons in the hippocampus and throughout the temporal lobe (Liu *et al.,* 2004; Rogawski *et al.,* 2003). KA-Rs are activated by agonists including kainate, which is a more potent agonist than glutamate and AMPA (Siegel *et al.,* 1994; Sekiguchi *et al.,* 1997). Other KA-R agonists have been identified including 5-tert-butyl-4-isoxazolepropionic acid (ATPA), which is a potent and selective agonist at GluR5 subunit-containing receptors (Clark *et al.,* 1997; Swanson *et al.,* 1998; Alt *et al.,* 2004). Some agonists are gamma-substituted glutamate analogues which are more selective for kainate than AMPA-Rs (Kew and Kemp, 2005; Sekiguchi *et al.,* 1997). Several competitive antagonists at KA-Rs have been identified, including quinoxalinediones (CNQX and NBQX), that show a micromolar affinity for AMPA/KA-Rs and an affinity for the glycine binding site of NMDA-Rs (Kew and Kamp, 2005; Traynelis *et al.,* 2010). Other studies have examined the effect of the selective KA-R antagonist (S)-1-(2-amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methylpyrimidine-2, 4-dione (ACET) on altered behavioural phenotypes in a genetic model of NMDA-R hypofunction where ACET reversed the deficits in pre-pulse inhibition produced by mutation (Duncan *et al.,* 2012).

2- mGluRs

The presence of glutamate receptors that are not ligand-gated ion channels but are coupled directly to the second messenger system by G-proteins was first detected in the mid-1980s (Conn and Pin, 1997). It has become clear that glutamate can activate a family of receptors termed metabotropic glutamate receptors (Sladeczek *et al.,* 1985; Sugiyama and Hirono, 1987). These receptors are GTP-binding protein (G-protein)-linked receptors and participate in a wide variety of CNS functions, as well as in the modulation of synaptic plasticity, by regulating postsynaptic protein synthesis through the second messenger system (Conn and Pin, 1997). All mGluRs have a large N-terminal extracellular domain named the Venus flytrap domain (VFD) which contains the glutamate-binding site, seven transmembrane domains (TMD), and an intracellular C-terminal domain that is variable in length between mGluRs (Conn and Pin, 1997). The C-terminus is subject to alternative splicing, regulation by phosphorylation, and modulatory protein-protein interactions (Conn and Pin 1997; Niswender and Conn, 2010) (*see Fig 6)*. Conformational changes induced by ligand binding are propagated from the VFD via cysteine-rich domains (CRDs) to the C-terminal domain (Niswender and Conn, 2010).

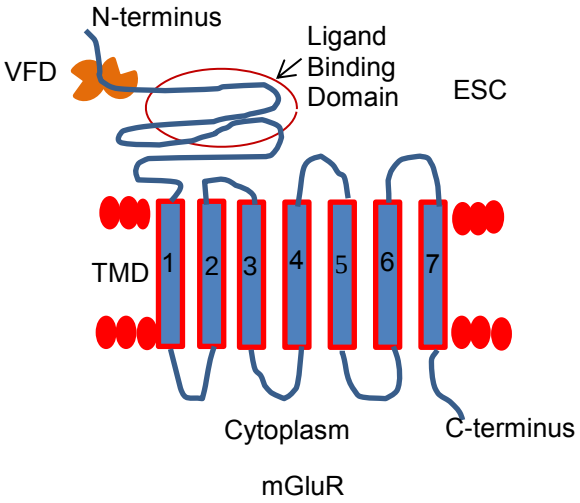


Figure 6- Pharmacological structure of mGluRs.

All mGluRs have a large N-terminal extracellular domain named the Venus flytrap domain (VFD), which contains the glutamate-binding site (adapted from Conn *et al.,* 2009).

The expression of mGluRs has been evaluated in developing white matter in rat and human brains (Jantzie *et al.,* 2010). The efficacy and potency of glutamate at mGluRs depends mainly on the concentration of extracellular Ca^{2+} (Emile *et al.,* 1996). Quisqualate, glutamate and ibotenate are agonists at mGluRs (Conn and Pin, 1997). A specific antagonist for mGluR1a is α-methyl-4-carboxyphenylglycine (MCPG) and phenyl-glycine derivatives (Thomsen et al., 1994; Ferraguti et al., 1994). An α-methyl-L-CCG-I (MCCG-I), 2S, 4S- 2-amino-4-(4,4-diphenylbut-1-yl)-pentate-1, 5-dioic acid (ADPD) is potent antagonist at Group-II mGluRs (Thomsen et al., 1996; Wermuth *et al.,* 1996).

White matter injury and the role of GluRs

Apart from the corpus callosum, the white matter of the mammalian CNS contains no neuronal soma (Baltan, 2009). In humans, white matter and grey matter constitute an equal proportion of the brain – approximately 50% each – while in rodents, white matter comprises only 10% of the brain volume (Baltan *et al.,* 2008). This means that white matter injury will be a more important component of CNS injury in humans than in rodents, which might explain why many experimental protective therapies used in clinical trials have failed (Baltan, 2009; Del Zoppo, 1995; Dirnagl, 2006). Loss of blood supply results in early ischaemic events that occur in all regions of the CNS including white matter, grey matter and the retina (Hansen, 1985). The events that follow loss of blood perfusion are as follows: depletion of ATP leads to the failure of the ATP-dependent-Na^+-pump; this results in the disruption of the ionic balance across the cell membrane and an increase in the extracellular K^+, which in turn leads to membrane depolarisation and a conduction block (Jonas *et al.,* 1991; Fern and Ransom, 1997). Both white matter and grey matter suffer irreversible injury when subjected to anoxia/ischaemia for a sufficient period, but the mechanism of the injury differs between the two tissue types – although it is mainly a Ca^{2+}- dependent processes in both tissues (Stys *et al.,* 1992). The anoxic/ischaemic injury of grey matter involves a Ca^{2+} influx across the cell membrane through excitotoxin-gated channels and voltage-gated Ca^{2+}-channels (Choi, 1985; Krieglstein *et al.,* 1989; Weiss *et al.,* 1990). In a study using *in vitro* RONs, extracellular Ca^{2+} was shown to be an important mediator of CNS white matter injury (Ransom *et al.,* 1990; Stys *et al.,* 1990; Waxman *et al.,* 1991).

Stys *et al.* (1992) suggested that Na^+ influx through the voltage-gated Na^+-channel plays a critical role in white matter injury, resulting from a persistent influx of intracellular Na^+ and subsequently promoting the reverse operation of the Na^+/Ca^{2+} exchanger, which in turn leads to the influx of damaging Ca^{2+}. However, anoxic injury to developed white matter not only dependent on the presence of extracellular Ca^{2+}, but the degree of injury is largely dependent on the amount of Ca^{2+} influx (Brown *et al,* 2001). This may contribute to the production of reactive oxygen species and nitric oxide inside the mitochondria, and consequently, lead to necrosis and apoptosis (Kann and Kovacs, 2007) *(see Fig 7).* These injury pathways are suggested by experiments in which RONs were perfused with a zero-Na^+ solution, or a zero-Ca^{2+} solution which resulted in marked protection of the axon from ischaemic-induced permanent failure (Fern and Ransom, 1997). Fern and Ransom (1997) found that blocking Na^+-channels (TTX) or the Na^+/Ca^{2+} exchanger (bepridil) also had a marked protective effect from injury (Fern and Ransom, 1997).

In recent years three mechanisms of anoxic/ischaemic injury in white matter have been implicated (Baltan, 2009) (*see Fig 8)*:

(1): An ionic mechanism that targets axons due to the failure of ionic homeostasis and results in the toxic accumulation of intracellular Na^+ and Ca^{2+} due to a reverse Na^+/Ca^{2+} exchange and the opening of calcium channels (Stys *et al.,* 1990; Fern *et al.,* 1995; Wolf *et al.,* 2001; Tekkök and Goldberg, 2001; Baltan, 2009);

(2): An excitotoxicity mechanism that involves the over-activation of AMPA/KA-Rs, leading to oligodendrocyte damage (Matute *et al.,* 1997; Li *et al.,* 1999; Rosenberg et al., 1999; Tekkök *et al.,* 2007; Baltan, 2009; Dewar *et al.,* 2003);

(3): An oxidative mechanism that leads to the formation of free radicals mediated through glutamate competing with the cysteine pump, and involves mainly white matter constituents (Oka *et al.,* 1993; Baltan, 2009). For this reason, a block of the AMPA/KA-Rs, the removal of extracellular Ca^{2+}, a block of glutamate reversal, and the prevention of free radical formation can all preserve the function of myelinating axons (Baltan, 2009; Follet *et al.,* 2000; Pitt *et al.,* 2000; Smith *et al.,* 2000). It was previously hypothesised that oligodendrocyte AMPA/KA-Rs-induced excitotoxicity is the mechanism for white matter injury (Zhang *et al.,* 2013).

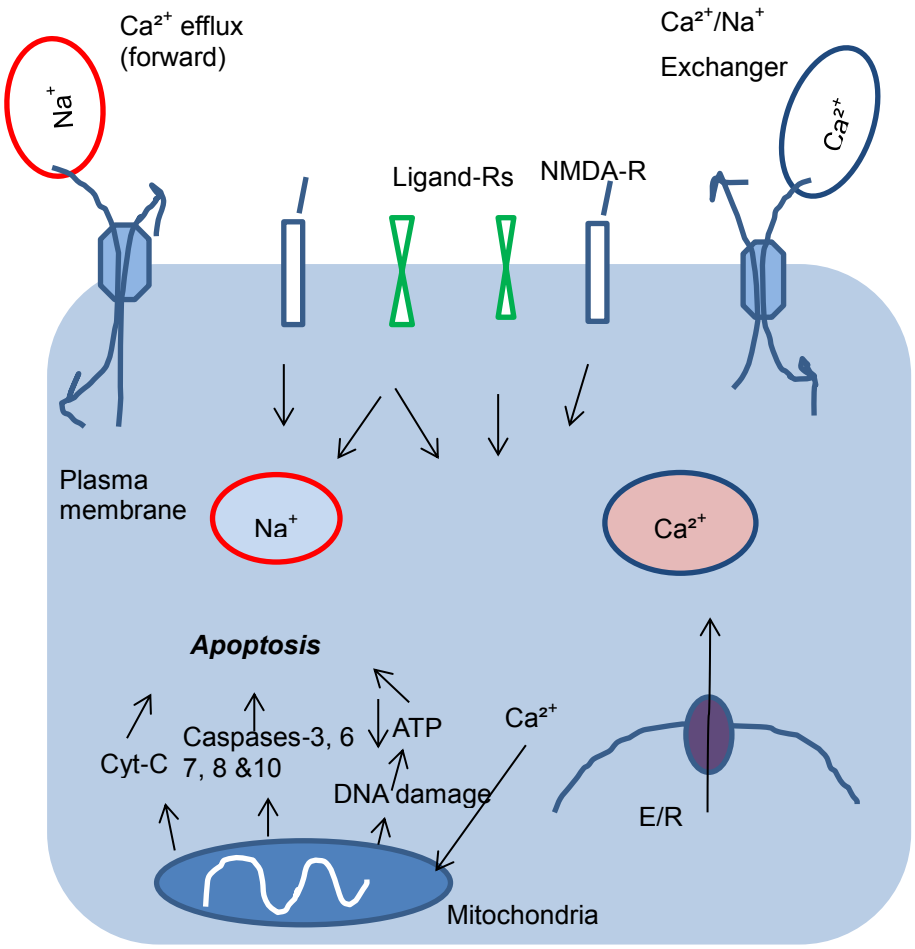


Figure 7- The role of NMDA-Rs and the Na^+/Ca^{2+} exchanger in white matter injury.

The sustained activation of the NMDA-Rs leads to Ca^{2+} overload inside the mitochondria. The increase in Ca^{2+} concentration can be caused by the Ca^{2+}/Na^+ exchanger during injury, and both result in processing death cascades by the mitochondria.

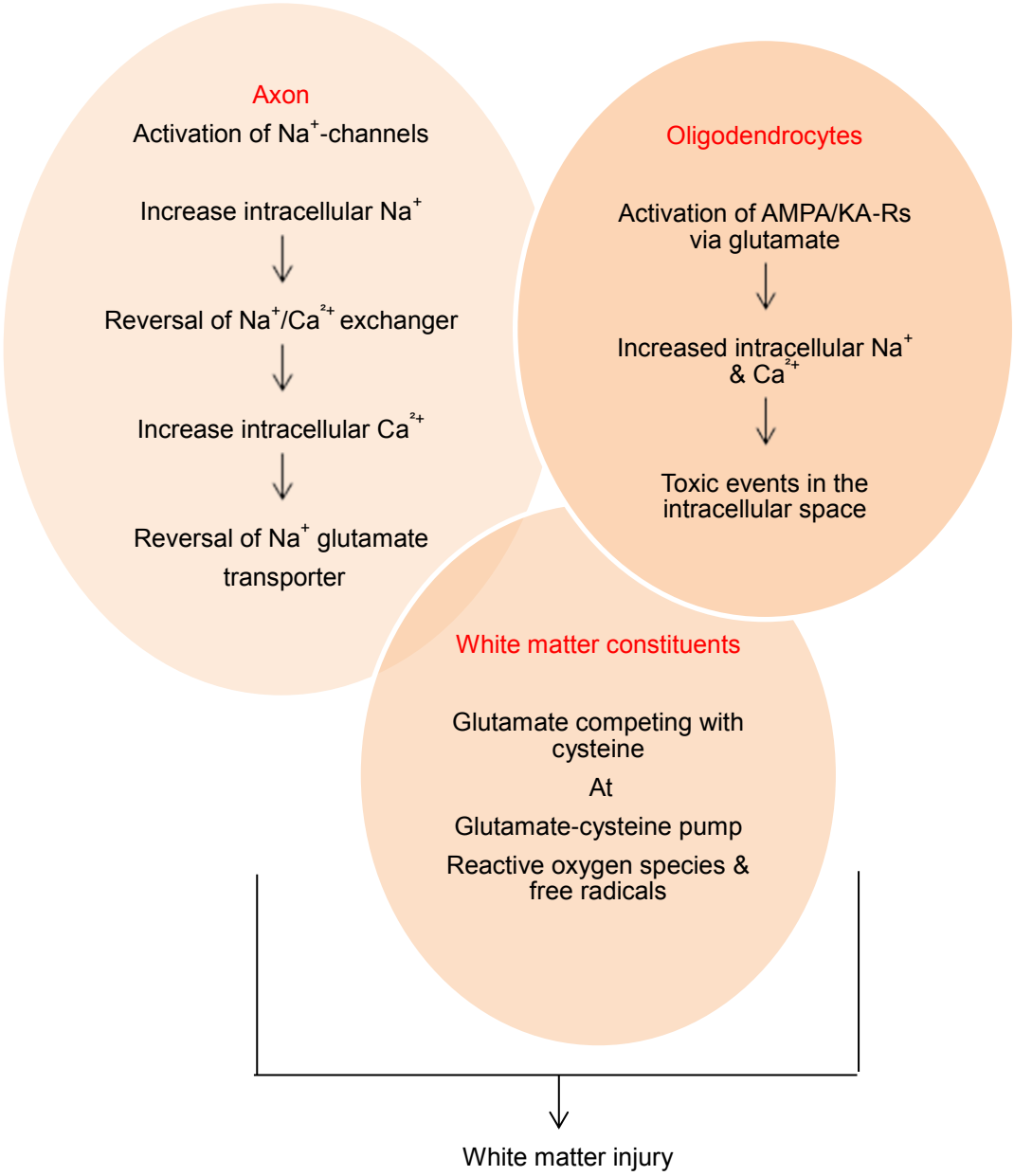


Figure 8- Three mechanisms of anoxic/ischaemic injury in white matter.

The ionic mechanism targets the axon, excitotoxic mechanism targets the oligodendrocytes, and oxidative mechanism targets the white matter constituents (adapted from Baltan, 2009).

Recently, the activation of NMDA-Rs expressed on the oligodendrocytes and the myelin sheath has been shown to play an important role in mediating the Ca^{2+}-dependent injury of central white matter (Stys and Lipton, 2007). NMDA-Rs are expressed by oligodendrocytes at all stages of development, and on myelinating processes in mature oligodendrocytes (Karadottir *et al.,* 2005; Micu *et al.,* 2006; Salter and Fern, 2005). These NMDA-Rs are activated by the accumulation of extracellular glutamate in various conditions of ischaemia such as stroke, MS and PVL (Karadottir *et al.,* 2005; Micu *et al.,* 2006; Salter and Fern, 2005; Werner *et al.,* 2001; Back *et al.,* 2007). This leads to oligodendrocyte damage, which depends entirely on Ca^{2+} overload in the cytoplasm (Micu *et al.,* 2006; Salter and Fern, 2005).

Several studies were conducted which attempted to treat white matter ischaemia by the blocking of NMDA-Rs. Micu *et al.* (2006) and Salter and Fern (2005) used memantine as a treatment for oligodendrocyte damage due to the activation of NMDA-Rs. However, Karadottir *et al.* (2005) found that oligodendrocyte NMDA-Rs are different in molecular structure from most neuronal NMDA-Rs, where they are formed from NR1, NR2c andNR3; so it is uncertain whether memantine alone could be effective in blocking the activation of NMDA-Rs by glutamate. Micu, *et al.* (2006) used adult RONs as a model to study the role of NMDA-Rs in chemical ischaemia; they found that NMDA-Rs mediate the influx of Ca^{2+} inside the central myelin during ischaemia. They also used an AMPA/KA-R antagonist (NBQX), which prevented significant increase in Ca^{2+} inside the oligodendroglial cell bodies, and reduced the increase in Ca^{2+} in the myelin only modestly (Micu, *et al.,* 2006). This increase in Ca^{2+} inside the myelin was completely abolished by

the introduction of the NMDA-R antagonist MK-801, which greatly reduced the damage to the myelin (Micu, *et al.,* 2006). Bakiri, *et al.* (2008) studied the role of NMDA-Rs during central white matter ischaemia; they used the same preparation that has been found to activate NMDA-Rs during ischaemia and is known to cause damage to the structure of the myelin. NBQX alone was used as a treatment, which resulted in no significant effect on the recovery of the CAPs post-ischaemia; in contrast, blocking NMDA-Rs with either MK-801 alone, or with the combination of NBQX and memantine, significantly improved the recovery (Bakiri, *et al.,* 2008). They concluded that the blocking of NMDA-Rs by memantine is considerably more voltage-dependent than with MK-801. During ischaemia, glutamate activates AMPA/KA-Rs on the oligodendrocytes, with consequent depolarisation that can decrease the blockade effect of memantine on NMDA-Rs, but have much less of an effect on the blockade of NMDA-Rs by MK-801(Bakiri, *et al.,* 2008). Therefore, a combined memantine + AMPA/KA-R blocker, or selective NMDA-R blocker (MK-801) alone, are potentially useful therapies for white matter diseases (Bakiri *et al.,* 2008).

Interestingly, white matter injury is a multifaceted phenomenon; so in addition to the role of GluRs in its pathogenesis, age is also an important factor. CNS white matter undergoes some changes in molecular architecture due to the aging processes and this plays a critical role in ischaemic injury (Ay *et al.,* 2005; Schaller, 2007). Baltan *et al.* (2008) studied ischaemic mechanisms in young and old adults using MONs as a model, and found that older adults are more susceptible to ischaemic injury than younger adults. Additionally, the degree of ischaemic injury damage to white matter in older

adults is greater than that in younger adults (Baltan *et al.* 2008). Furthermore, they revealed that the mechanism of injury in older adults is mainly dependent on glutamate excitotoxicity (Baltan *et al.* 2008). There are several age-related changes in white matter that might contribute to the increased susceptibility of axons to ischaemia such as a change in Na^+ concentration and a decrease in Na^+-ATPase performance (Scavone *et al.,* 2005). Another factor that increases the susceptibility of aging white matter to ischaemia is the changes in the mitochondrial dynamics that lead to depletion in ATP production followed by oxidative stress and the formation of free radicals (Schaller, 2007). Oxidative stress is considered the second most predominant pathway of injury in aging white matter, because the antioxidant detoxification mechanisms are progressively reduced with time (Scavone *et al.,* 2005). Other factors that might contribute to glutamate excitotoxicity in developed white matter are glutamate transporters. Physiologically, glutamate uptake from the extracellular space by glutamate transporters is an important step in the prevention of excessive glutamate signalling that leads to the over-activation of GluRs in white matter, as well as the prevention of excitotoxicity (Matute, 2011; Rothstein *et al.,* 1996). Domercq *et al.* (2005) showed that the inhibition of glutamate uptake in oligodendrocyte cultures increases glutamate levels and causes excitotoxicity, which is prevented by AMPA and KA receptor antagonists. Moreover, the altered activity of glutamate-producing enzymes such as glutaminase in microglia, and the decreased expression of glutamate transporters such as EAAT-1 and EAAT-2 in oligodendrocytes, possibly leads to increased levels of extracellular glutamate, resulting in excitotoxic changes and glial death (Matute, 2011).

These findings highlight the possibility that treatment strategies derived from experimental works on young adults may be ineffective in the clinical treatment of older adults. Therefore, a better understanding of the mechanism of injury of central white matter is important in developing potential therapeutic approaches for several brain disorders such as stroke and PVL.

Neurotransmitters other than glutamate and their role in white matter injury

Other than glutamate, several neurotransmitters have action upon white matter.

1- ɣ-amino-butyric acid (GABA)

GABA is the major transmitter for rapid inhibitory synaptic transmission in the brain (Olsen and DeLorey, 1999). Glutamate and GABA serve to regulate the excitability of all neurones in the CNS, and therefore both are implicated as important mediators of many physiological as well as serious pathophysiological events that cause brain dysfunction and disability. GABA was discovered in the 1950s by Eugene Roberts and J. Awapara. It is synthesised by a specific enzyme, glutamic acid decarboxylase (GAD), from L-glutamate (Lee *et al.,* 2011) (*see Fig 9)*. Cultured human astrocytes express the mRNA and proteins for the GABA synthesising enzyme (GAD), the GABA metabolising enzyme GABA-transaminase (GABA-T), and the

$GABA_{A,}$ and $GABA_B$ receptors (Lee *et al.,* 2011). Cultured microglia does not express GAD, but they do express GABA-T and the $GABA_A$ and $GABA_B$ receptors (Lee *et al.,* 2011)

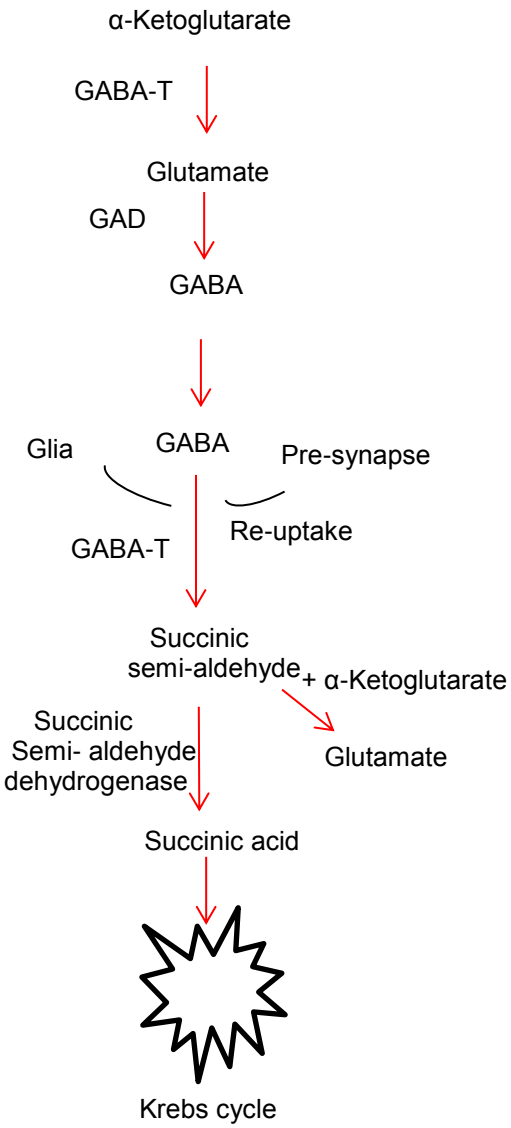


Figure 9- Synthesis and metabolism of GABA.

GABA is synthesised from α-ketoglutarate, which is formed from glucose metabolism via the Krebs cycle. The α-ketoglutarate is transaminated by the enzyme GABA-T into glutamate. Glutamate is metabolised by the GAD enzyme to GABA, which is consequently metabolised to succinic semi-aldehyde by the enzyme GABA-T. Succinic semi-aldehyde in combination with α-ketoglutarate results in the formation of glutamate, though succinic semi-aldehyde might be metabolised to succinic acid which is required in the Krebs cycle (adapted from Erlander and Tobin, 1991; Paul, 2000).

These findings indicate that astrocytes have GABAergic properties, while microglias have GABAceptive properties (Lee *et al.,* 2011). Reports suggest that astrocytes express multiple receptors designed to facilitate GABA release through Ca^{2+}-dependent and Ca^{2+}-independent mechanisms (Anderson and Swanson, 2000; Hamilton and Attwell, 2010). Heja *et al.* (2009) showed that the receptor-mediated release of GABA is a Ca^{2+}-dependent mechanism, whereas GABA release from astrocytes by GABA-transporters is a Ca^{2+}-independent mechanism which may be coupled with glutamate release. The Ca^{2+}-dependent release of GABA has been demonstrated in the adult brain, with less evidence in the developing brain (Saransaari and Oja, 1997; Kontro and Oja, 1987). During the neonatal period, GABA release is entirely Ca^{2+}-independent, and becomes Ca^{2+}-dependent at later stages of development (Balcar *et al.,* 1986). However, GABA release is markedly affected by Na^{+} and Cl^{-} removal, indicating that GABA release in ischaemia may be mediated partially by Na^{+} and Cl^{-}-channels (Saransaari and Oja, 1992; Bernath, 1992).

This anion-channel-mediated release is probably attributable to cell swelling and leakage through disordered membrane structures (Saransaari and Oja, 2008). GABA release during ischaemia has been suggested to be either neuroprotective by inducing the hyperpolarisation of neurones or reducing the release of the excitotoxic neurotransmitter glutamate, or to be neurotoxic by enhancing Cl^{-} influx into neurones that contribute to cellular swelling and neuronal death (Allen *et al.,* 2004).

GABA is an endogenous agonist for the three subtypes of GABA receptors (GABA-Rs); $GABA_A$, $GABA_B$, and $GABA_C$ (Chebib and Johnston, 1999). $GABA_A$-Rs are one of the large superfamilies of ligand-gated ion channels which include iGluRs, nicotinic-cholinergic receptors and glycine receptors (Barnard *et al.,* 1987; Squires *et al.,* 1983). They have 5 polypeptide subunits organised at the central ion pore of the channel (α, β, ɣ, ƍ, & P); these subunits have been cloned, and there are 19 mammalian genes encoding for GABA-R subunits (α_{1-6}, β_{1-3}, γ_{1-3}, ƍ, ε, ϕ, π, & P_{1-3}) (MacDonald and Olsen, 1994; Whiting, 2003). $GABA_A$-Rs are expressed on cells of oligodendrocyte lineage (Aoki, 1992; Belachew and Gallo, 2004), and on the astrocytes of neonatal optic nerves (Butt and Jennings, 1994). Immunohistochemical studies conducted on mouse brains have provided growing anatomical evidence that white matter interstitial cells comprise GABAergic interneurons (Engelhardt *et al.,* 2011). Such studies have also revealed that α_1-subunits are widely expressed in the brain, whereas other subunits are expressed in distinct populations of neurones (Barnard *et al.,* 1987). Remarkably, the α_6-subunit is only expressed in a single neuron type, the cerebellar granule neuron, whereas the α_5 and ƍ subunits are expressed in the hippocampus and cortex (Luddens *et al.,* 1990; Olsen and Sieghart, 2009; Glykys and Mody, 2007). The co-expression of α and ɣ subunits are of particular importance in determining the ligand-gating as well as the pharmacological properties of $GABA_A$-Rs (Bormann, 1988; Clarkson, 2012). Synaptic $GABA_A$-Rs are formed from the α_{1-3}, β_{1-3}, and γ_{1-3} subunits, and they are considered to be the site of action for a range of clinically important medicines such as benzodiazepines, neurosteroids and anaesthetics;

whereas extra-synaptic $GABA_A$-Rs are formed of α_{4-6}, β_{1-3}, and γ_2 or δ (Clarkson, 2012). Moreover, when the β_1-subunit is co-expressed with an α_1-subunit, they produce receptors with a high affinity for GABA; whereas the co-expression of an α_2 subunit with a β_1-subunit leads to the decreased affinity of GABA-Rs for GABA (Bormann, 1988). GABA-Rs composed of α_3 + β_1 + α_2 subunits have greater responses to benzodiazepines than GABA-Rs with α_1 or α_2 subunits (Luddens and Wisden, 1991). Muscimol is a selective and potent GABA agonist and is isolated from the hallucinogenic mushroom *Amanita muscaria*, but it is not a substrate for the GABA transporter (Curtis *et al.,* 1970). Picrotoxin and t-butylbicyclophosphorothionate (TBPS) are non-competitive $GABA_A$-R antagonists; they do not bind directly to the recognition site on GABA-R, but to a separate recognition site within the receptor complex (Squires *et al.,* 1983). In contrast, bicuculline is a competitive antagonist which directly binds to GABA-R and reduces both the frequency and the mean open time of the GABA-gated Cl^--channel (Squires *et al.,* 1983). $GABA_A$-Rs have multiple allosteric binding sites for a variant range of drugs which can modulate the inhibitory action of GABA positively or negatively (Paul, 2000; Squires *et al.,* 1983).

The second type of GABA-Rs is $GABA_B$-Rs, which are metabotropic trans-membrane receptors linked via G-proteins to K^+-channels (Lee *et al.,* 2011). The activation of $GABA_B$-Rs in the brain leads to an increase in K^+-channel conductance and consequently the hyperpolarisation of the neuronal membrane (Bormann, 1988; Bowery, 1993). Post-synaptic $GABA_B$-Rs are indirectly coupled to K^+-channels via G-protein, whereas pre-synaptic $GABA_B$-Rs are directly linked to K^+-channels (Andrade *et al.,* 1986). The first

selective agonist for $GABA_B$-Rs identified was baclofen, which is centrally active. Phaclofen, a derivative of baclofen, exhibited a weak effect as an antagonist of $GABA_B$-Rs (Bowery, 1993). However, experimentally studied $GABA_B$-R antagonists have not resulted in intense behavioural effects such as the seizures that occur following the administration of $GABA_A$-R antagonists to laboratory animals indicating that $GABA_A$-Rs are tonically activated, whereas $GABA_B$-Rs are activated under defined physiological circumstances (Bormann, 1988; Bowery, 1993; Paul, 2000). Determining the composition and pharmacology of GABA-Rs may lead to the development of novel therapies for use in CNS ischaemia such as stroke (Costa *et al.,* 2004).

It has been reported that cerebral ischaemia is the major cause of death worldwide, and the concentration of glutamate and other amino acids either excitatory or inhibitory is increased during ischaemia (Hutchinson *et al.,* 2002). The role of the excitatory amino acid glutamate and its receptors has been extensively studied during ischaemia, but the role of GABA and its receptors is less well established (Choi, 1992). A few investigators have focused on the role of the inhibitory amino acid GABA in the pathogenesis of neuronal death during ischaemia (Choi, 1992). The GABAergic system produces opposing effects to that of the glutamatergic system, so may be of particular importance in cerebral ischaemia (Lees, 1997). In ischaemia, the extracellular concentration of GABA increases to approximately 50 times the normal level and remains elevated for about 30 min during the period of reperfusion (Schwartz *et al.,* 1995; Inglefield *et al.,* 1995). Several mechanisms are implicated in the elevation of extracellular GABA concentrations in acute ischaemia (Schwartz-Bloom and Sah, 2001). These

include a decrease in ATP due to blood loss and a subsequent increase in intracellular Ca^{2+} that triggers a series of cellular events. These include the activation of phospholipase-A2, proteases and the accumulation of eicosanoids, arachidonic acid, and the release of reactive oxygen species that leads to a change in the Cl^--gradient, with a consequent increase and decrease in the uptake of GABA from different neuronal regions (Bazan 1970; Chan *et al.,* 1983; Rego *et al.,* 1996; Saransaari and Oja, 1998; Globus *et al.,* 1991; Phillis *et al.,* 1994). Other mechanisms that are involved in the accumulation of extracellular GABA are depolarisation-induced Ca^{2+}-dependent vesicular release, and a depolarisation-induced Ca^{2+}-independent reversal of GABA transporters (Burke and Tyalor, 1992; Phillis *et al.,* 1994).

Cellular mechanisms generated by ischaemia have been shown to modulate GABA-R function, since the accumulation of extracellular GABA causes the constant exposure of GABA-Rs to a high concentration of GABA with the consequent down-regulation of receptors (Alicke and Schwartz-Bloom, 1995). Furthermore, the expression of the α_1 and α_2 mRNA of GABA-Rs is decreased during acute ischaemia, especially in the hippocampus areas (CA1 andCA2) and the dentate gyrus (Alicke and Schwartz-Bloom, 1995). It is not recovered post-ischaemia which may be why the neurones in these areas are more sensitive to ischaemia compared to the interneurons of the CA3 region (Johansen *et al.,* 1983; Nitsch *et al.,* 1989; Crain *et al.,* 1988). The increase in extracellular GABA leads to an increase in tonic inhibition that minimises neuronal damage by supressing neuronal excitability and helps to regulate neuronal action potential firing; this increase in tonic inhibition lasts for two weeks after a stroke (Clarkson *et al.,* 2010).

The decrease in GABA-R function is aggravated by a reduction in the Cl^--gradient during ischaemia, which subsequently leads to a decrease in inhibitory post-synaptic potentials (IPSPs) (Luhmann *et al.,* 1995). A decrease in the extracellular Cl^- concentration is due to the hypofunction of outward Cl^- co-transporters and the activation of inward Cl^- co-transporters (Inglefield and Schwartz-Bloom, 1998). However, the influx of Cl^- through GABA-gated Cl^--channels is prevented by the GABA-gated Cl^--channel blocker picrotoxin (Inglefield and Schwartz-Bloom, 1998). The increase in the extracellular GABA concentration leads to an increase in tonic inhibition, which counteracts the increase in extracellular glutamate and helps regulate neuronal action potential firing as an endogenous protective mechanism; this subsequently leads to a decrease in neuronal injury (Schwartz-Bloom and Sah, 2001).

These findings raise the possibility of using GABA-R agonists as a treatment for brain ischaemia to balance the excessive glutamergic excitation which is the pivotal event leading to cell death (Shuaib and Kanthan, 1997). Neuro-protection can be achieved in rats, mice, gerbils and rabbits by GABA agonists, GABA modulators, GABA transporter inhibitors, and transaminase inhibitors (Shuaib and Kanthan, 1997). However, several studies show that GABA-R agonists exacerbate neuronal death in the neonatal period by inducing excitotoxicity and depolarisation (Erdo *et al.,* 1991; Lukasiuk and Pitkanen, 2000). The activation of $GABA_A$-Rs in central white matter during development, however, produces a partial and reversible nerve block, an effect associated with an increase in extracellular K^+ as well as axonal depolarisation (Sakatani *et al.,* 1994). Chen *et al.* (1999) found in an *in vitro*

study that there is a difference between adult and neonatal neurons: neonatal neurons have a more positive reversal potential for Cl^-, and exposure to GABA will therefore depolarise the cells and cause excitotoxicity; though they remarked that the combination of both $GABA_A$ and glycine antagonists decreases neuronal injury during ischaemia. Other *in vitro* studies (Obata, 1997; Ikonomidou *et al.,* 2000) have shown that GABAergic drugs can cause excitation in neonates, and that muscimol and diazepam can cause excitatory effects in neonatal mice in contrast to their sedative effects on young and adult mice; a single dose of diazepam injected into the neonatal rat can lead to apoptosis. However, another group found that GABAergic drugs might cause neuronal death even in adults. The possible explanation concerns two issues: the first is the extent of the depolarising shift in the equilibrium reversal of Cl^- (E_{C1}) (when shifted to a more positive value than the resting membrane potential); and the second is the severity of the ischaemia (Schwartz-Bloom and Sah, 2001). If the ischaemia is severe enough, it will lead to a greater accumulation of intracellular Cl^- and a subsequent large depolarising shift of E_{C1} to the spike threshold, which in turn will lead to neuronal firing and cell death (Schwartz *et al.,* 1995). The significance of the expression of GABA-Rs in developmental central white matter reveals the potential role of these receptors in mediating ischaemic injury in neonates (Constantinou and Fern, 2009).

2- Adrenergic receptors (ARs)

The catecholamines (adrenaline and noradrenaline) are endogenous agonists for a family of GPCRs known as ARs, which are linked to trimetric G-proteins. There are two categories of receptors, α and β, each of which has two subtypes: α_1, α_2 and β_1, β_2 (Molinoff, 1984).

2-1- α-ARs

The α-ARs of subtypes α_1 and α_2 were identified in the 1970s (Berthelsen and Pettinger, 1977). The α_1 subtypes are post-synaptic, and the α_2 subtypes are pre-synaptic (Bylund, 1992). However, a recent study showed that the α_2 subtypes are also post-synaptic and non-synaptic (Chen *et al.,* 2011). The α_1 subtype contains three subunits ($\alpha_{1A,}$ $\alpha_{1B,}$ and α_{1D}), and the α_2 subtype also contains three subunits ($\alpha_{2A,}$ $\alpha_{2B,}$ and α_{2C}) (Morrow and Creese, 1986). The α_1-ARs activate adenylyl cyclases (AC) and result in an increase in cAMP levels, activation of phospholipase-A2 and D, and the release of arachidonic acids (Johnson and Minneman, 1987; Han *et al.,* 1987). All three subunits ($\alpha_{1A,}$ $\alpha_{1B,}$ and α_{1D}) are able to release Ca^{2+} from intracellular stores as well as increase intracellular Ca^{2+} by gating voltage-gated Ca^{2+}-channels (Harrison *et al.,* 1991). Gilbert et al. (1984) showed that functional adrenoceptors are expressed on cells of oligodendrocyte lineage. Neonatal optic nerve astrocytes express adrenergic-α-receptors, which are capable of elevating intracellular Ca^{2+} (Duffy and MacVicar, 1995). Several studies have shown that α_{1A}-mRNA is expressed in the rat cerebral cortex and thalamus, and α_{1B}-mRNA is expressed in the hippocampus (Lomasney *et al.,* 1991; Perez *et al.,* 1991). Another study demonstrated the presence of

α_{1D}-mRNA in the rat cerebral cortex, brain stem, hippocampus, vas deferens, heart and spleen, while in the human brain it was found in the cerebral cortex and cerebellum (Johnson and Minneman, 1987). Various physiological functions are controlled by α_1-ARs such as smooth muscle contraction, increase in cardiac output (CO), and blood pressure (BP) (Thomas *et al.,* 1995). In addition, α_1-ARs modulate metabolic responses such as glycogenesis and gluconeogenesis in the liver, decrease in pancreatic secretions, and increase in salivary as well as sweat gland secretions (Thomas *et al.,* 1995).

The α_2-ARs mediate their effects by interacting with the $G_i/G_\circ$ family of heterotrimeric G-proteins (Goldenstein *et al.,* 2009). However, all three subunits of α_2-ARs share multiple signalling pathways (Lomasney *et al.,* 1991). They inhibit AC through G_i, which results in a decrease in cAMP; they also inhibit voltage-gated sensitive Ca^{2+}-channels through the $G_\circ$ family, and activate K^+-channels, which results in the reduction of neurotransmitters and hormone release (Lomasney *et al.,* 1991). All α_2-ARs are widely distributed throughout the CNS, and they modulate the autonomic nervous system functions pre-synaptically by a reduction in sympathetic outflow that results in decreased BP and CO (Zeng & Lynch, 1991; Lockette *et al.,* 1995). The $\alpha 2_B$-mRNA is expressed in basal ganglia, the thalamus and hippocampus, while α_{2C}-mRNA is present in the cerebral cortex, basal ganglia, brain stem, pons and middle brain (Bylund, 1992). Sanders *et al.* (2005) found that, α_1, α_2, and β-ARs are expressed in the corpus callosum and anterior commissure at gestational day 20. Among α-AR agonists, phenylephrine is selective for α_1-ARs, whereas clonidine is selective for α_2-ARs. Recently, SDZNVI 085 was

used as an antidepressant and selective α_1-ARs agonist (Bylund, 1992). SB216469 is an α_1-ARs antagonist used in the treatment of benign prostatic hyperplasia (BPH) (Bylund, 1992). All three subunits of the α_2-ARs are stimulated by the agonists UK14304, clonidine and apraclonidine (Lomasney *et al.,* 1991; Perez *et al.,* 1991). Prazosine as an antagonist has a higher affinity for α_{2B} and α_{2C}, whereas BAM1303 and WB4101 have a higher affinity for α_{2C}, and BRL44408 is more potent at α_{2A} (Lomasney *et al.,* 1991; Perez *et al.,* 1991).

2-2- β-ARs

β-ARs share structural similarities with the bacterial rhodopsin and G-protein receptors, including a long intracellular C-terminus and a short extracellular N-terminus (Sun *et al.,* 2006). The glycosylation sites are found in the N-terminus, whereas the C-terminus is involved in the interaction of the receptor with GTP-binding proteins (Han *et al.,* 2009). The occupancy of the receptor by agonists leads to the activation of the beta-adrenergic receptor kinase (BARK), which in turn leads to the phosphorylation and desensitisation of the receptor (Morrow and Creese, 1986). As mentioned above, β-ARs have two subtypes, β_1 and β_2; but another subtype, β_3, has been identified by quantitative autoradiography techniques which are pharmacologically distinct from the β_1 and β_2 subtypes (Gustafson *et al.,* 1989). Most of the therapeutic efficacy of the β-AR agonists and antagonists is directed toward PNS rather than CNS dysfunction (Nishigaya *et al.,* 1991). Isoprenaline is a synthetic agonist, and propranolol is an antagonist, which both act at β-ARs (Swanson *et al.,* 1990).

High concentrations of noradrenaline cause neuronal damage by increasing the sensitivity of pyramidal neurones to the excitatory neurotransmitter glutamate, which leads to cellular Ca^{2+} influx, increased free radicals and oxidative damage (Zhang and Kimelberg, 2005; Nishigaya *et al.,* 1991; Siesjo, 1988). As mentioned previously, high intracellular Ca^{2+} triggers catabolic intracellular processes by the activation of lipid peroxidases, proteases and phospholipases, which leads to increases in free radical formation and subsequent cellular degeneration and even cell death (Zhang and Kimelberg, 2005). Several studies have provided considerable evidence that α_2-AR agonists can protect the brain from ischaemic injury, but the exact mechanism of protection is not completely understood (Gustafson *et al.,* 1989). Normally, α_2-ARs act as auto-inhibitory receptors in regulating noradrenaline levels in the brain, so the activation of these receptors by noradrenaline inhibits its further release during nerve stimulation (Langer, 1974; Dixon *et al.,* 1979). In models of cerebral ischaemia, reports indicate potent neuroprotective effects of α_2-ARs in the myelinated optic nerve; whereas in the pre-myelinated optic nerve there is evidence for the β-AR-mediated modulation of excitability, which disappears as the nerve matures (Honmou and Young, 1995). However, during cerebral ischaemia, the noradrenaline level reaches nearly 40 times the normal value; this is extremely neurotoxic and exacerbates neuronal damage (Globus *et al.,* 1989; Gustafson *et al.,* 1991).

Dexmedetomidine is a newly discovered α_2-AR agonist which achieves a neuroprotective effect by decreasing the neuronal damage in the gerbil model of global cerebral ischaemia (Kuhmonen *et al.,* 1997). The underlying mechanism of this protection involves a reduction in axonal Na^+ and Ca^{2+} loading either through the axonal α_2-G-protein modulation of Na^+-channels and AMPA-Rs, or through the α_2-ARs of astrocytes (Stys, 2004; Nikolaeva *et al.,* 2009). In addition, dexmedetomidine increases glutamine disposal by oxidative metabolism in astrocytes and consequently decreases the availability of glutamine as a precursor of glutamate (Huang *et al.,* 2000). Clonidine, another α_2-AR agonist used experimentally, enhances neuronal survival in rat cerebral ischaemia (Hoffman *et al.,* 1991). The activation of α-ARs is known to evoke Ca^{2+} rises inside the astrocytes of neonatal rat optic nerves (Khorchid *et al.,* 2002). However, the over-activation of adrenoceptors leads to excitotoxicity and subsequent glial cell injury (Khorchid *et al.,* 2002).

The involvement of β-ARs in cerebral ischaemia is complex, and there is a debate as to whether the activation of β_1-ARs or β_2-ARs is protective (Amory *et al.,* 2002; Capraro *et al.,* 1984). The activation of β_1-ARs in neonatal rat optic nerves increases the amplitude of the CAPs in a Ca^{2+}-dependent manner, but it does not induce changes in the extracellular K^+ (Honmou and Young, 1995). Clenbuterol is a β_2-AR agonist which displays neuroprotection in cerebral ischaemic models by increasing the expression of nerve growth factor (NGF) and activating astrocytes (Latchaw *et al.,* 1985; Andre *et al.,* 1999). Another study reported that β_2-AR agonists increase heat shock protein 72 (HSP72) levels, which block both necrotic and apoptotic cell death, in models of cerebral ischaemia (Yenari *et al.,* 2005; Giffard and

Yenari, 2004). A further study demonstrated that propranolol, esmolol, and landiolol are β-AR antagonists that have a protective effect against brain ischaemia (Standefer and Little, 1986). Although the mechanisms underlying the protective effects of β-ARs are unknown, Chin *et al.* (1996) and Heneka *et al.* (2003) reported that catecholamines play a key role in cerebral ischaemia through the induction of HSP72; they showed that catecholamines regulate intracellular and extracellular HSP72 by α_1-ARs rather than β_2-ARs. However, the significance of the toxic effects of adrenoceptor activation in developmental central white matter may be high (Constantinou and Fern, 2009).

3- Glycine receptors (GlyRs)

Glycine is a simple amino acid which acts as a neurotransmitter at the inhibitory synapses in the adult CNS (Betz and Laube, 2006), and as an excitatory neurotransmitter during embryonic development and at birth (Garcia-Alcocer *et al.,* 2008). It activates strychnine-sensitive GlyRs and promotes the expression of GABA-Rs (Carpenter *et al.,* 1988). GlyRs belong to the pentameric nACh-R superfamily, along with $GABA_{A/C}$-Rs (Betz and Laube, 2006). Two types of GlyRs have been identified; $GlyR_{\alpha}$ and $GlyR_{\beta}$ (Akagi *et al.,* 1989; Morales *et al.,* 1994), and four different gene encoding $GlyR_{\alpha}$ subunits (α_1-α_4) have been identified in vertebrates called GIra1-4 (Matzenbach *et al.,* 1994; Laube *et al.,* 2002). Only the single gene-encoding $GlyR_{\beta}$, called GLrb, has been identified in mammals (Laube *et al.,* 2002; Lynch, 2004). The sequence homology over $GlyR_{\alpha}$ subunits are > 80 % and contain major determinants of agonists and antagonist binding (Garcia-

Alcocer *et al.,* 2008). In addition, the $GlyR_{\beta}$ subunit has a heterologous expression which makes it unable to result in glycine-activated currents; this causes the $GlyR_{\alpha}$ subunits to be essential for the assembly of functional GlyRs (Garcia-Alcocer *et al.,* 2008). The individual contribution of GlyRs in the brain and spinal cord, as well as in several areas of the embryonic rat brain, has been determined by the molecular cloning of the genes encoding the GlyR subunits ($\alpha_{1\text{-}4}$, and β) (Akagi *et al.,* 1989; Charrier *et al.,* 2006; Garcia-Alcocer *et al.,* 2001). During early development, the activation of both GlyRs and $GABA_A$-Rs increases Cl^--conductance and excitatory potentials (Chen *et al.,* 1996; Flint *et al.,* 1998; Virginio and Cherubini, 1997). The excitatory function of GlyRs during development is important for synaptogenesis, as the increase in intracellular Ca^{2+} caused by these receptors is crucial for the correct formation of post-synaptic glycinergic membrane specialisation (Kirsch and Betz, 1998). GlyRs cause ionic changes and the release of Ca^{2+} in immature cells; they also produce molecular signals for appropriate neuronal development (Flint *et al.,* 1998). In addition, they have an important role during ontogeny (an organism`s life span): this role was determined when the antagonist strychnine induced severe neurotoxicity and abnormalities such as anencephaly when administered to pregnant rats (Garcia-Alcocer *et al.,* 2005).

GlyRs are expressed on cells of oligodendrocyte lineage (Aoki, 1992; Belachew and Gallo, 2004; Gilbert *et al.,* 1984). Immuno-histochemical study has revealed that the mRNAs of the GlyR subunits are expressed in the cerebellum and especially in the Purkinje and granular cells during all stages of development (Garcia-Alcocer *et al.,* 2008), as well as in the external and

internal granular layers during early development (Garcia-Alcocer *et al.,* 2008). This suggests that the inhibitory transmission in these cells might be generated by GlyRs as well as $GABA_A$-Rs (Dugue *et al.,* 2005). Glycine is released into the synaptic cleft when the glycine-containing vesicle fuses with the pre-synaptic membrane in a Ca^{2+}-dependent manner; it then binds to the post-synaptic GlyRs due to increasing Cl^--conductance and the depolarisation of the post-synaptic cells (Betz and Laube, 2006). The binding of glycine to GlyRs is antagonised by strychnine, a convulsive alkaloid derived from the Indian tree *Strychnos nux vomica,* which is widely used to distinguish glycinergic from GABAergic inhibition (Betz and Laube, 2006).

The activation of GlyRs produces very powerful depolarisation on the membrane potential of neonatal optic nerve axons (Simmonds, 1983). Constantinou and Fern (2009) showed that the activation of either GlyRs or $GABA_A$-Rs induces depolarisation in neonatal astrocytes and the oligodendroglial line due to the opening of chloride conductance. There is no evidence of elevating Ca^{2+} inside the glia following the activation of either GlyRs or $GABA_A$-Rs (Constantinou and Fern, 2009). Micro-dialysis measurement study has revealed that the extracellular concentrations of glycine and GABA are increased in central white matter during ischaemia (Shimada *et al.,* 1993).

4- Acetylcholine receptors (ACh-Rs)

Acetylcholine (ACh) is a fast-acting neurotransmitter in the CNS and PNS (Picciotto *et al.,* 2012). It was the first neurotransmitter identified in 1914 by Henry Hallett Dale and was confirmed by Otto Loewi; both accordingly received the Nobel Prize for Medicine in 1936 (Cherry, 2013). ACh acts as a neuromodulator in the brain by changing the state of a group of neurons in response to alterations in environmental conditions (Picciotto *et al.,* 2012). Furthermore, ACh influences synaptic transmissions in the brain, alters neuronal excitability and induces synaptic plasticity (Yu and Dayan, 2005). The two primary sources of ACh in the brain include cholinergic projection neurons that are found in nuclei throughout the brain, such as the pedunculopontine (PPTg) and laterodorsal tegmental (LDTg) areas and the basal forebrain (BF); and local interneurons (Ren *et al.,* 2011; Zaborszky, 2002). ACh released from both groups of cholinergic cells mediates its action through ionotropic nicotinic receptors (nACh-Rs) and metabotropic muscarinic receptors (mACh-Rs) (Picciotto *et al.,* 2000; Wess, 2003). The ligand-gated ion channel nACh-Rs has been characterised in both the CNS and skeletal muscles; there are five subunits of nACh-Rs (2α, β, *∂*, and ɣ), and each subunit consists of four trans-membrane segments (Wonnacott and Barik, 2007). The extracellular N-terminus contains a cys-loop which is two cysteine residues from a disulphide bond; it is implicated in the transduction of agonist binding into channel openings (Sine and Engel, 2006). Nicotine acting at α_7-containing nACh-Rs can enhance hippocampal LTP (Welsby et al., 2006). The $\alpha_4\beta_2$ nACh-R subunit has a high affinity for nicotine and forms > 90% of nicotine-binding sites in the brain; these have a low affinity for ACh

and high Ca^{2+} permeability (Moroni *et al.,* 2006; Tapia *et al.,* 2007). Both nACh-Rs and mACh-Rs have a role in hippocampal synaptic plasticity, which is mediated through intracellular signalling pathways downstream from both receptors (Mckay *et al.,* 2007; Berg and Conroy, 2002). It has been demonstrated that both types of these receptors can alter the subsequent response of both GABAergic and glutamatergic neurons in the hippocampus to excitatory inputs (Drever *et al.,* 2011). The stimulation of nACh-Rs that are expressed on glutamatergic neurones in the ventral tegmental area (VTA) can lead to LTP in various brain areas (Mansvelder and McGehee, 2000). One fundamental action of ACh through nACh-Rs is to regulate the timing of Cl^- transporter expression, which is necessary for the ability of GABA to hyperpolarise and therefore inhibit central neurones (Liu *et al.,* 2006). Recently, several studies have reported that nACh-Rs induce the maturation of both GABAergic and glutamatergic synapses, thus emphasising the fundamental role of ACh in signalling in synaptic development (Lozada *et al.,* 2012). Functional nACh-Rs are expressed on the astrocytes of developmental white matter (Duffy and MacVicar, 1995; Mantyh *et al.,* 1995), on oligodendrocyte lineage (Aoki, 1992; Belachew and Gallo, 2004), and on oligodendrocyte precursors (Rogers *et al.,* 2001). Electrophysiological study has indicated that nACh-Rs are also expressed on rat retinal ganglion cells during development (Lipton *et al.,* 1987).

ACh is the endogenous agonist for all nACh-R subtypes. Carbamoylcholine is made by the modification of ACh to carbamate and is used as a muscarinic agonist because it lacks affinity at $\alpha_4\beta_2$ and α_7 (Jensen *et al.,* 2003). Nicotine is an antagonist at the α_9 and $\alpha_9\alpha_{10}$ subtypes, whereas it is an agonist at all other subtypes of nACh-Rs (Elgoyhen *et al.,* 2001). Anatoxin A is about 8 times more potent than ACh and activates neuronal nACh-R subtypes at sub-micromolar concentrations (Swanson *et al.,* 1986). The efficacy of nicotine for eliciting Ca^{2+} responses by nACh-Rs is more potent than that of ACh, and this nicotine-mediated Ca^{2+} response is diminished during the process of myelination (Swanson *et al.,* 1986). Such nicotine responses are blocked by d-tubocurarine and mecamylamine (Freund *et al,* 1990). Nicotine causes a rapid reduction in the CAP amplitude in neonatal optic nerves that could be partially prevented by d-tubocurarine, suggesting an activity-dependent release of ACh which leads to the activation of axonal nACh-Rs (Edwards and Cline, 1999).

There are competitive and non-competitive antagonists of nACh-Rs such as d-tubocurarine (d-TC), which is a non-selective antagonist that predominantly blocks muscular nACh-Rs; and dihydro-β-erythroidine (DHβE), which is a pure competitive antagonist at neuronal nACh-Rs, mainly at human and rat $\alpha_4\beta_2$ and $\alpha_3\beta_2$ (Mourot *et al.,* 2008). Mecamylamine is a non-competitive antagonist at neuronal nACh-Rs; 10 µM mecamylamine is used to achieve a complete block *in vitro* (Jensen *et al.,* 2005; Chavez-Noriega *et al.,* 1997). A high concentration of mecamylamine (100 µM) can inhibit NMDA-Rs. Interestingly; MK-801 is an antagonist at NMDA-R and can be used as a neuronal nACh-R antagonist, especially at the $\alpha_4\beta_2$ subunits.

Several observations have suggested that nACh-Rs have toxic effects on white matter, which are consistent with the expression of the receptors in developing white matter (Kawai *et al.,* 2007). Zhang *et al.* (2004) have shown that the activation of nACh-Rs produced a non-reversible injury characterised by a partial conduction block and glial cell pathology in the neonatal rat optic nerve. These receptors cause a Ca^{2+} influx inside the axons, but the decline in the CAP is a Ca^{2+}-independent effect (Zhang *et al.,* 2004) In addition, nACh-R activation can also modulate axonal excitability in the mature myelinated axons of the thalamo-cortical pathway (Kawai *et al.,* 2007). Constantinou and Fern (2009) found that the activation of nACh-Rs in neonatal rat optic nerves for 80 min produced a non-reversible decline in the CAPs. This effect was associated with astrocyte injury as well as oligodendroglial damage and the absence of any morphological changes in the axons, which suggests that it is the glial injury that underlies the non-reversible recovery in the CAPs evoked by 80 min exposure to nACh-R activation (Constantinou and Fern, 2009).

Maternal smoking is considered to be one of the risk factors for perinatal white matter brain injury (Froen *et al.,* 2002), as early exposure to nicotine can cause selective white matter abnormalities (Abdel-Rahman *et al.,* 2005; Froen *et al.,* 2002; Jacobsen *et al.,* 2007). Nicotine also affects the development of the adolescent brain, by affecting the cholinergic neurotransmission and cell signalling (Abreu-Villaca *et al.,* 2003); it might also alter the timing of the switch in the action of GABA from excitation to inhibition (Liu *et al.,* 2006). These toxic effects are consistent with the expression of both nACh-Rs in developmental central white matter (Swanson

et al., 1987; Zhang *et al.,* 2004). This suggests that the clinical significance of the damaging effect of nACh-Rs activation in central white matter is high (Constantinou and Fern, 2009).

PVL

Periventricular leukomalacia is a form of brain damage that involves the periventricular white matter of the brain in premature infants with a peak incidence between 23 and 32 weeks of gestation, and is the leading cause of permanent neurological disability (Petersen *et al.,* 1990). In 1962, the term PVL was coined by Banker and Larrouche when they discovered the necrosis of the white matter of the lateral ventricles in premature infants. The pathogenesis of PVL involves several interacting factors that can include maternal infection with the overproduction of cytokines, and inflammation (Degos *et al.,* 2008; Zammit *et al.,* 2011); perinatal hypoxia/ischaemia is thought to be the primary cause in most cases (Follett *et al.,* 2004). During hypoxia/ischaemia, the glutamate level is increased in the extracellular space, which leads to the activation of NMDA-Rs and non-NMDA-Rs (Alix, 2006). The over-activation of these receptors causes a toxic influx of Ca^{2+} inside the cell, leading to the damage of the progenitor cells of late oligodendrocytes and even death (Alix, 2006). Oligodendrocyte progenitor cells are the most sensitive type of cells in the brain to ischaemic injury and their role in PVL is well defined (Zammit *et al.,* 2011).

Two components of PVL have been identified: a focal necrosis of all cell types present within a core region; and diffuse injury affecting only the developing oligodendroglia, which leads to hypo-myelination (Volpe, 2001) (*see Fig 10)*. Back *et al.,* (2002) studied the susceptibility of early oligodendrocyte progenitor cells, late oligodendrocyte progenitor cells, and immature oligodendrocytes to ischaemic injury in P2 mice by using brain slices of the corpus callosum. They found that early oligodendrocyte progenitor cells appeared hypertrophic with thick processes, whereas late oligodendrocyte progenitor cells were completely degenerated, and their density was higher than that of immature oligodendrocytes (Back *et al.,* 2002). This confirmed that early oligodendrocyte progenitor cells are more resistant to ischaemic insult, while the sensitivity of late oligodendrocyte progenitor cells is the main leading cause of preferential white matter injury in neonates (Volpe *et al.,* 2011). Some studies have shown that developing oligodendroglia express GluR2-lacking AMPA-Rs that are highly permeable to Ca^{2+} (Burnashev *et al.,* 1992; Pellegrini-Giampietro, 1992). Follett *et al.* (2004) showed that the expression of AMPA/KA-Rs on developing oligodendrocytes increases between 23 and 32 weeks of gestation, which is coincident with the risky time for PVL. The activation of AMPA/KA-Rs on pre-oligodendrocytes leads to an increase in the intracellular Ca^{2+} concentration, followed by necrosis and even cell death (Itoh *et al.,* 2000; Itoh *et al.,* 2002; Matute *et al.,* 2002). *In vitro* studies have shown that a GluR antagonist, topiramate, results in the prevention of ischaemic damage to both axons and oligodendrocytes during the developmental stage (McCarren and Goldberg, 2007). This protective effect of AMPA/KA-R antagonists during

hypoxia/ischaemia has been demonstrated both *in vivo* and *in vitro* (Follett *et al.,* 2004).

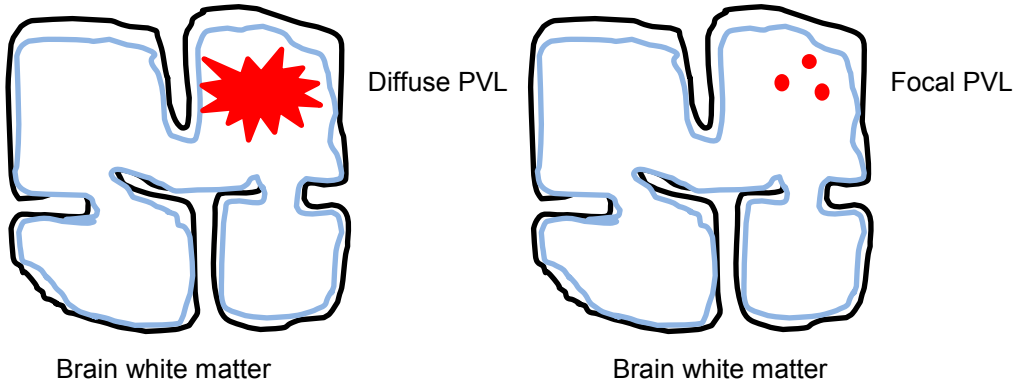


Figure 10- Illustration of two types of PVL lesions.

There are local and diffuse lesions of PVL in CNS white matter.

Recently, NMDA-Rs have been demonstrated in both neonatal and mature oligodendrocyte processes (Salter and Fern, 2005; Micu *et al.,* 2006). These receptors play a role in PVL due to their permeability to Ca^{2+} and consequent excitotoxicity (Karadottir *et al.,* 2005; Salter and Fern, 2005; Micu *et al.,* 2006). Pre-myelinated axons are predominantly sensitive to glutamate-induced excitotoxicity at sites of contact with the oligodendroglial processes that contain NMDA-Rs (Fern *et al.,* 1998; Alix and Fern, 2009).

Membrane depolarisation during oxygen and glucose withdrawal in neonatal white matter causes the excessive release of glutamate, which leads to an over-influx of Na^{+} and Ca^{2+} inside the cell via NMDA-Rs (Degos *et al.,* 2008). Therefore, NMDA-R antagonists for glutamate are potent neuroprotective agents in numerous experimental animal models of neonatal brain lesions (Johnston *et al.,* 2002). NMDA-Rs also play a key role in brain development and proliferation, and in neuronal differentiation (Lujan *et al.,* 2005). Therefore, the blocking of NMDA-Rs at different stages of development might have an adverse effect on brain development in neonates (Degos *et al.,* 2008).

Previous studies have revealed that a non-competitive NMDA-R antagonist, memantine, has a protective role in ischaemia in developing rodents without any adverse effects (Chen *et al.,* 1998). Memantine is used for the treatment of dementia, or Alzheimer`s disease, and is clinically well tolerated; this advantage is due to its rapid off-rate binding kinetics (Chen *et al.,* 1998; Chen *et al.,* 1992; Lipton, 2006). In addition, there has been no teratogenicity effect reported in pregnant rats and rabbits for memantine, and it does not appear to have any long-term effect on myelination (Manning *et al.,* 2008). Unlike memantine, the non-competitive NMDA-R antagonist MK-801 is potent and highly protective in white matter injury but poorly tolerated, and leads to substantial cell death by apoptosis in rats during post-natal growth (Degos *et al.,* 2008; Manning *et al.,* 2008). Recently, neurotransmitters other than glutamate have been shown to have actions on neonatal rat optic nerve axons (Constantinou and Fern, 2009). Constantinou and Fern (2009), tested the injury capacity of a standard 80 min perfusion of

glycine GABA, nACh, and adrenoceptor activation in P10 RONs, they revealed the potential role for non-glutamate-mediated neurotransmitters-mediated injury in developing white matter injury. However, the most efficacious neuroprotective strategies in treating PVL remain a matter of debate, while understanding the pathophysiology in immature white matter will progressively lead scientists toward the further development of effective therapeutic interventions in the future (Hagberg *et al.,* 2002).

Chapter II

Methods

Animals and tissue dissection

Adult rat (Wistar) and mouse (balb-c)

Adult rats and mice were sacrificed by cervical dislocation in accordance with the regulations of the British Home Office. Optic nerves were dissected between the optic disc and optic chiasm and then placed in artificial cerebrospinal fluid (aCSF). The rodent optic nerve is quite easy to dissect because it is opaque, long and thick; dissections typically took less than 2 min.

Perinatal Wistar rat

Perinatal rats were used between P0-P4, an age when the optic nerve is pre-myelinated (Hildebrand and Waxman, 1984; Fern *et al.,* 1998). The optic nerve is translucent at this age because the axons lack a myelin sheath. As a result, the dissection was difficult compared to adult rodents and typically took 5-10 min. The perinatal rats were decapitated and dissected following the same procedures used for adult rodents without any differences.

All dissected nerves were placed in an interface perfusion chamber (Harvard Apparatus Inc. *(see Fig 12)* where they were allowed to equilibrate for an hour before being inserted into the electrodes. This was done to give the nerve time to recover from the trauma of the dissection and so to regain homeostatic control over the intracellular and extracellular ionic environment.

Solutions and Drugs

The following solutions were used in the experiments and prepared in mM concentrations:

Normal (aCSF)

Na^+ 126, K^+ 3, Ca^{2+} 2, Mg^{2+} 2, Cl^- 129, HCO_3^- 26, SO_4^{2-} 2, $H_2PO_4^-$ 2, dextrose 10.

PH 7.45 bubbled with 5% CO_2 / 95% O_2.

Zero-Ca^{2+}

Na^+ 126, K^+ 3, Mg^{2+} 2, Cl^- 129, HCO_3^- 26, SO_4^{2-} 2, $H_2PO_4^-$ 2, Dextrose 10

See text for different ethylene glycol-bis [β-aminoethyl ether]-N,N,N'N'-tetra acetic acid (EGTA) concentrations used for zero- Ca^{2+}.

Zero-Na^+ (choline substitute)

K^+ 3, Ca^{2+} 2, Mg^{2+} 2, Cl^- 129, HCO_3^- 26, SO_4^{2-} 2, $H_2PO_4^-$ 2, dextrose 10, choline 124.

Zero-Na^+ ([N-methyl-d-glutamine] substitute ($NMDG^+$))

K^+ 3, Ca^{2+} 2, Mg^{2+} 2, Cl^- 129, HCO_3^- 26, SO_4^{2-} 2, $H_2PO_4^-$ 2, dextrose 10, $NMDG^+$124.

When oxygen-glucose deprivation was required (OGD), glucose was omitted from solutions, and bubbled with 95% N_2, 5% CO_2.

Osmolarity was periodically measured and maintained at 310-320 mOsm using NaCl or sucrose as required. Drugs are shown in Table-I and where typically made up as stock solutions Where some experiments done in the dark.

Drugs used in electrophysiology experiments are shown in the following table.

Table 1

Name	**Source**	**Solvent**	**Concentration**
Kynurenic acid (4-Hydroxyquinoline-2-carboxylic acid)	Sigma	Water	100 μM
Glycine	Tocris	Water	10 μM
Memantine hydrochloride	Tocris	Water	1 μM
Ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA)	Acros Organics	Water	50 μM
Kainaic Acid	Tocris	Water	30 μM
(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten5,10-imine maleate (MK-801)	Sigma	Water	1 μM
N-methyl D-aspartate (NMDA)	Tocris	Water	1 mM
Strychnine hydrochloride	Sigma	Water	2 μM
Picrotoxin	Tocris	Water	100 μM
Mecamylamine	Tocris	Water	10 μM
Propranolol hydrochloride	Sigma	Water	10 μM
Phentolamine hydrochloride	Tocris	Water	10 μM

Electrophysiology

Electrophysiology is the study of the electrical properties of biological cells and tissues. The RON (Stys *et al.,* 1991), MON (Brown et al., 2003), and recently hamster optic nerve (James *et al.,* 2010) have been the models of choice in studying the pathophysiology of white matter using electrophysiological recording. For this study, I used an isolated rodent optic nerve suction electrode preparation to study the pathophysiology of CNS white matter. This technique employed capillary glass electrodes, which are hollow tubes filled with aCSF (Stys *et al.,* 1991; James *et al.,* 2010). These glass electrodes are used to record the numerical sum of action potentials arising from individual axons within a nerve bundle, termed the compound action potential (CAP). In this technique the nerve is inserted into a glass electrode filled with a saline and connected to a stimulus unit at one end. A similar electrode is connected to an amplifier at the opposite end, allowing a post-stimulus CAP to be recorded when the nerve is stimulated. As originally described, recording of CAPs by suction electrodes frequently varied over time during control experiments, and an electrical model was developed in order to correct the instability of suction electrode recordings (Stys *et al.,* 1991). As the adult RON consists of more than 100.000 axons surrounded by glial cells (Foster *et al.,* 1982; Fukuda *et al.,* 1982), the whole nerve is considered as a single equivalent fibre used in this model (Stys *et al.,* 1991). The intracellular potential of this single equivalent fibre very near the inserted end of the recording electrode is the Ve, while the intracellular potential outside the recording electrode is the Vm, both potentials are recorded with

respect to ground (Stys *et al.,* 1991). Following stimulation, the Vm will depolarise and the resulting difference between the Vm and the Ve generates the Vo, which is the output voltage sensed by the recording electrode; Vo is positive in this instance (Stys *et al.,* 1991). As the Vm repolarises it becomes more negative than the Ve, which is now depolarised by the propagation action potential, and a negative potential is recorded. Once both the Vm and the Ve have returned to the resting potential, no signal is recorded and hence the Vo is zero (Stys *et al.,* 1991).

The resistive elements along the path of action potential propagation influence the size of the recorded signal – that is, the Vo. The internal resistance of the equivalent fibre, Ri, defined as including the series membrane resistance at both entry and exit points of the current, and the lumped resistance of the external space, Ro which defined as a parallel combination of resistances formed by the fluid layer at the nerve/electrode junction, connective tissue sheath, glial cells and the extracellular space) (Stys *et al.,* 1991). The equation is as follows (Cohen, 1970; Stys *et al.,* 1991):

Vo= Ro (Vm - Ve)/ Ri.

However, Vo can be increased by increasing Ro, that is achieved by shaping the mouth of the recording electrode to tightly fit the nerve (Connors *et al.,* 1982).

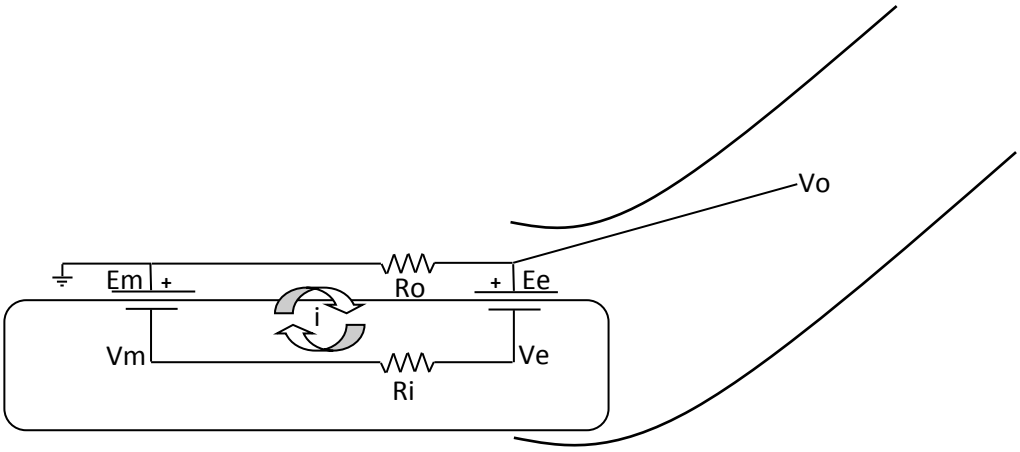


Figure 11-The electrical model of the suction electrode used by Stys et al. (1991)

The Vo is the voltage change across the Ro recorded by the by the electrode. Em is the trans-membrane potential of the nerve away from the recording electrode; Ee is the equivalent potential at the inserted end. The figure is from Stys *et al.* (1991).

In the current study, I used this technique to illustrate the effects of OGD upon rodent optic nerves. The procedure involved inserting the optic nerves into the electrodes for the entire length of the experiments. To ensure an accurate comparison between the CAP areas (for adult rodents) at the two time points, Stys *et al.* (1991) created a means of approximating the Ro; as the Vo is directly proportional to the Ro, Ro provides a means for fair comparison. As Ro is in parallel with the Ri, so, it cannot be measured as a discrete entity (Stys *et al.,* 1991). The latter showed that a value for the Ro can be calculated by measuring the Rp, which is the combined parallel resistance of the Ro and the Ri. This is achieved by using a constant current to generate a baseline resistance, Rb, at the fluid/electrode interface of the recording electrode without a nerve present in the electrode lumen (Cummins *et al.,* 1979). The RON can then be inserted into the electrode and the procedure repeated several times while the nerve is slowly inserted

further into the electrode, causing the resistance to increase. As the Rb does not change, it is then subtracted from the values obtained to give the Rp (Stys *et al.,* 1991). As the CAP area is a linear function of the Rp, readings of the area can be taken and divided by the Rp to give a fair comparison of RON function before and after hypoxia (Stys *et al.,* 1992; Fern *et al.,* 1995). A series of recordings should be taken to battle the effects of the stimulus artefact which may also vary over time, causing a shift in the offset of the CAP area vs. the Rp plot (no alteration in the slope of the plot is observed as the artifact is independent of the Rp). The stimulus artefact can result in significant errors in the estimate of the CAP area or amplitude, because it represents an additional signal, summed with the CAP (Stys *et al.,* 1991). The artefact had a different configuration depending on the nerve position in respect to the ground (Stys *et al.,* 1991). A subsequent study reported that similarly stable recordings could be achieved by leaving the electrodes in place, allowing the continuous recording of the CAP during OGD (Fern *et al.,* 1998; Brown *et al.,* 2001). The impedance of the recording electrode was monitored in these experiments and was found to remain constant throughout the time. The data presented in this thesis were acquired using a continuous recording technique (Fern *et al.,* 1998; Hughes *et al.,* 2001; Allen *et al.,* 2006).

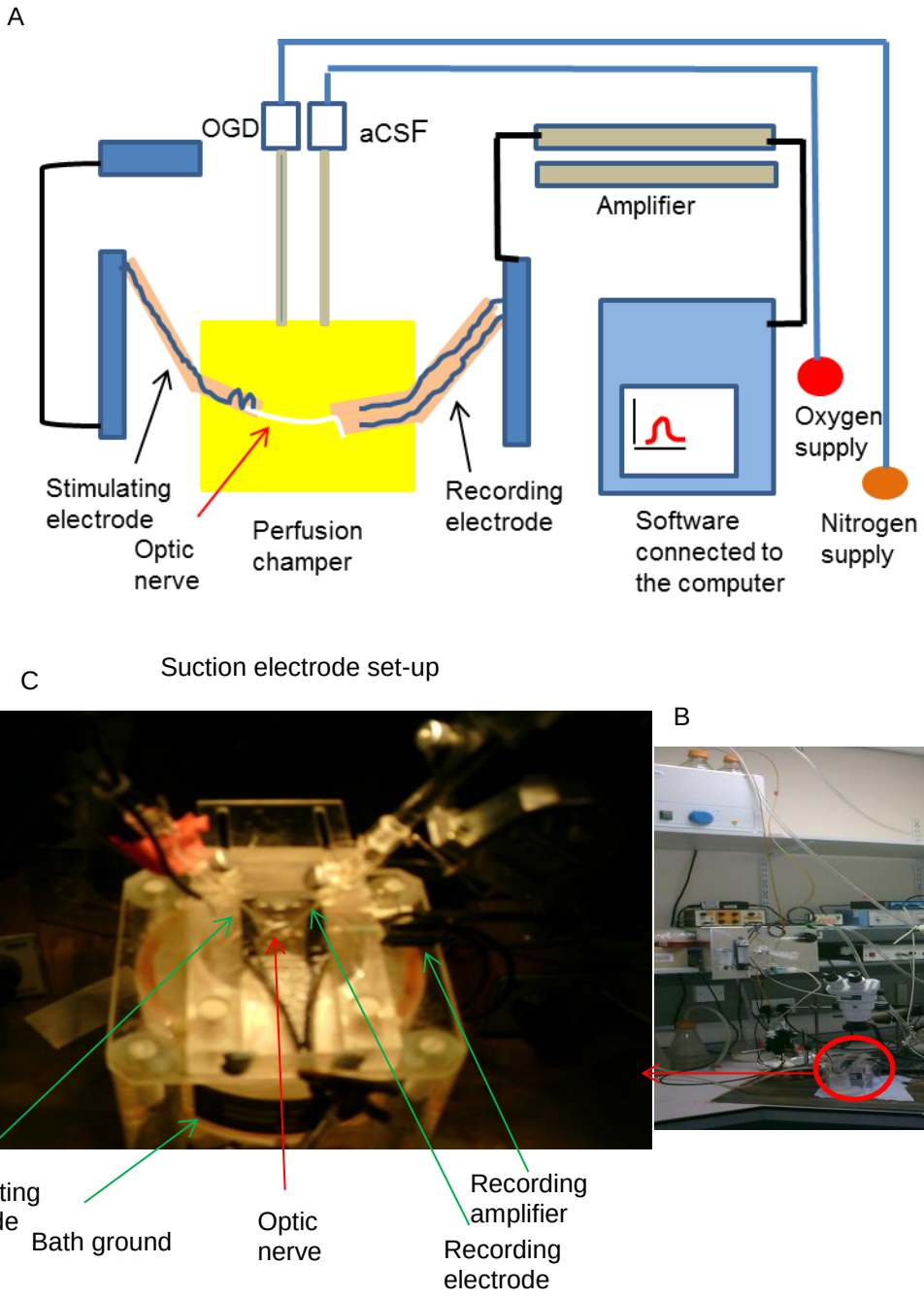


Figure 12- Electrophysiological recording set-up.

A&B: is the illustration of the electrophysiology technique. The nerve is inserted into a glass electrode filled with a saline and connected to a stimulus unit at one end as seen in Fig C. C: is the suction electrode set-up. The optic nerve (white colour) is placed on the nylon sheath of the bath (yellow colour in A, and black colour in C) after dissection where perfused with fluid connected to the bath with perfusion tubes (tan colour in A), and with gases coming through the gas tubes (red colour for the oxygen and orange colour for the nitrogen).

Experimental protocol

After dissection, the RONs were placed in an interface perfusion chamber (Harvard Apparatus Inc.) where they were allowed to equilibrate for one hour before being inserted into the electrodes. This was done to permit the RON time to recover from the trauma of the dissection and so recover homeostatic control. The RONs were perfused with aCSF at a rate of 1-2ml/min. The temperature was maintained at 37˚C throughout the time of the experiment (101C temp controller, Warner etc.), and the RONs oxygenated with 95%O_2/5%CO_2, which was passed through aCSF and through a water chamber for warming and humidification.

The electrodes:

The electrode is made by the gentle heating of the end of a small glass capillary until a constriction is formed. Further heating of the tube several millimetres up the barrel allows the tube to bend under its own weight, forming a periscope shape, which facilitates the insertion of the nerves (Stys *et al.,* 1991), and tightly fitted around the adult and neonatal RONs. It was important to ensure a good fit, to prevent moving the RONs during an experiment, and thus avoiding a change in the shape of the recorded CAP. The silver wires are put in chlorine bleaching agent over night after removing the plastic layer that isolates the silver wire. This chloriding of the silver allows for stable signals over long periods of recording. The stimulating electrode had a chlorided silver wire in the lumen and another wrapped around the barrel near the end of the electrode to provide the current return. The CAPs were recorded from a second glass electrode with

chlorided silver wire running through the lumen. A third electrode, the subtraction electrode, was placed next to the recording electrode to reduce the stimulus artefact and electrical noise. The electrodes were connected via a high impedance head-stage (Axon Instruments) to an amplifier (Axon Instruments, Cyber Amp 320) which subtracted the signal from the subtraction electrode from the signal originating from the recording electrode.

Upon starting an experiment, CAP recordings were made under control conditions (normal aCSF and 95%O_2/5%CO_2) for the first10 min to ensure an environment conducive to stable recording had been achieved. To simulate ischaemia RONs were exposed to a perfusate which had been continuously bubbled with a 95%N_2/5%CO_2 mixture and from which glucose was omitted for 60 min in adult nerves. Following OGD, exposure of adult RONs to normal aCSF for 60 min was allowed as previous studies have shown that during this time the RON achieves its maximum functional recovery (Stys *et al.,* 1990; Fern *et al.,* 1998); while in neonatal rats, the OGD time and post OGD employed was a 90 min. When the experiment solutions and gases were changed, it took about 2 min for the new solutions to reach the interface perfusion chamber, this time recorded as the time of the solutions and gases were physically swapped. It has been shown by previous studies using a similar experimental design that the gaseous environment changes from normoxic to anoxic (and vice versa) in approximately 2 min (Stys *et al.,* 1990; Stys *et al.,* 1992).

Observations were made from electrical stimulus evoked compound action potentials (CAPs) using glass electrodes. The relationship between increasing stimulus intensity and the CAP is proportional. Increasing the stimulus current will result in an increase in the CAP up to maxima (James *et al.,* 2010). CAPs for nerves younger than P15 were elicited by square constant-voltage stimulus pulses of 150-600 μsec duration which were delivered by an isolated stimulus unit at one minute intervals (unless otherwise stated). For older nerves pulses of 50 μsecs duration were used, the discrepancy being due to the high input resistance and capacitance of the younger axons which are smaller and non-myelinated. The CAPs were elicited by 125% supra-maximal stimuli applied to the proximal end of the RON by stimulating electrode. The recorded signal was amplified ten times, filtered at 800Hz (low pass) in experiments using immature RONs or 10000 Hz for more mature RONs and acquired at 25000 Hz for a period of 40 milliseconds every 60 seconds (unless otherwise stated). The data were transferred to computer where it was exported to excel and analysed using Signal software (Cambridge Electronic Design Ltd 2002). CAP loss is an indication for a loss of axonal function (Fern *et al.,* 1998), which is well correlated with pathological changes in the axonal structures such as, loss of axonal integrity, mitochondrial damage, dissolution of microtubules and neurofilaments (Alix and Fern, 2009).

Technical problems

There are some technical problems that affect the recording of the CAP during the experiments. (1): air bubble inserted into the electrodes and prevents the signal conduction. The bubble is removed by suction and the fluid inside the electrode that has a bubble using suction tube, and then re-fill the electrode with aCSF and then recording can be started. (2): The glass electrode might break during recording of CAP and leads to cancellation of the experiment. A new electrode is made by gentle heating of the glass capillary tubes until the end of the glass bends to a degree that facilitates the insertion of the nerve. (3): The silver wire might separates and stop signal conduction. Re-fixation of the wire is needed by ironing. (4): Gas perfusion might empty during the experiment and affect the result of CAP recording. (5): Blocking of perfusion tubes connected to the perfusion champer during CAP recording due to the precipitations from the solutions of frequent experiments which lead to dryness and death of the nerve and with sudden cancellation of the recording. Rig cleaning protocol is done after each day at the end of the experiments.

Rig cleaning protocol:

Materials:

- Sodium phosphate tribasic ($Na_3Po_{4.}$ 12 H_2O).
- Dilute Hcl solution (0.1% dilution (10 ml Hcl into 990 ml H_2O)-water first then acid.
- H_2O from pure water filter.

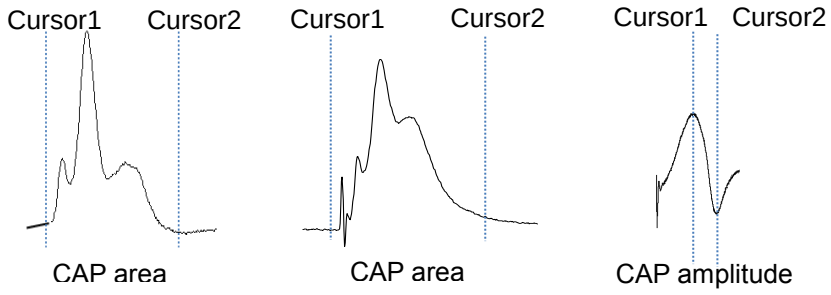
Methods:

- Rinse tubing with 500ml H_2O
- Run through pipes 200ml phosphate solution.
- Rinse with 500ml H_2O.
- Run 200ml of Hcl through tubing.
- Rinse well with 500ml H_2O.
- Finally do not leave fluid in tubing-make sure tubing is as dry as possible.

Data Analysis

For experiments involving adult RONs, the area under the triphasic "supra-maximal" CAP was used as a quantitative estimate of all the contributing axons in a nerve, and to assess changes in the number of action potentials (Stys *et al.,* 1991; Cummins *et al.,* 1979) (*see the outline below*). The amplitude of the biphasic CAP, calculated from the maxima of the positively deflected peak minus the minima of the negatively deflected peak, was used as a measure of the functional integrity of neonatal RONs (Fern *et al.,* 1998) (*see the outline below*). Attempts to use the area of the CAP were not made due to interference from the stimulus artefact.

Data are expressed as mean ± SEM, significance determined by Bonferroni`s Multiple Comparison Test or ANOVA as appropriate.



Outline illustrating measurement of the CAP area or amplitude in adult or neonatal rodents. An area under the curve is used to calculate the CAP in adult rodents, generally, cursor1 is placed before the first peak and cursor2 is placed after the third peak, in some few experiments when the plot is represented by a lot of noise especially when the artefact is close to the first peak, the cursor1 is placed before the artefact and the noise

disappeared. The amplitude of the biphasic CAP of neonatal RONs, calculated from the maxima of the positively deflected peak minus the minima of the negatively deflected peak.

Electron microscopy (EM)

Ultrastructure was examined to show the axonal and glial injury in P0 RONs following regular OGD, OGD + picrotoxin; OGD + strychnine; OGD + mecamylamine; and OGD + propranolol and phentolamine; in comparison to control conditions. After dissection, the nerves were put in separate containers (35.0/10mm petri dishes) with their own solutions and were maintained at 37° for 90 min of OGD. All samples were then fixed with the following steps and washed in Sorenson's buffer: 2% formaldehyde/ 4% glutaraldehyde/ 0.1M sodium cacodylate buffer (2 mM $CaCl_2$, PH 7.4), which was supplied by the EM lab and kept refrigerated overnight.

1- Washed three times 20 min with 0.1M sodium cacodylate and stored over the weekend at 4ºC.
2- Washed three times 20 min in distilled de-ionised water.
3- Secondary fixation with 1% osmium tetroxide / 1.5% potassium ferricyanide in DDW for 3 hrs.
4- Washed three times 20 min in distilled de-ionised water.
5- Tertiary fixation done in 2% aqueous uranyl acetate for 1 hr. at 4ºC.
6- Washed twice for 10 min in distilled de-ionised water.

7- 30% ethanol for 15 min, then 50% ethanol for 15 min and 70% ethanol for 15 min.

8- 70% ethanol and stored overnight at 4ºC, then 90% ethanol for 30 min.

9- 100% analytical grade ethanol for 30 min, this step repeated three times.

10- Propylene oxide per 10 min, repeated twice.

11- 2 Propylene oxide: 1 modified Spurrs Viscosity Resin (Hard Formula) for 90 min.

12- 1 Propylene oxide: 1 Modified Spurrs Viscosity Resin for 60 min.

13- 1 Propylene oxide: 2 modified Spurrs Viscosity Resin for 60 min.

14- 100% Modified Spurrs Viscosity Resin for 30 min, then 100% modified Spurrs Viscosity Resin overnight.

15- Fresh modified Spurrs Viscosity Resin for 3 hrs. (repeated twice)

16- Embed and polymerase at 60 ºC for 16 hrs. And examined with a Joel 100 CX electron microscope.

Electron-micrographs were collected blind by Robert Fern. The fixation steps were done by EM technicians while I assisted and observed. For morphometric analysis and viability scoring, axons within a minimum of three grid sections each from three RONs were outlined by hand (Image-J software, NIH) and the axon area and perimeter measured. The mean axon diameter was taken as the mean of the longest and shortest widths. Grids

were randomly selected and all identifiable axons within the area were included. Axon viability scores were assigned blind using the following scoring system. Axons were given one point for each of three well-established indicators of viability: i) the presence of an intact axolemma, ii) the presence of microtubules, and iii) the presence of a debris-free axoplasm. Axons that showed all three attributes were therefore given a viability score of "3", and axons with none were given a score of "0".

Immunohistochemistry

Immunohistochemistry was used to detect an antigen (protein) in biological tissues via antigen-antibody interactions. Antibodies are produced by inoculating an animal, often a mouse or a rabbit, with an antigen leading to the generation of antibodies against the foreign protein or peptide. There are two types of antibodies, monoclonal and polyclonal; monoclonal antibodies recognise only one epitope on an antigen while polyclonal antibodies recognise multiple epitopes on any one antigen (Frank, 2002). I was primarily looking for the expression of NMDA-Rs on both P0 and P8 RONs to support the hypothesis that both myelinated and non-myelinated central white matter injury is partially a glutamate-dependent process.

RONs were dissected into 0.1M phosphate buffered saline (PBS) immediately after the animals were killed according to UK Home Office regulations. Neonatal RONs aged between P0-P4 were then fixed for thirty minutes in 4% paraformaldehyde in 0.1M PBS at room temperature, while those from adult RONs were fixed for one hour. Subsequently, RONs from neonatal and adult animals were cryoprotected in 20-25% sucrose and

freeze-sectioning. 20µm cryostal sections were subsequently blocked for 60 min in 0.1M PBS containing 10% goat serum (DakoCytomation) and 0.5% Triton X (Sigma; PBGST) for two hours. The sections were then incubated in this solution overnight at 4˚C with the primary antibodies. Antibodies raised against neurofilament-200 (NF-H) and neurofilament-70 (NF-L) (Chemicon; 1:200 and 1:100 respectively) were used. Next, the sections were washed three times in PBGST for 5 minutes each time, after which the secondary antibodies, diluted in PBGST, were applied for one hour. For double staining, the sections were incubated overnight with the above antibodies plus the following primary antibodies:

1. Mix mouse NG2+ chondroitin sulphate proteoglycan – dilution 1:500 and 1:100

2. Mix mouse monoclonal anti-NG2+ antibody-dilution – dilution1:200

3. Conjugated mouse monoclonal anti-glial fibrillic acid protein (Cy3 Anti-GFAP) – dilution 1:100

4. Unconjugated mouse monoclonal anti-glial fibrillic acid protein (Cy3.5 Anti-GFAP) – dilution 1:100

5. O4 (Oligodendrocyte): anti-oligodendrocyte marker O4 phycoerythrin conjugated mouse IgM- 1:200

6. Neurofilament-Heavy (NF-H [200KDa]): Mouse monoclonal anti-NF-H – dilution 1:200

7. Neurofilament-Light (NF-L [70KDa]): Mouse monoclonal anti-NF-L – dilution 1:100

Primary antibodies against glutamate NMDA receptor subunits:

10. Rabbit polyclonal anti-NMDA receptor (NR1) C2 variant- dilution 1:200, 1:500

Following this, the sections were washed in PBGST for 3×15 min. The secondary antibodies were applied onto sections (the concentrations are given below) and incubated for 2 hrs at a temperature of -4◦C. Primary antibody omission controls were performed.

1. Alexa Flour 568 (Cy3.5) Goat anti-rabbit IgG (Molecular probes, OR, USA) – dilution 1:1000

2. Alexa Flour 488 (FITC) Goat anti-mouse IgG (Molecular probes, OR, USA) – dilution 1:1000

3. Alexa Flour 568 (Cy3.5) Goat anti-mouse IgG (Molecular probes, OR, USA) – dilution 1:100

The sections were washed for 5×15 minutes in PBGST and then mounted with 22×40 mm cover slips using mounting solution, and imaged by using an IX70 scanning laser confocal microscope with Fluoview (Olympus, Japan) software.

IHC fixation

2 % Paraformaldehyde (1L) was prepared in the fume hood; 20 g paraformaldehyde was added to 400 ml 0.2 M Na_2 HPO_4 in the fume hood. The mixture was continuously heated until dissolved, and then was cooled. 100ml 0.2M Na_2 HPO_4 was added, and finally 500 ml distilled H_2O was added.

Analysis of immunohistochemistry data:

Primary antibody omission controls were blank when collected at the same image setting as used for positive staining (see chapter V). Images were collected using an Olympus Fluoview IX70 confocal microscope using a x60 objective. The intensity levels of neurofilament staining were assessed by mean pixel intensity levels within the standard region of interest performed using Metamorphic software (Universal Imaging Corporation). Random fields of view (FOV) were taken from a minimum three-nerve section in at least four different optic nerves and was averaged using the identical image acquisition setting.

Chapter III

OGD-induced injury in myelinated axons: Electrophysiological features

Introduction

The brain constitutes 2 % of the body`s mass and entails 20 % of its resting energy consumption, this reflects the high metabolic demands of neurons (Sokoloff, 1960; Attwell and Laughlin, 2001). The local and global connections of the brain are divided into distinct white matter and grey matter regions; this division reduces the conduction delay and energy use in the brain (Wen and Chklovskii, 2005). The energetics of grey matter have long been studied in contrast to white matter energetics which are poorly understood (Attwell and Laughlin, 2001; Lennie, 2003; Nawroth *et al.,* 2007; Alle *et al.,* 2009). Yet white matter forms half of the human brain; its plasticity is involved in the memory and learning mechanisms, therefore, any energy disruption can cause vascular dementia (Laughlin and Sejnowski, 2003; Ullen, 2009; Fields, 2010; Englund, 2002; Patel and Markus, 2011). White matter function is a more energy-efficient process than grey matter function, consuming ≤ 50 % less energy than the latter (Harris and Attwell, 2012); in addition, mitochondria in optic nerve axons may possibly sustain action potential firing rates with a reasonable density of glucose transporters in the nodal membrane without the need for energy transfer from oligodendrocytes (Harris and Attwell, 2012). Therefore, the function of the CNS myelinated axons may be maintained by mitochondrial oxidative phosphorylation based on glucose influx at nodes of Ranvier (Harris and Attwell, 2012). These findings could explain why grey matter is more sensitive to ischaemic injury than white matter; in addition, it exhibits a more rapid irreversible loss of

functions than in white matter (Fern and Ransom, 1997). However, loss of blood supply in both white matter and grey matter results in a collapse in ATP concentration, followed by failure in the Na^+-K^+ pump that leads to a disturbance in ionic homeostasis (Clarke and Sokoloff, 1994). This is followed by the un-compensated redistribution of K^+ into the extracellular space and subsequent membrane depolarisation and the loss of action potential conduction (Kass and Lipton, 1989; Clarke and Sokoloff, 1994). Although, the pathogenesis of white matter injury in adult includes several interacting factors; hypoxia/ischaemia is thought to be the primary cause in most cases (e.g. stroke).

There is contradictory evidence regarding the mechanism of OGD-induced central white matter injury; some studies indicate a role for NMDA-R and others are not consistent with this conclusion. One early paper, (Ransom *et al.,* 1990), showed that ketamine, an NMDA-R blocker, is protective against OGD in adult RON. Bakiri *et al.* (2008) showed that memantine, another NMDA-R blocker is also protective in this preparation. Micu *et al.* (2006) used adult RON and showed that Ca^{2+} increase in myelin during ischaemia is mediated by NMDA-Rs, which is greatly reduced by broad spectrum NMDA-R antagonists, such as MK-801. Activation of these receptors during ischaemia can elevate [Ca^{2+}] and damage the myelinating processes of oligodendrocytes, suggesting that these receptors are a novel therapeutic objective for preventing white matter injury (Lipton, 2006; Matute, 2006; Salter and Fern, 2005). Blocking of NMDA-Rs alone using MK-801 or combined memantine together with an AMPA/KARs antagonist (NBQX), significantly improved recovery in RON (Bakiri *et al.,* 2008). However, Tekkök

et al. (2007) and Baltan *et al.* (2008) reported no similar protection of MON using NMDA-R block. One explanation for this contradiction might be a species difference, or physical differences in the preparations such as nerve diameter. I have addressed this by examining OGD-induced injury in both preparations using the same recording system and testing the effects of NMDA-R activation during OGD. MON was found to be more resistant to OGD than RON, an effect that was abolished by NMDA-R activation, consistent with a role for NMDA-R in white matter injury in both species.

Basic ionic mechanisms of injury were investigated by the second paper showed that ischaemic injury of white matter is critically a Ca^{2+}-dependent process (Stys *et al.,* 1990). The latter used *in vitro* RON as a model of white matter anoxic injury and concluded that white matter injury depended on persistent membrane Na^{+} conductance. This led to influx of Na^{+} promoting reverse operation of Na^{+}/Ca^{2+} exchanger; consequently increasing Ca^{2+} influx via the Na^{+}/Ca^{2+} exchanger (Stys *et al.,* 1992). Therefore, blocking of Na^{+}/Ca^{2+} exchanger, Ca^{2+}-channels or Na^{+}-channels produced a protective effect against central white matter injury (Stys *et al.,* 1992; Fern *et al.,* 1993). In addition, acid-sensing ion channels (ASICs), which are newly discovered channels expressed on the mammalian CNS, these channels are highly permeable to Ca^{2+}, and mediate excitotoxicity during ischaemia in the brain (Barber *et al.,* 2003). However, this mechanism of Ca^{2+}-induced excitotoxicity is a glutamate-independent process that triggers both necrotic and apoptotic cell death (Stys *et al.,* 1992; Krishtal, 2003). Therefore, blocking of these channels produces a neuroprotection against brain ischaemia so proposing a

new therapeutic target for stroke therapy (Stys *et al.,* 1992; Xiong *et al.,* 2000).

Besides to the arguments regarding the mechanism of OGD-induced injury in developed brain, white matter of older rodents becomes intrinsically more susceptible to these conditions, suggesting the mechanism of white matter injury changes as a function of age (Baltan, 2009). The ageing process alters the relationship between axons and myelin as well as the molecular architecture of the axons themselves, and the risk for ischaemic stroke increases considerably with age (Peters and Sethares, 2002; Hinman *et al.,* 2006; Baltan *et al.,* 2008). As seen in Chapter VI, the accumulation of extracellular Ca^{2+} ions is an important step in the development of ischaemic injury in immature white matter, and the removal of Ca^{2+} and even Na^{+} from the perfusion protects white matter function; the removal of such ions does not ameliorate injury in older white matter, which indicates that the role of the ionic pathway in aging white matter during ischaemia is diminished (Baltan *et al.,* 2008). There are several factors in aging white matter that contribute to glutamate excitotoxicity, such as a reduction in antioxidant detoxification and the inhibition of glutamate uptake by glutamate transporters (Domercq *et al.,* 2005); *see general introduction*. However, as the death of the oligodendrocytes and the axonal damage remain the most common targets of ischaemic injury in older white matter, therefore, a detailed understanding of such a mechanism is necessary in order to meet the challenge of developing effective therapeutic strategies for treating stroke patients.

Results

Evoked CAP recording during normal conditions

The rodent RONs are considered to be an ideal model to investigate the function of CNS and the mechanism of injury, because they are completely white matter tracts, and they are devoid of the complications of neuronal cell bodies, the retinal ganglion cells (Ransom *et al.,* 1997). Remarkably, the number of axons per RON are 120,060 (Hughes, 1977), and per MON are 24,068 (Allen *et al.,* 2006). The functions of rodent optic nerves under normal conditions were investigated using electrophysiological recording. Under control conditions, axon function (measured as the area under the CAP) remained relatively stable for more than 120 min in both adult RONs and MONs at 37°C (111.60 ± 13.3% of the initial value, (n=19) & 90.4± 5.5% of the initial value, (n=12), respectively; *(see Fig 13 & 14 respectively).* The CAPs of RONs and MONs are qualitatively similar and each had three discreet peaks (Stys *et al.,* 1991; Brown *et al.,* 2003).

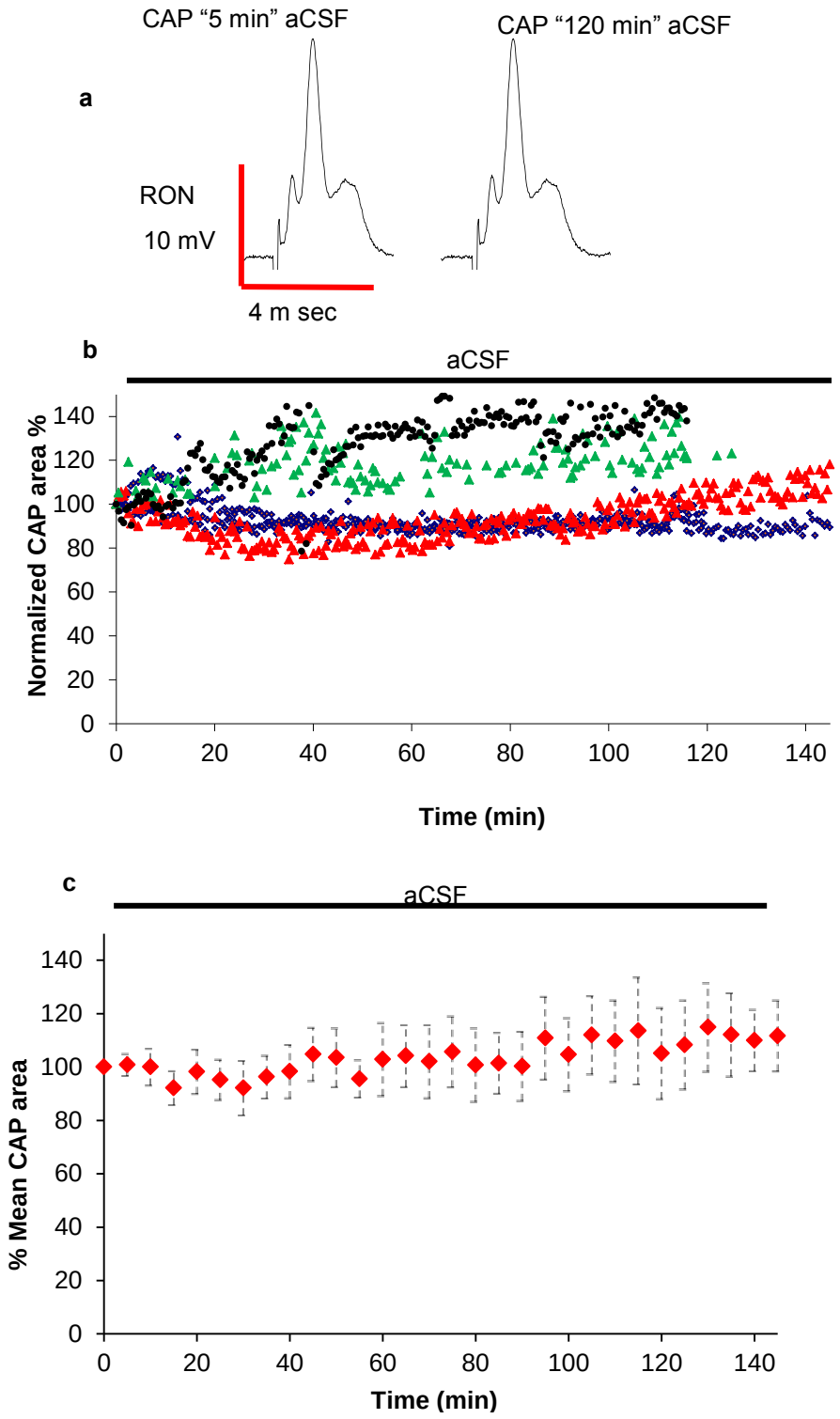


Figure 13- Control recording of the CAP from adult RON is stable and has three components.

a, A representative example of CAPs recorded from a single control experiment at the beginning and at the end of the experiment. No change in the CAP wave form is observed**. b**, Plot of CAP area against time (example of4 single control experiments) (green plot is an example of (Fig a) CAP. **c**, The mean percentage of CAP area showing stable recordings against time. CAPs are normalized to 100 % at zero time, at 70 min is (101.95 ± 13.7), & at 145 min is (111.60 ± 13.3), n=19 nerves. Error bars are SEM.

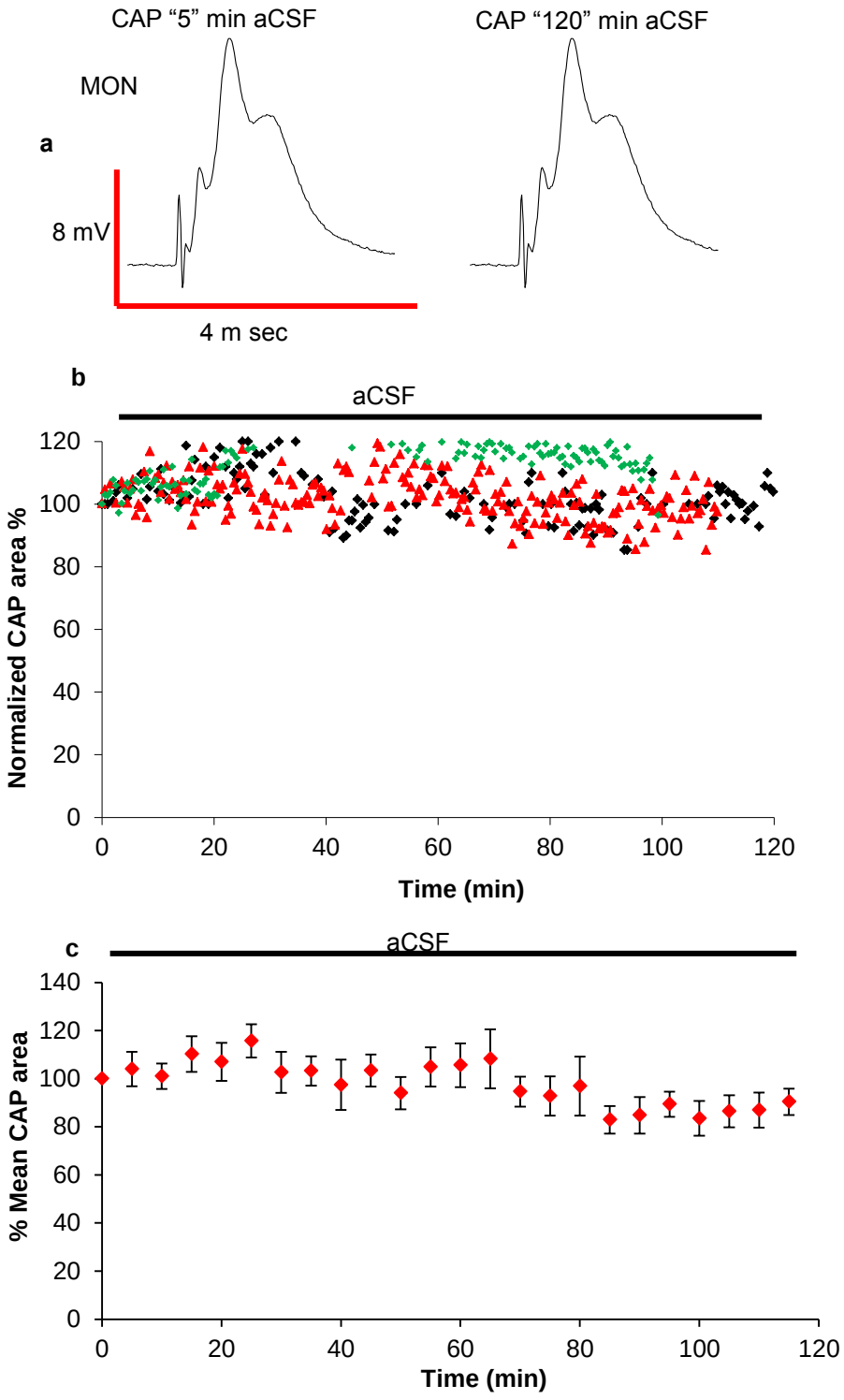


Figure 14- Control recording of the CAP from adult MON is stable and has three components.

a, A representative example of the CAP recorded from a single control experiment at the beginning and at the end of the experiment. No change in the CAP wave form is observed. **b**, Data plot of three single control experiments against time showing no changes in the CAP(black plot is an example of the CAP in (a).**c**, Mean data is plotted over time showing stable CAP area for 120 min. CAP is normalized to 100 % at zero time, CAP at 70 min is (94.6 ± 6.2), & at 120 min is (90.4± 5.5), n=12. Error bars are SEM.

Adult RON is more susceptible to 60 min OGD than MON

Prior studies of the mechanisms of white matter injury in the optic nerve have used anoxia as the insult (Stys *et al,* 1992). Recently, ischaemia (i.e. OGD) has been recognised as being more clinically relevant than anoxia (Baltan, 2007). RON has been widely used as a model to study white matter injury due to anoxia (Stys *et al.,* 1990; Stys *et al.,* 1992), aglycemia (Brown *et al.,* 2001) and ischaemia (Garthwaite et al., 1999). MON, however, has important advantages over RON as an *in vitro* model for studying the mechanism of white matter injury (Tekkök *et al.,* 2007) (*see the discussion*).

In this study, the effects of 60-min durations of OGD on an adult rodent's optic nerve excitability and integrity were determined. The RON CAP recovered to 16.03 ± 3.4% of the initial value, (n= 8; P<0.0001 vs control) after switching back to normal conditions following 60 min OGD. The MON CAP recovered to 36.1 ± 6.0% of the initial value, (n= 7; P<0.0001 vs control) when normal conditions were restored following 60 min OGD. In both RONs and MONs, the CAP fell rapidly after the initiation of the ischaemic conditions, with partial recovery following the re-institution of normal conditions *(see Fig 15 & 16 respectively).* There is a significant difference between the two species in response to the same period of OGD, the recovery in the CAP post OGD in MON is more significant than the recovery in the CAP post OGD in RON (P<0.0001 MON vs RON), (*see Fig 20 b)*. Typically, the MON CAP recovered the first and second peaks following restoration of normal conditions and the higher level of recovery suggest that the smallest axons with the thinnest myelin sheath are resistant to ischaemia

(Baltan *et al.,* 2008) and that MONs, but not RONs, can recover substantial electrical activity after ischaemia (Tekkök *et al.,* 2003).

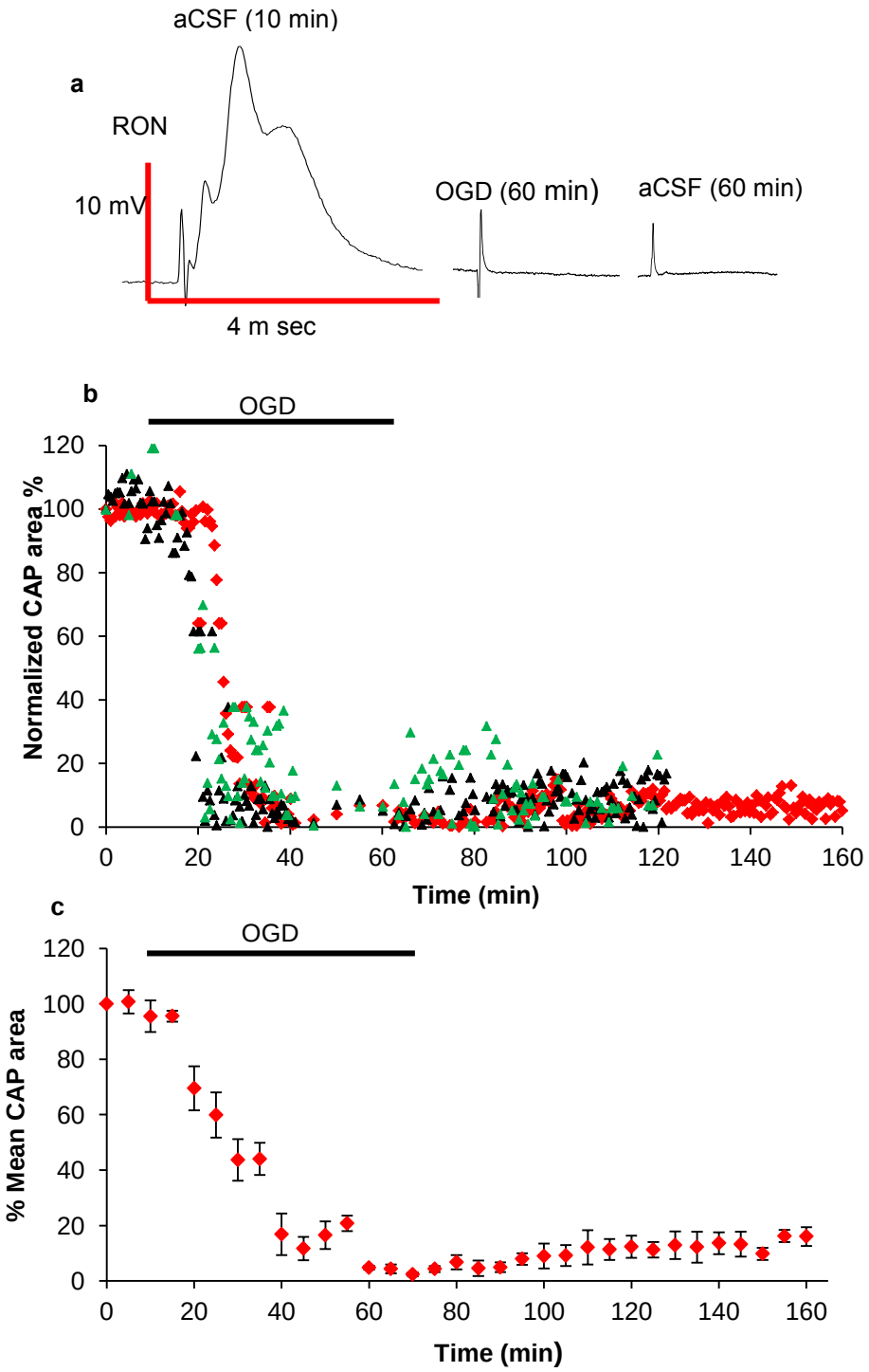


Figure 15- Adult RON CAP showed a significant decline following 60 min OGD.

a, A representative CAPs recorded from adult RON under normal conditions for 10 min, during OGD for 60 min which showed a significant fall in the CAPs, and during re-perfusion with normal conditions for 60 min which showed large conduction failure in adult RON. **b**, plot showing the time course of action potential conduction in adult RON monitored as the area under the CAP before, during, and after 60 min OGD (three single experiments). **c**, Mean data, showing the changes in the CAP area before, during, and after the effect of 60 min OGD. CAP normalized to 100% at zero time, CAP at the end of OGD is (2.3±0.7), & at the end of re-introduction of normal conditions is (16.03 ± 3.4). n=8. Error bars are SEM.

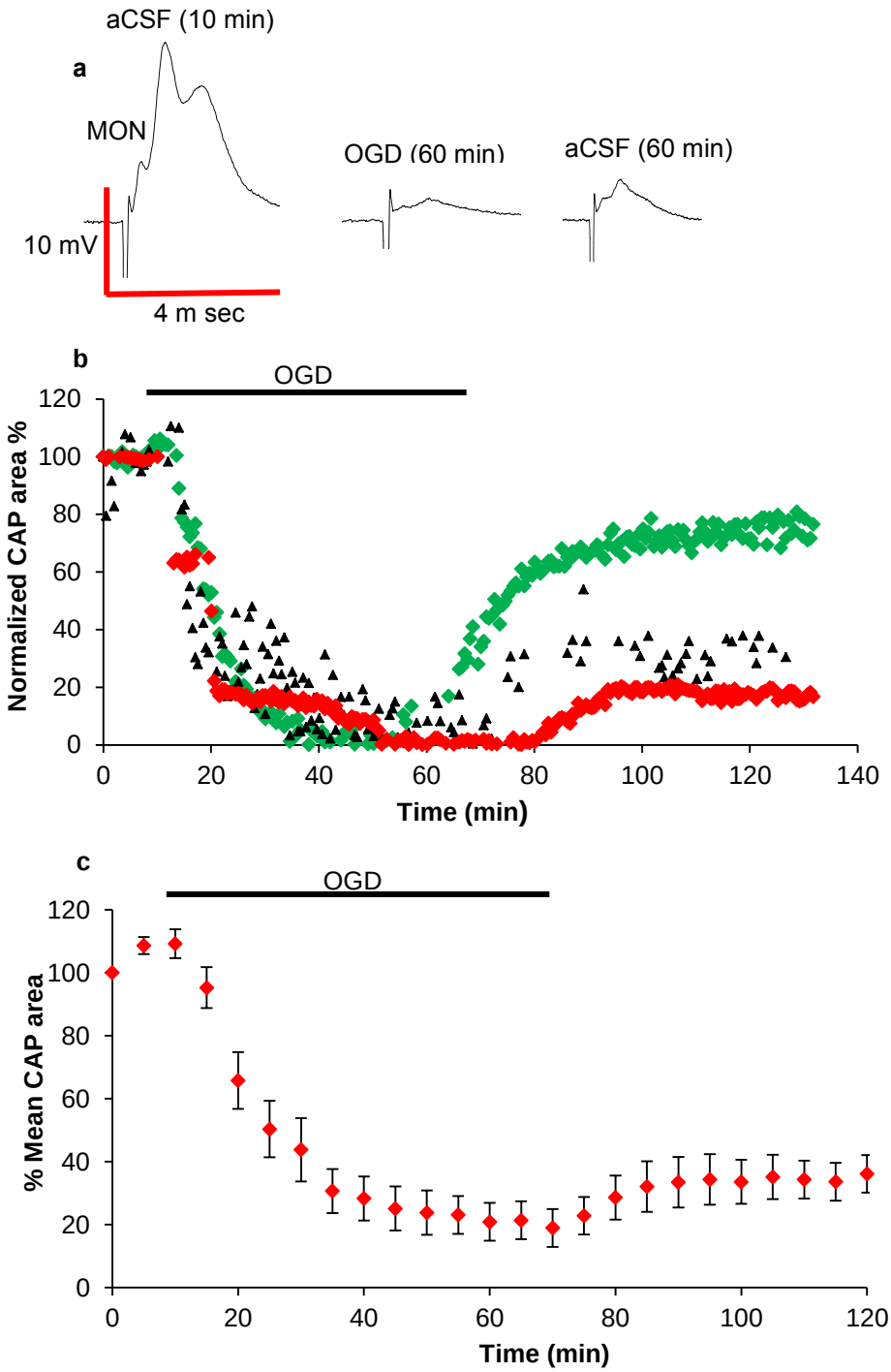


Figure 16- Effect of OGD (60 min) on Adult MON CAP.

a, A representative CAPs recorded from adult MON under normal conditions for 10 min, during OGD (60 min) which showed a significant fall in the CAP, and during restoration of normal conditions which showed a recovery to 36.1 ± 6.0 following restoration of normal conditions for 60 min. **b**, plots of three single experiments showing the changes in the CAP before, during, and after exposure to 60 min OGD (black plot is an example of CAP in (a)). **c**, Mean data plotted against time showing the changes in the CAP area before, during, and after exposure to 60 min OGD. CAP is normalized to 100% at zero time, CAP at the end of OGD is (18.9 ± 6.0), & at the end of re-introduction of normal conditions is (36.1 ± 6.0), n=7. Error bars are SEM.

Application of glutamate agonists increases white matter injury in the presence of OGD in adult rodents

The role of NMDA-Rs during OGD-induced white matter injury in adult rodents was investigated using NMDA-R agonists. It was found that OGD supplemented by the addition of 1mM NMDA + 10µM glycine produced irreversible loss in the CAPs in both RONs and MONs. The recovery in the CAP was significantly different from OGD alone in MONs but not in RONs. In RONs and MONs, the recovery was 7.1 ± 3.2% of the initial value, (n=7) & 6.1 ± 3.2% of the initial value, (n=10), respectively; ($P<0.0001$ vs OGD alone in MON) *(see Fig 17 & 18; 19 & 20 respectively).*

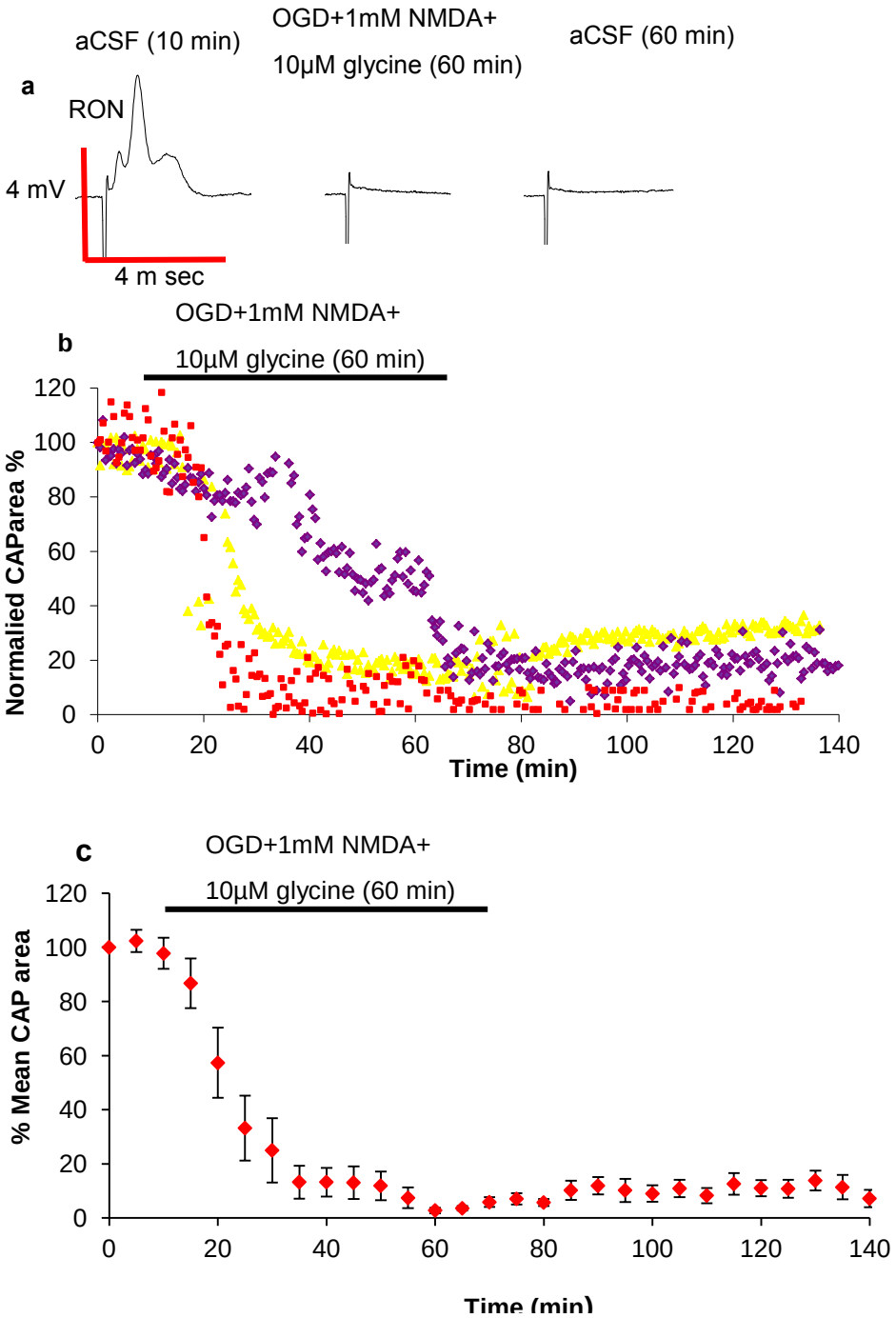


Figure 17- OGD (60 min) co-perfused with NMDA-R agonists NMDA (1mM) and glycine (10µM) had no significant effect compared to OGD alone in adult RON.

a, A representative CAP recorded under normal conditions for 10 min, during OGD (co-perfusion with 1mM NMDA +10µM glycine) which showed a large fall in the CAP, and during reperfusion with normal conditions for 60 min which showed irreversible recovery in the CAP. **b**, plots of CAP area during 60 min activation of NMDA-R + OGD (three single experiments)(red plot is an example of CAP in (a). **c**, Mean data, showing the changes in the CAP area before, during, and after exposure to OGD co-perfused with NMDA-R agonists (1mM NMDA + 10µM glycine). CAP is normalized to 100% at zero time, CAP at the end of OGD + NMDA+ glycine is (5.8 ± 1.8), & at the end of restoration of normal conditions is (7.1 ± 3.2), n=7. Error bars are SEM.

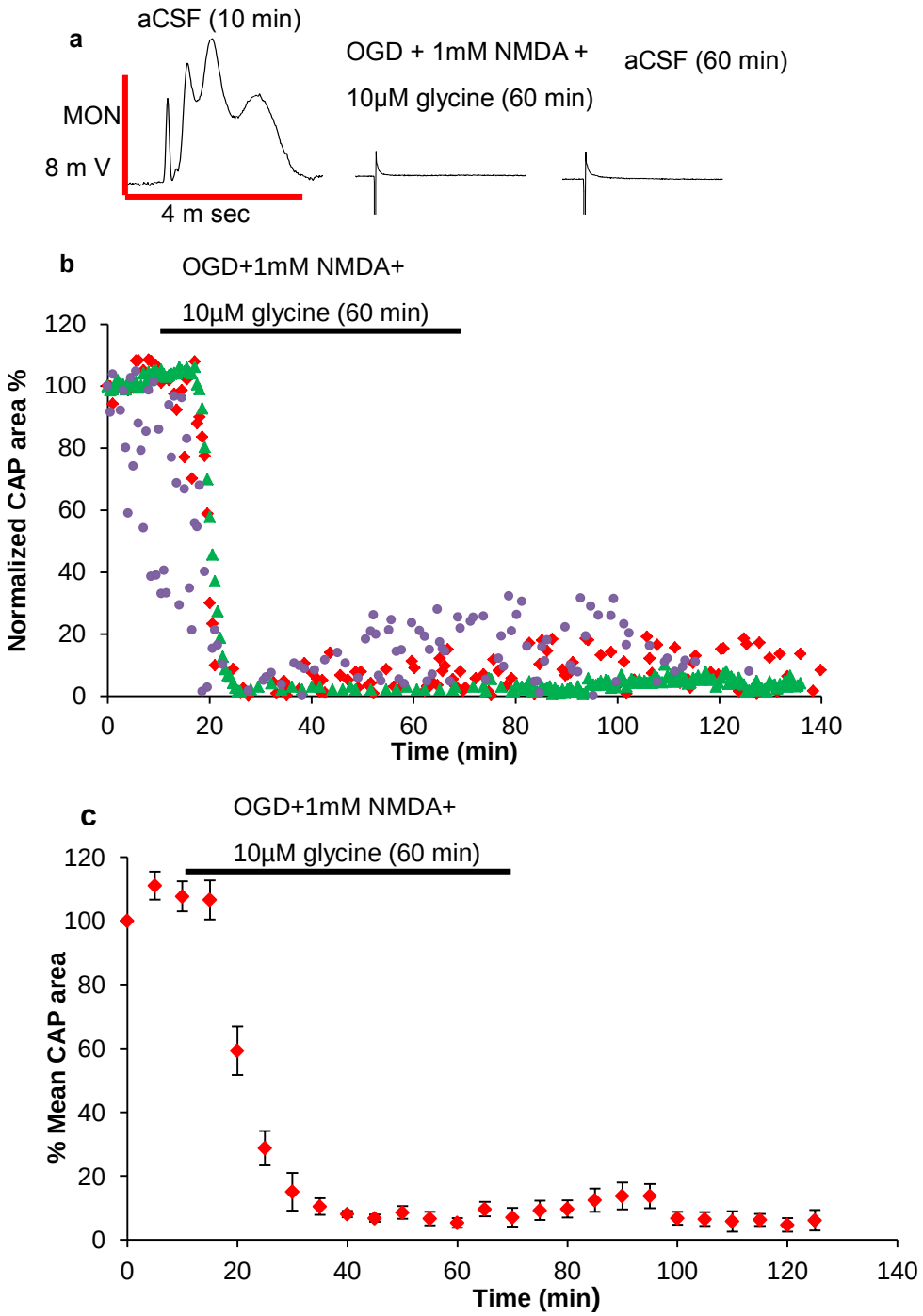


Figure 18- NMDA-R activation potentiated the effects of OGD in adult MON.

a, A representative CAP recorded during normal conditions for 10 min, during activation of NMDA-Rs in case of OGD co-perfused with 1mM NMDA + 10µM glycine for 60 mi which showed a large fall in the CAP, during re-perfusion with normal conditions for 60 min which showed failure in CAP recovery. **b**, plots of three single experiment showing the changes in the CAP before, during, and after 60 min activation of NMDA-R + OGD, (red plot is an example of CAP in (a)). **c**, Mean data, showing the changes in the CAP area before, during, and after exposure to OGD co-perfused with 1mM NMDA + 10µM glycine. CAP is normalized at zero time, CAP at the end of OGD+ NMDA + glycine is (7.0 ± 2.9), & at the end of re-introduction of normal conditions is (6.1 ± 3.2), n=10. Error bars are SEM.

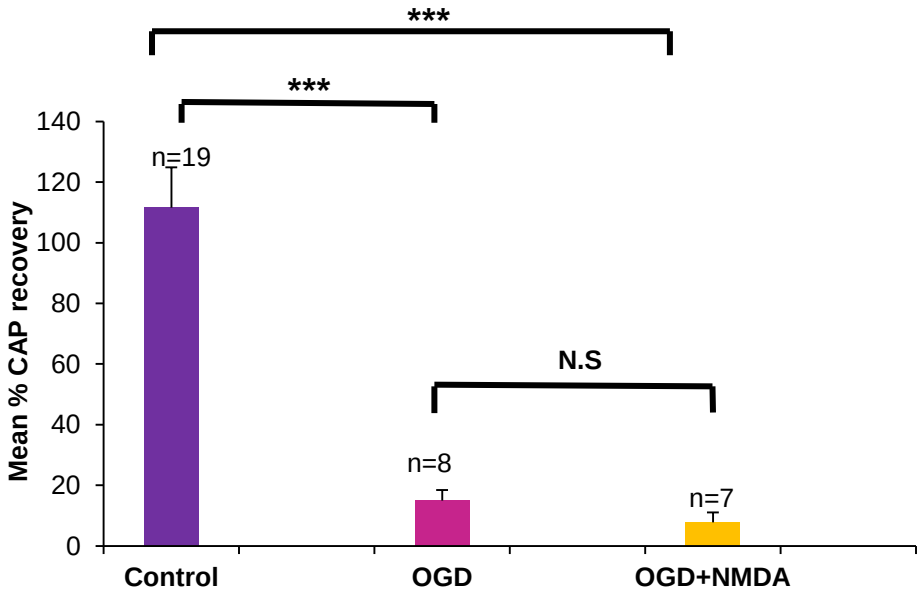


Figure 19- Summary of changes in CAP area of adult RON.

The CAP was significantly reduced compared to control following exposure to OGD alone or OGD + NMDA. There was no significant difference between OGD alone and OGD+ NMDA. ***=p<0.0001. Error bars are SEM.

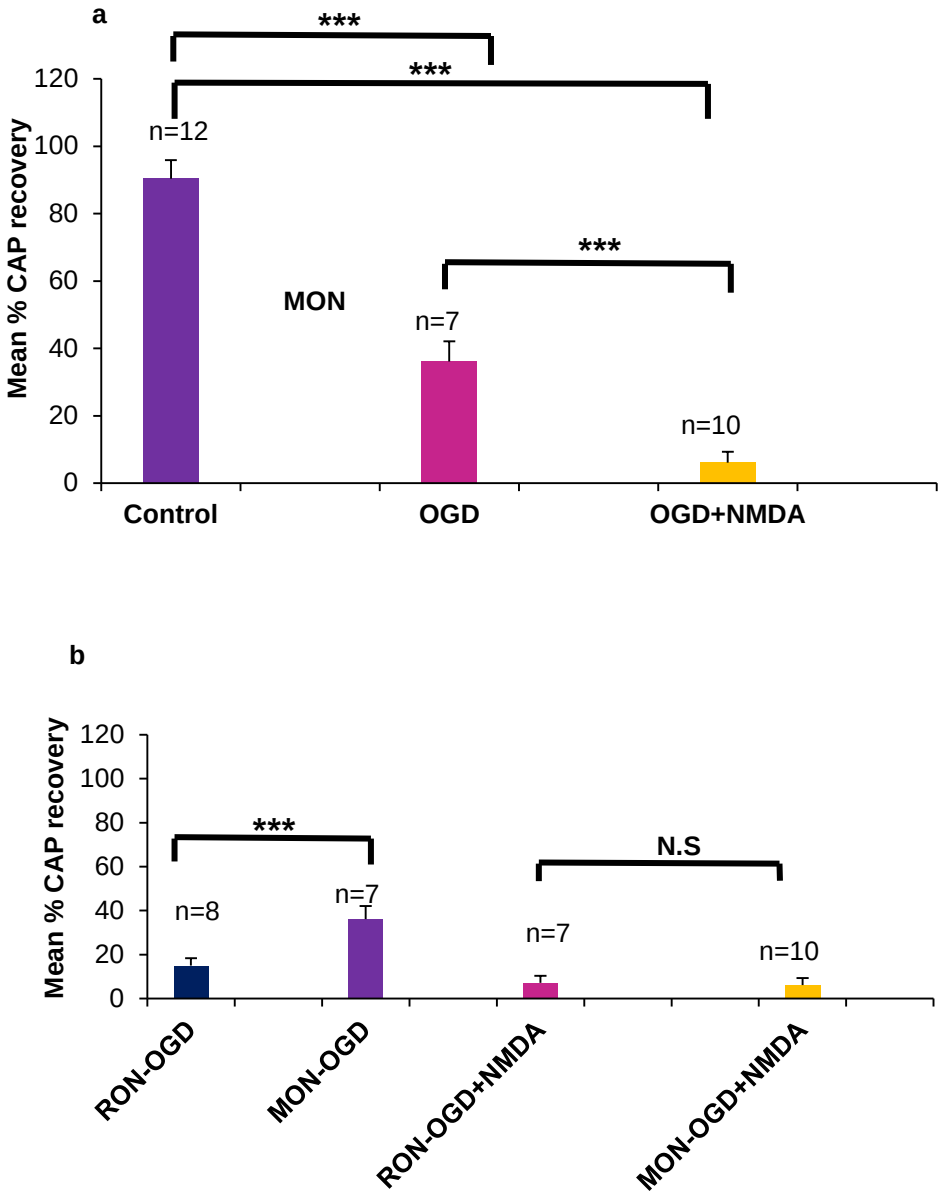


Figure 20- Summary of changes in the CAP area of adult MON.

a, CAP area of adult MON during normal conditions, after exposure to 60 min OGD, and 60 min OGD with over-activation of NMDA-Rs. NMDA-Rs over-activation during OGD reduced the CAP recovery significantly**.** The CAP was significantly reduced compared to control following exposure to OGD alone or OGD + NMDA. **b,** Compared recovery in the CAPs between adult RONs and MONs during OGD, and OGD + NMDA+ glycine. CAPs recovery post OGD is significant in MON compared to RON. CAPs recovery in MON post OGD alone is significant compared to OGD + NMDA + glycine (a). CAP recovery post OGD + NMDA + glycine in RON is not significant compared to CAP recovery post OGD + NMDA + glycine in MON (b). ***=p<0.0001. Error bars are SEM.

Discussion

Axonal injury is responsible for a significant proportion of the disability associated with stroke and other neurological diseases that affect white matter (Tekkök *et al.,* 2007). The pathophysiology of white matter injury is finally attracting attention, and studies to date indicate that the process is unexpectedly complex (Stys *et al.,* 1990; Ransom *et al.,* 1990; Tekkök and Goldberg, 2001; Wrathall *et al.,* 1992). In this chapter, the axonal injury induced by combined oxygen and glucose withdrawal was investigated, and the role of NMDA-Rs in a purely myelinated white matter tract using electrophysiological recording of CAPs. Recordings were made from adult rodents, demonstrating similar CAP profiles for both RONs and MONs. The CAPs were qualitatively similar, composed of three peaks of which the second was the largest, and they were stable for more than 120 min maintained in an isolation perfusion bath. The majority of previous studies which have used the CAP to monitor axon conduction used the entire CAP area since there is no significant difference in the response of each of the peaks (Brown *et al.,* 2003; Fern *et al.,* 1996; Garthwaite *et al.,* 1999; Stys *et al.,* 1990; Sugioka *et al.,* 1995), and I also used the entire CAP area to monitor axon conduction in adult rodent optic nerves.

Adult rat and mouse optic nerves are almost 100% myelinated and both have been successfully used to investigate the pathophysiology of ischaemic injury (Baltan, 2009). Interestingly, RONs and MONs showed variable sensitivity to a 60 min period of OGD. The CAPs of both species falls rapidly but the extent of CAP recovery following OGD is significantly lower in RONs with very limited recovery in comparison to MONs. Tekkök *et al.* (2003) found that the RON and MON were different in size; the RON has a diameter that is 2.3 times larger than MON diameter (650 ±60.2 μm vs 281 ±29.9 μm). This may indicate that the more slowly conducting fibres with smaller diameters in the MON are better able to survive ischaemia (Tekkök *et al.,* 2003). Stys *et al.* (1990) used isolated RONs and showed that axon function was lost within a few min during anoxia; Tekkök *et al.* (2003) used MONs to verify these in a second species and to prepare for future genetic studies which favour mouse models. They were surprised to discover a major difference in the response of the MON to anoxia; which was partially resistant in comparison to the RON (Tekkök *et al.,* 2003). They also discovered that, once they increased the bath glucose concentration threefold, the RON gave a similar response to the MON, suggesting that spatial diffusion of glucose into the larger RON may account for the difference (Tekkök *et al.,* 2003). These results are similar to the findings in this study; however this study is the first to compare the sensitivity of these species to combined oxygen and glucose withdrawal, a more sophisticated model of ischaemia than the anoxia previously studied. A glucose diffusion-based explanation for my findings can be ruled out since both preparations experience zero-glucose conditions, yet the species difference persists.

An alternative hypothesis is that as axon diameter decreases, energy demands lessen due to increased intermodal distance and the axon is more tolerant to ischaemia. This argument is relevant since MON axons are smaller in diameter than RON axons (Allen *et al.,* 2006). The results are also consistent with the idea that a low level of ATP production either from anaerobic metabolism of glucose or aerobic metabolism of energy stores could have a significant impact on the ability of CNS white matter to survive energy deprivation (Fern *et al.,* 1998). During anoxia, a small amount of ATP generated by glycolysis in the axons of white matter can contribute functional maintenance in the axons (Fern *et al.,* 1998); while in the absence of glucose axons can access energy from other energy reserves such as amino acids and phospholipids (Siesjo, 1988). MON astrocytic glycogen is broken down to lactate and transported out of the astrocyte into the extracellular space, from which it can be taken-up by axons and metabolized under conditions of aglycemia or increased tissue energy demand (Brown and Ransom, 2007). The axons of central white matter can maintain their function for a further 20 min or more with astrocytic glycogen (Brown and Ransom, 2007). Under OGD conditions, however, anaerobic glycolysis can occur by utilising glycogen stores.

Tekkök and Goldberg (2001) found that OGD for 30 min in adult mice corpus callosum slices caused irreversible loss of CAPs followed by loss of oligodendrocytes and disruption of axonal neurofilaments. This OGD-induced axonal injury was blocked by application of NBQX which resulted in a full recovery of axonal conduction after OGD (Tekkök and Goldberg, 2001). During OGD, therefore, glutamate excitotoxicity through AMPA/KA-Rs on

oligodendrocytes was an important mechanism for white matter injury (Tekkök and Goldberg, 2001). Previous studies showed that axons can be injured directly by ionic mechanisms leading to toxic accumulation of extracellular Ca^{2+} (Fern *et al.,* 1995; Ouardouz *et al.,* 2003; Stys *et al.,* 1990; Wolf *et al.,* 2001); while oligodendrocytes and the myelin they manufacture can be injured by glutamate (Alberdi *et al.,* 2002; Li *et al.,* 1999; Matute *et al.,* 1997), and by AMPA-R agonists in culture (Matute, 1998; Yoshioka *et al.,* 1995), *in vivo* (Dusart *et al*., 1992; McDonald *et al.,* 1998), *in situ* (Li and Stys, 2000), and *in vitro* (Fern and Moller, 2000; McDonald *et al.,* 1998). Therefore, either AMPA/KA-R blockade or removal of Ca^{2+} can preserve oligodendrocytes and axonal structure and function (Tekkök and Goldberg, 2001). Glutamate however, also activates unusual NMDA-Rs in oligodendrocytes that are blocked weakly by Mg^{2+} (Karadottir *et al.,* 2005). These NMDA-Rs are expressed at all stages of oligodendrocyte development and on the myelinating processes of mature oligodendrocytes (Karadottir *et al.,* 2005; Micu *et al.,* 2006; Salter and Fern, 2005), which are activated in conditions of energy deprivation (Karadottir *et al.,* 2005; Micu *et al.,* 2006; Salter and Fern, 2005), such as stroke (Dewar *et al.,* 2003) and PVL (Volpe, 2001).

The role of NMDA-Rs in adult white matter ischaemic injury is controversial. Some authors, using mainly mouse preparations, report that NMDA-R block is not protective against injury (Tekkök and Goldberg, 2001; Gallo *et al.,* 1994; Patneau *et al.,* 1994). Other workers find significant protection using NMDA-R blockers (Ransom *et al.,* 1990; Bakiri *et al.,* 2008; Micu *et al.,* 2006), mainly using RON. The findings in this chapter suggest that the elevated ischaemic tolerance of mouse axons compared to rat and the absence of protection of the axons by NMDA-R blockers is in fact correlated. By enhancing NMDA-R activation rather than attempting to block the receptor, I have been able to eliminate the species difference in ischaemic sensitivity. The data are best explained by a lower level of NMDA-R activation during ischaemia in the mouse, possibly due to its smaller diameter leading to wash-out of glutamate.

RONs and MONs were subjected to OGD supplemented by GluR agonists (1mM NMDA + 10µM glycine). The major finding of these tests was an increase in the degree of the CAP loss in both RONs and MONs. The loss in the CAP was qualitatively similar between both RONs and MONs, but the difference was significant in the case of MONs exposed to OGD + 1mM NMDA + 10µM glycine compared to OGD alone. My results showed that completely myelinated central white matter, whatever the species; suffer irreversible injury due to over- activation of NMDA-Rs during ischaemia. These results are consistent with the findings in previous studies that NMDA-Rs can be activated by glutamate in conditions of energy deprivation in white matter (Karadottir *et al.,* 2005; Micu *et al.,* 2006; Salter and Fern, 2005).

Another study showed that OGD in adult MON produced ionic disruption in axons and glia that primed white matter for glutamate-induced excitotoxicity (Tekkök *et al.,* 2007). However, this glutamate excitotoxicity is possibly mediated by over-activation of NMDA-Rs in both RONs and MONs. My results, and others, indicate that NMDA-Rs give sensitivity to acute injury in different ages and species, and represent an important target for drug development in a variety of brain disorders (Salter & Fern, 2005).

In summary, this chapter describes the injury mechanisms responsible for the loss of axon function following combined oxygen and glucose withdrawal in the myelinated RONs and MONs. It is argued that the contribution to the injury cascade is made by the over-stimulation of NMDA-Rs, while a harmful Ca^{2+} influx into the cell is mediated by NMDA-Rs causing excitotoxicity; this indicates that excitotoxicity is a constant in the development of ischaemic white matter pathology, from PVL in the neonate to stroke in later life. Therefore, further experiments are needed in order to establish a complete developmental picture of OGD-mediated injury and the effects of NMDA-R block upon post-OGD recovery in myelinated white matter.

Chapter IV

OGD-induced injury in pre-myelinated central axons:

Introduction

Ischaemic injury to the perinatal brain has become an issue of significant clinical interest, especially since injury to developing white matter termed PVL is the leading cause of death and neurological disability such as cerebral palsy and cognitive impairment in survivors who are born before the 32nd week of gestation (Degos *et al.,* 2008). PVL is categorised by damage to axons and death of glia, features that appear very early within 4 hours of the onset of injury (Fern, 2011); cerebral palsy affects between 1.5 and 2.5 per 1,000 live births a year (Armstrong, 1993; Kuban and Leviton, 1994; Volpe, 1992). The neonatal CNS, compared to the adult, is generally more resistant to oxygen and glucose withdrawal (Duffy *et al.,* 1975); however, prolonged exposure of the neonatal brain to these conditions can result in extensive CNS damage, and the white matter structures are selectively vulnerable (Paneth *et al.,* 1994). There are several factors implicated in the pathophysiology of PVL, but hypoxic-ischaemic insult remains the main cause of this disorder (Inder *et al.,* 2002; Laudenbach *et al.,* 2001). Recently, it has been reported that axonal injury following ischaemia is an early sign of both types of PVL (focal and diffuse lesions, *see general introduction*) (Alix and Fern, 2009). Diffuse injury of the oligodendroglial population and subsequent hypo-myelination is the most common pathological feature of PVL (Paneth *et al,* 1990; Iida *et al.,* 1995). Late oligodendrocyte progenitors and oligodendrocyte precursors are present during the time of peak vulnerability of developing white matter to ischaemic injury (Back *et al.,* 2001). The remarkable sensitivity of these differentiating cells to ischaemia

and oxidative stress has been demonstrated by various studies, as their anti-oxidative defences are limited (Back *et al.,* 2002; Fern and Moller, 2000). However, developing white matter injury under oxygen and glucose withdrawal has been shown to be dependent on extracellular glutamate build-up and subsequent sustained activation of GluRs permeable to Ca^{2+}, followed by the induction of excitotoxic cascades (Deng *et al.,* 2003; Salter and Fern, 2005). Manning *et al.* (2008) reported that hypoxia-ischaemia results in glutamate accumulation in developing white matter under pathophysiological disorders from multiple sources. These include the vesicular release of glutamate from axons and the reversal of glutamate transporters that lead to the over-activation of AMPA/KA-Rs as well as NMDA-Rs. Although the blocking of GluRs achieves a degree of neuroprotection during ischaemia, the specific aspects of such neuroprotection remain a matter of debate (Hagberg *et al.,* 2002). The NMDA-R antagonist MK-801 has been used experimentally, and there is strong evidence of its protective qualities against ischaemia; but it is poorly tolerated in contrast to the non-competitive NMDA-R antagonist memantine, which shows neuroprotective activity and is clinically well tolerated (Robinson and Keating, 2006). There is no neuroprotective drug to block the effect of glutamate completely; however, this might be because of multiple difficulties linked to drugs used in clinical trials such as pharmacokinetic problems, side effects and lack of efficacy (Lees, 1997).

A number of neurotransmitters other than glutamate have also been shown to have actions upon developing white matter (Constantinou and Fern, 2009). The significance of the expression of neurotransmitter receptors other than glutamate in developing white matter such as GABA, glycine, nACh and adrenergic receptors reveals the potential role of these neurotransmitters to mediate ischaemic injury in neonates (Constantinou and Fern, 2009). The increase in extracellular glutamate concentration during ischaemia is accompanied by changes in the concentration of other amino acids and by increase in GABA concentration (Clarkson *et al.,* 2010; Alonso-Alonso *et al.,* 2007). Even though there are no effective treatments for PVL discovered yet, several basic questions continue to be strongly debated; for example, whether several drugs must be used in combination to efficiently block the leading mechanisms underlying PVL.

In this chapter, the effects of both iGluRs and other neurotransmitter receptors are examined in the neonatal optic nerve under normal conditions and OGD.

Results

Control recording of CAPs in P0-P4 RONs

Optic nerves from this preparation are in the same late pre-myelination state present in the mid-gestation foetus subjected to PVL. CAPs recorded from nerves taken from animals of P0-P4 are biphasic and stable for 180 min under control conditions at 37C°. Extracellular CAPs in neonatal optic nerves are evoked and recorded with suction electrodes, and the axon function measured between the peak of the largest positive component and the peak of the largest negative component. The mean CAP amplitude at the end of 3 hours was 107.05 ± 5.5% of the initial value, (n=13); *(see Fig 21).*

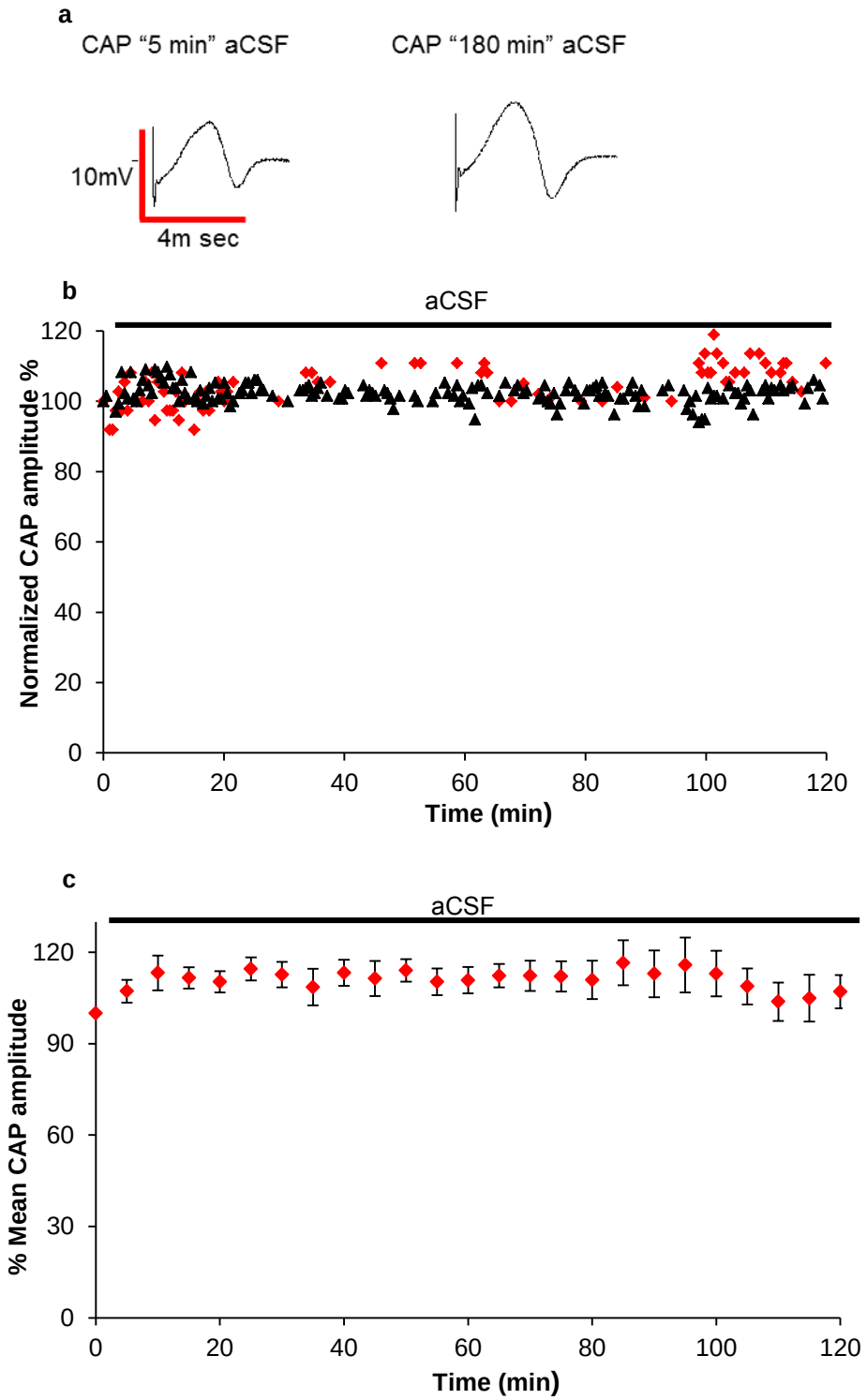


Figure 21- Biphasic and stable CAP (control) recording from P0-P4 RONs.

a, Representative CAPs recorded at the beginning and at the end from a single experiment. **b**, CAP amplitude of two single experiments plotted against time. **c**, Mean data of CAP amplitude of P0-P4 RONs plotted against time showing stable recording for two hours. CAP is normalized to 100% at zero time, CAP at 70 min is (112.2 ± 5.0), and at 120 min is (107.05 ± 5.5), n=13 nerves. Error bars are SEM.

OGD (60 min) in P0-P4 RONs

The standard 60-min period of OGD which is used to produce injury in adult RONs and MONs failed to result in a significant permanent loss of the CAPs in neonatal nerves. Acute exposure to a 60-min period of OGD in P0-P4 RONs produced very mild and transient changes in the CAP amplitude, followed by complete recovery after restoration of normal conditions to 94.47 ± 7.87% of the initial value, (n=3). The recovery in the CAPs recorded from P0-P4 RONs are compared to the recovery in the CAPs recorded from adult RONs and MONs after exposure to 60 min OGD, 94.47 ± 7.87% of the initial value, (n=3); 9.2 ± 18.2% of the initial value, (n=8); and 26.3 ± 4.4% of the initial value, (n=7), respectively *(see Fig 22). The results of these experiments show that*, *developing optic nerves are more resistant to shorter periods of OGD than optic nerves of developed brains.*

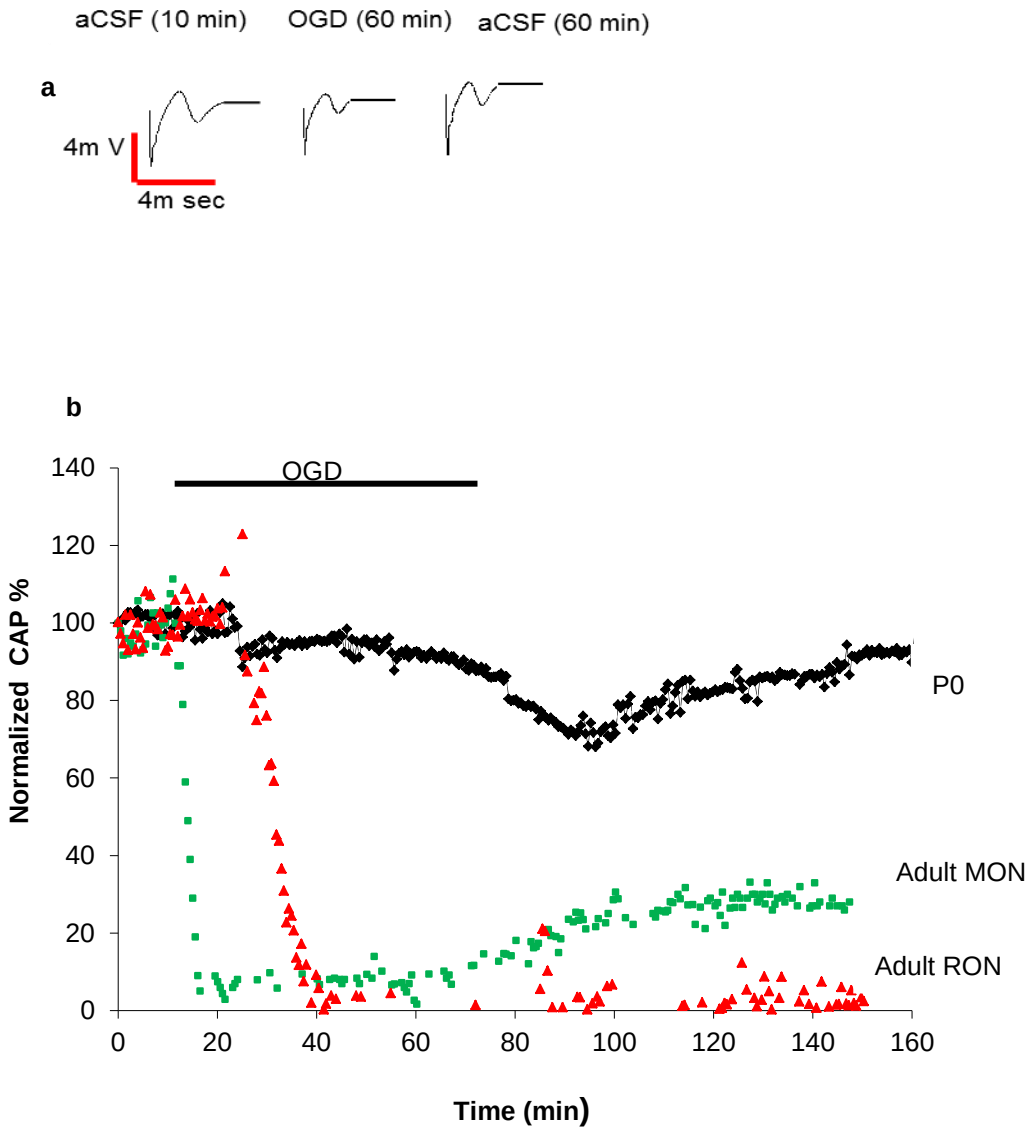


Figure 22- Standard (60 min) period of OGD failed to result in permanent loss of the CAP in P0-P4 RONs.

a, Representative CAPs recorded from a single experiment before, during, and after exposure to 60 min OGD. **b**, CAP area or amplitude plotted against time showing the comparative effect of 60 min exposure to OGD on P0-P4 RONs (black), adult MONs (green), or adult RONs (red). There is no significant loss of function in P0-P4 RONs, while the same period of OGD produces non-reversible conduction failure in adult RONs.

OGD (90 min) in P0-P4 RONs

Simulation of ischaemia by OGD for a 90-min period was sufficient to induce significant injury in developing white matter (P0-P4 RONs). The initial response to a longer period of OGD (90 min) was frequently an increase in the CAP amplitude over the first 10-20 min that could be as large as 111.0 ± 4.5% of the initial value. The CAP fell progressively after the initial rise, reaching 27.6 ± 5.5% of the initial value at the end of 90 min OGD. Unlike nerves from older rats and mice that are affected by exposure to shorter periods of OGD, the CAPs recorded from P0-P4 RONs did not recover significantly following the reintroduction of normal conditions and was 34.5 ± 3.9% of the initial value (n=8; $P<0.0001$ vs control); *(see Fig 23). It is clear that developing optic nerves are relatively more resistant to ischaemia than the optic nerves of the developed brain. However, prolonged exposure to these conditions can result in a significant injury.*

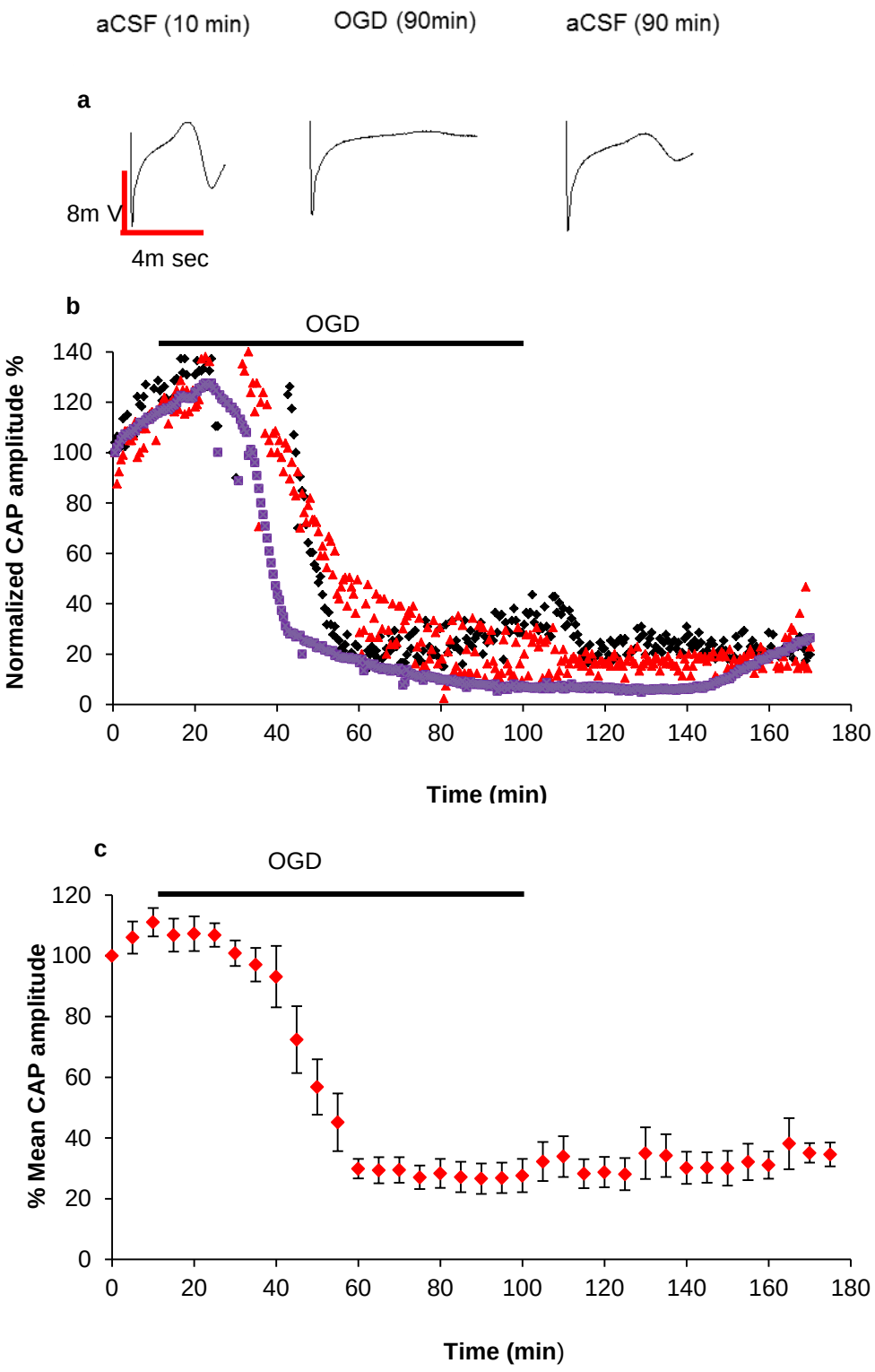


Figure 23- OGD (90 min) induced injury in P0-P4 RONs.

a, A representative CAP recorded from a single experiment during normal conditions for 10 min, at the end of 90 min OGD and after re-perfusion with normal conditions for 90 min. **b**, CAP amplitudes of three single experiments plotted against time. **c**, Data plot showing the changes in the CAP amplitude before, during, and after exposure to 90 min OGD. It showed low level of recovery in the CAP amplitude after re-perfusion with normal conditions. CAP did not reach to zero during 90 min OGD. CAP is normalized to 100% at zero min, CAP at end of OGD is (27.6 ± 5.5), and at the end of the experiment is (34.5 ± 3.9), n=8. Error bars are SEM.

Ischaemic injury is mediated by iGluRs in P0-P4 RONs

The activation of the non-NMDA type of GluRs located on the oligodendroglial somata is a central event in the ischaemic injury of white matter oligodendrocytes (Salter and Fern, 2005; Fern, 2011; Karadottir *et al.,* 2005; Micu *et al.,* 2006); while NMDA type GluR activation is a key step in the ischaemic injury of the processes of these cells (Salter and Fern, 2005). This event is linked to secondary damage to neighbouring small pre-myelinated axons in actively myelinating RONs (Alix and Fern, 2009). However, P0-P4 RONs used in this study are wholly pre-myelinated and contain no oligodendrocytes.

NMDA (1mM) + glycine (10µM) + OGD

Application of 1mM NMDA + 10µM glycine to 90 min OGD evoked a rise in the CAP amplitude within the first 10 min, then declined gradually and reached zero at the end of OGD with minor recovery after restoration of the normal conditions 12.4 ± 5.9% of the initial value, (n=6). Thus the recovery in the CAPs during OGD alone was significantly greater than the recovery in the CAPs during OGD co-applied with 1mM NMDA + 10µM glycine; ($P<0.0001$ vs OGD); *(see Fig 24 & 26). The results of this experiment show that, NMDA-Rs contribute to OGD-induced injury in pre-myelinated central axons.*

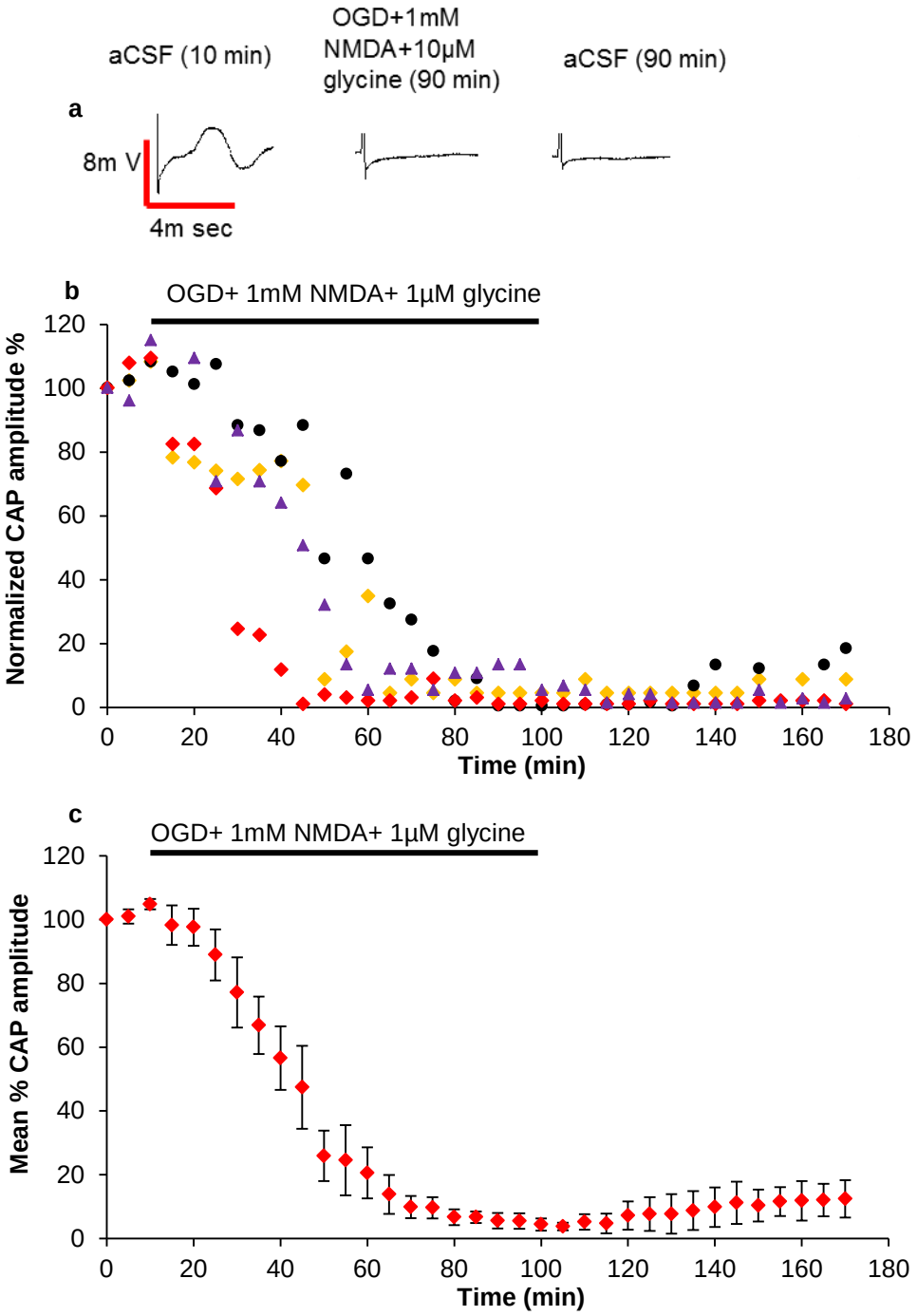


Figure 24- NMDA-R activation exacerbates acute CAP failure during OGD in P0-P4 RONs.

a, Representative CAPs recorded from a single experiment during normal conditions for 10 min, at the end of exposure to OGD +1mM NMDA +10µM glycine and, after restoring the normal conditions for 90 min. **b**, Plots of four single experiments showing that CAP raised in the 1st 10 min of OGD + 1mM NMDA +10µM glycine, then declined gradually and lost completely after restoring the normal conditions. **c**, Data plot showing the changes in the CAP amplitude before, during, and after exposure to 90 min OGD+1mM NMDA +10µM glycine . It showed low level of recovery in the CAP amplitude after re-perfusion with normal conditions. CAP is normalized to 100% at zero time, CAP at the end of OGD is (9.9 ± 3.5), and at end of the experiment is (12.4 ± 5.9), n=6. Error bars are SEM.

MK-801(1µM) + OGD

When P0-P4 RONs were exposed to the selective NMDA-R blocker MK-801(1µM) during OGD (90 min), the CAP amplitude fell to 58.0 ± 9.6% of the initial value, (n=8) at the end of OGD + MK-801. This compared to the CAP amplitude in the case of OGD alone which fell to 27.6 ± 5.5% of the initial value, (n=8) and OGD + NMDA + glycine to 9.9 ± 3.5%, (n=6). The CAP showed a significant recovery following re-perfusion with normal conditions to 82.2 ± 5.0% of the initial value in contrast to the CAP recovery in OGD alone and OGD + NMDA + glycine; ($P<0.0001$ vs OGD alone, and OGD + NMDA + glycine); *(see Fig 25 & 26). MK-801 is therefore highly protective against OGD-induced injury in developing white matter.*

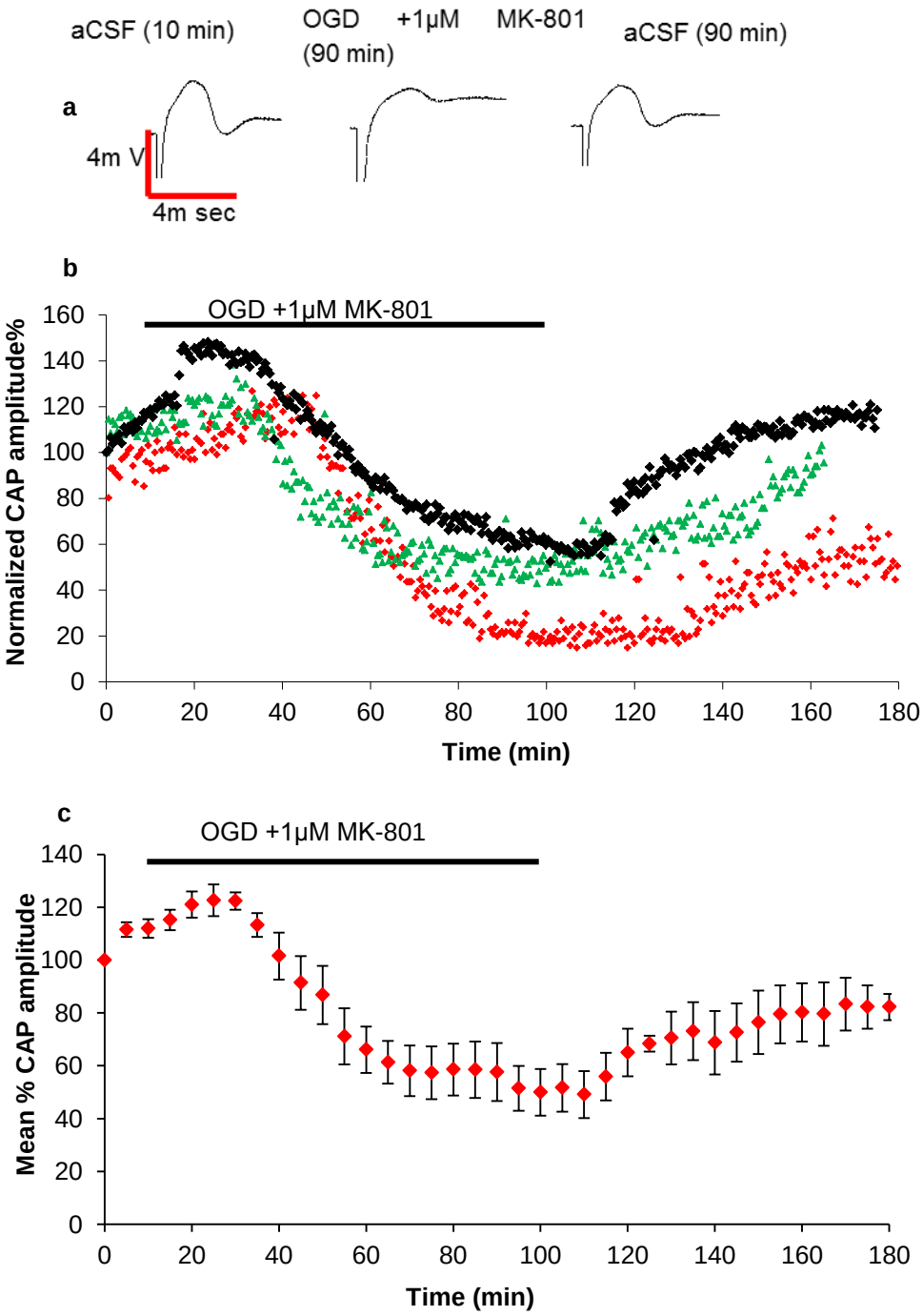


Figure 25- MK-801 (1μM) showed a protective effect during OGD for 90 min in P0-P4 RONs.

a, A representative CAP recorded from a single experiment during normal conditions for 10 min, during exposure to OGD for 90 min co-applied with 1μM MK-801 and after restoring the normal conditions for 90 min. **b**, CAP amplitude of three single experiments plotted against time showing the initial rise in CAP amplitude followed by a gradual decline Which frequently did not reach to zero in the presence of OGD + MK-801, followed by large recovery after restoring the normal conditions. **c**, Mean data showing the protective effect of MK-801 during acute ischaemia in P0-P4 RONs. CAP is normalized to 100 % at zero time, CAP at the end of 90 min OGD is (58.0 ± 9.6), and at the end of the experiment is (82.2 ± 5.0), n=8. Error bars are SEM.

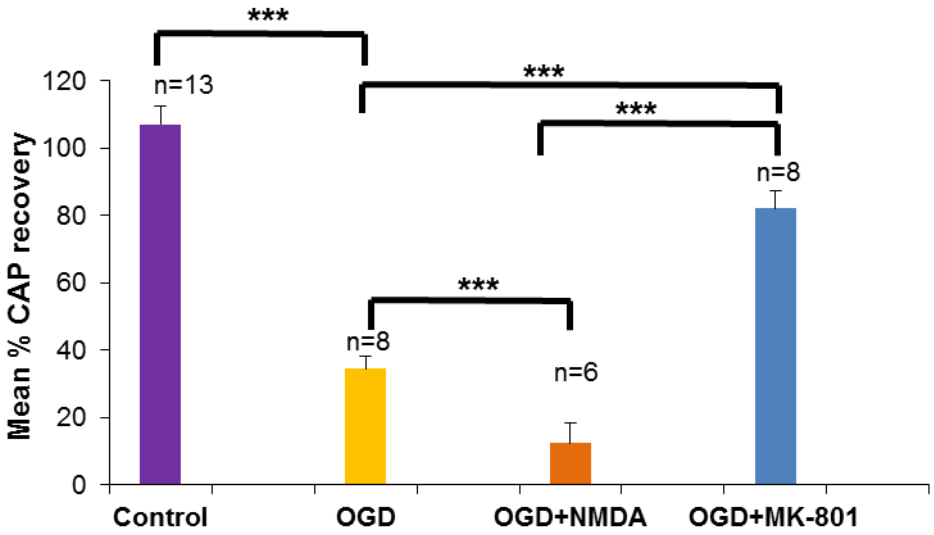


Figure 26- Histogram summary of mean recovery in the CAP amplitude under control conditions, OGD, OGD + NMDA + glycine and OGD + MK-801 in P0P4 RONs.

Histogram compares the effects of NMDA-R agonist and antagonist on the CAP amplitude recovery with normal conditions and OGD. CAP during control conditions is significant to the CAP recovery in case of OGD. There is significant comparison in the CAP recovery between NMDA-R antagonist MK-801, OGD alone and OGD+1mM NMDA +10μM glycine. The CAP recovery during OGD alone is significant to OGD + 1mM NMDA +10μM glycine. ***=p<0.0001. Error bars are SEM.

MK-801(1µM) + aCSF

When P0-P4 RONs were exposed to MK801 (1µM) in aCSF for a 90 min, the CAP amplitude fell gradually and reached to 58.0 ± 7.8% of the initial value at the end of 90 min, (n=8); and recovered to 67.1 ± 5.3% of the initial value after re-institution of normal conditions; (P<0.0001 vs control); *(see Fig 27& 29)*. *These results show therefore that the NMDA-R antagonist MK-801 is highly protective during OGD-induced injury in neonatal central white matter while being toxic under normoxic conditions.*

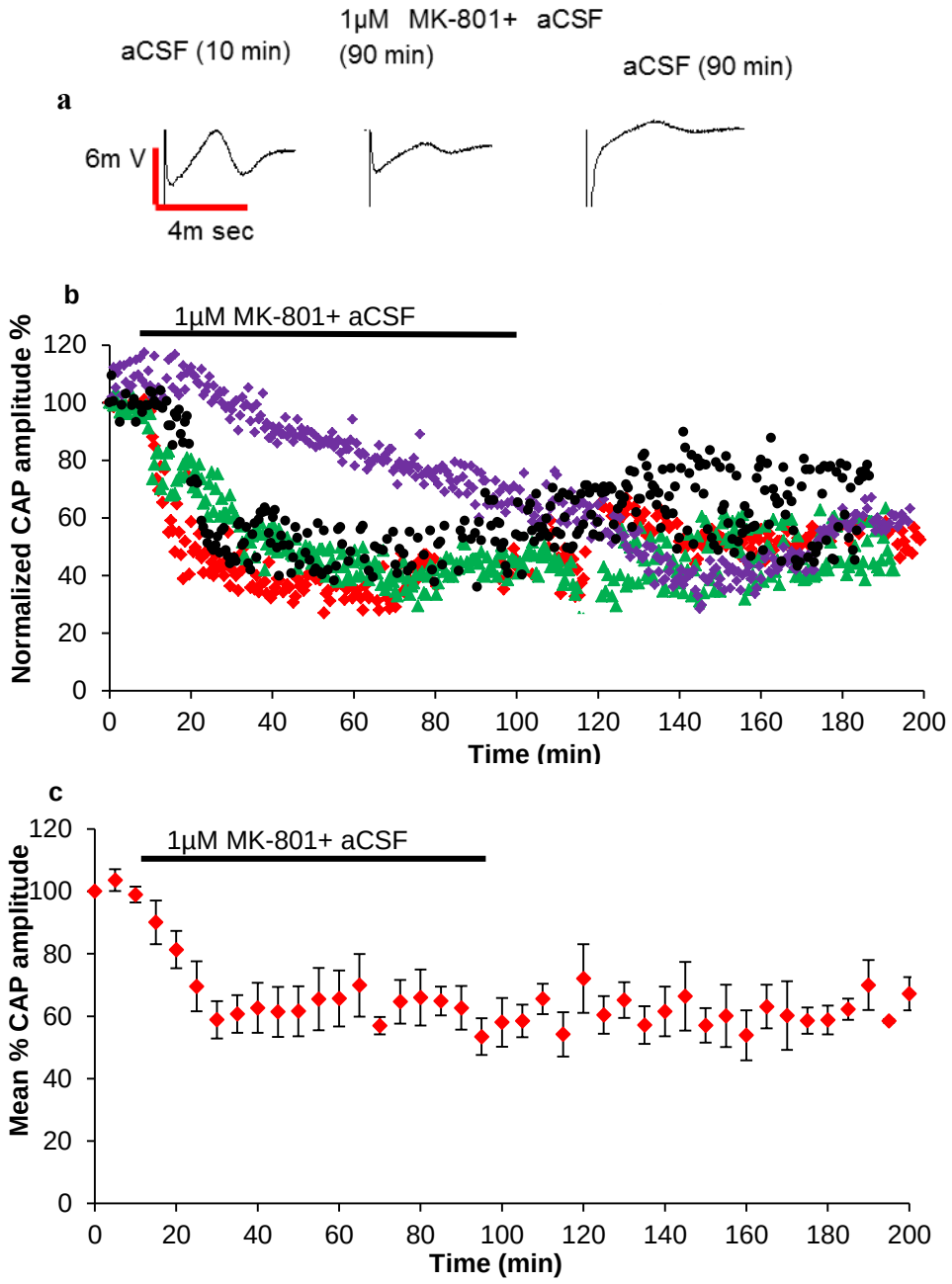


Figure 27- Application of NMDA-R blocker MK-801 (1µM) during control conditions evoked a gradual and irreversible decline in CAP amplitude in P0-P4 RONs.

a, A representative CAP recorded from a single experiment during normal conditions for 10 min, at the end of exposure to 90 min aCSF + 1µM MK-801 and following restoration of normal conditions for 90 min, respectively. **b**, plots of four individual experiments before, during and after the exposure to aCSF + MK-801. It shows variable responses but all experience a decrease in the CAP without complete recovery after restoration of normal conditions. **c**, Mean data showing a decline in the CAP amplitude during exposure to 90 min aCSF +1µM MK-801 without recovery to normal level following restoration of normal conditions. CAP is normalized to 100% at zero time, CAP at end of 90 min aCSF +1µM MK-801 is (58.0 ± 7.8) and at the end of the experiment is (67.1 ± 5.3), n=8. Error bars are SEM.

NMDA (1mM) + glycine (10µM) + aCSF

The mean CAP of P0-P4 RONs reached 51.4 ± 6.0% of the initial value at the end of 90 min of aCSF co-perfused with 1mM NMDA + 10µM glycine to selectively activate NMDA-Rs, and recovered to 67.3 ± 9.5% of the initial value, (n=8) following re-institution of normal conditions; ($P<0.0001$ vs control); *(see Fig 28& 29). The results of this experiment show that, NMDA-Rs contribute to OGD-induced injury in pre-myelinated central axons and that NMDA-R activation can be toxic under normoxic conditions.*

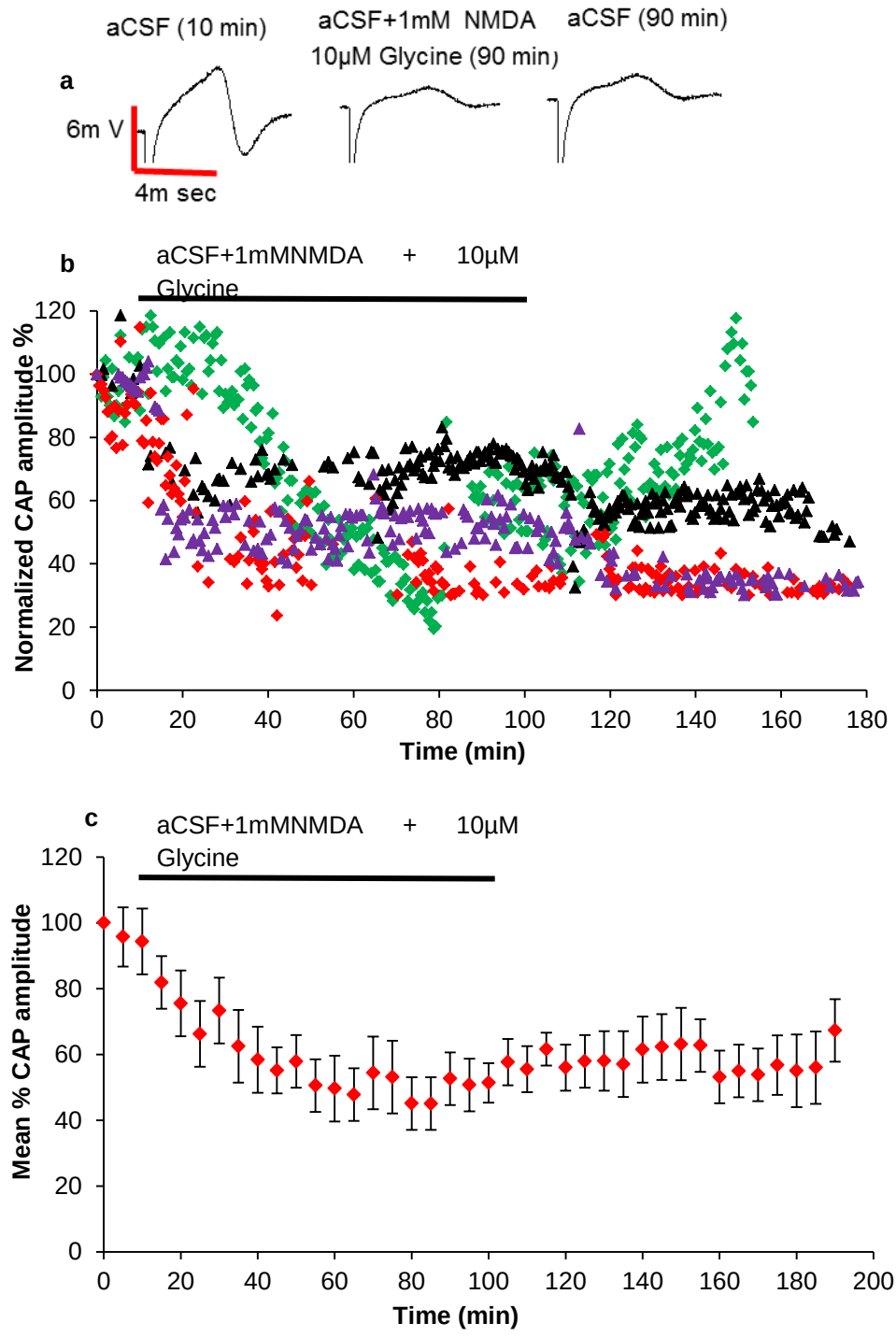


Figure 28- NMDA-R activation during control conditions has a significant effect upon CAPs in P0-P4 RONs.

a, A representative CAP recorded from a single experiment during normal conditions for 10 min, at the end of exposure to 90 min aCSF+1mM NMDA + 10µM Glycine and following restoration with normal conditions for 90 min. **b**, Plots of four individual experiments showed the changes in the CAP before, during and after NMDA-R activation during normal conditions. **c**, Mean data showed that activation of NMDA receptors during normal conditions had a significant effect upon the CAP. CAP is normalized to 100 % at zero time, CAP at the end of aCSF+1mM NMDA + 10µM Glycine is (51.4 ± 6.0), and at the end of the experiment is (67.3 ± 9.5), n=8. Error bars are SEM.

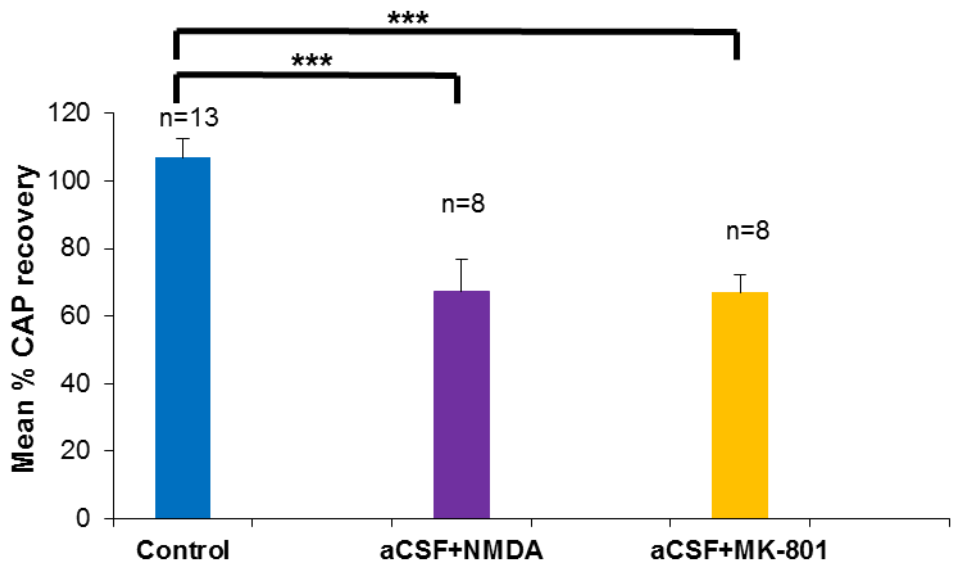


Figure 29- Histogram summarizing the effect of NMDA-R agonist and antagonist on the CAP amplitude during control conditions in P0-P4 RONs.

Histogram compares the effects of aCSF co-perfused with NMDA-R agonist or antagonist on the CAP amplitude recovery with control conditions. ***=p<0.0001. Error bars are SEM.

Application of memantine during OGD and control

The CAP amplitude in the case of OGD co-perfused with memantine fell to 18.2 ± 6.2% of the initial value, (n=6) at the end of OGD, and recovered to 19.9 ± 6.6% of the initial value following re-perfusion with normal conditions; *(see Fig 32 & 33).* MK801 is therefore protective against OGD despite its toxicity, while memantine is too toxic to provide any protection. When P0-P4 RONs were exposed to memantine (1µM) in aCSF; the CAP amplitude fell gradually and reached 62.4 ± 9.0% of the initial value, (n=6) and recovered to 70.8 ± 2.1% of the initial value; (P<0.0001 vs control); *(see Fig 30& 31). As a result, both NMDA-R over-activation and NMDA-R block is acutely toxic at this point in white matter development. It is therefore clear that an NMDA GluR block is toxic to P0-P4 RONs under normoxic conditions. MK-801 is protective against OGD-induced injury in pre-myelinated white matter, while memantine is not protective against OGD-induced injury in pre-myelinated white matter.*

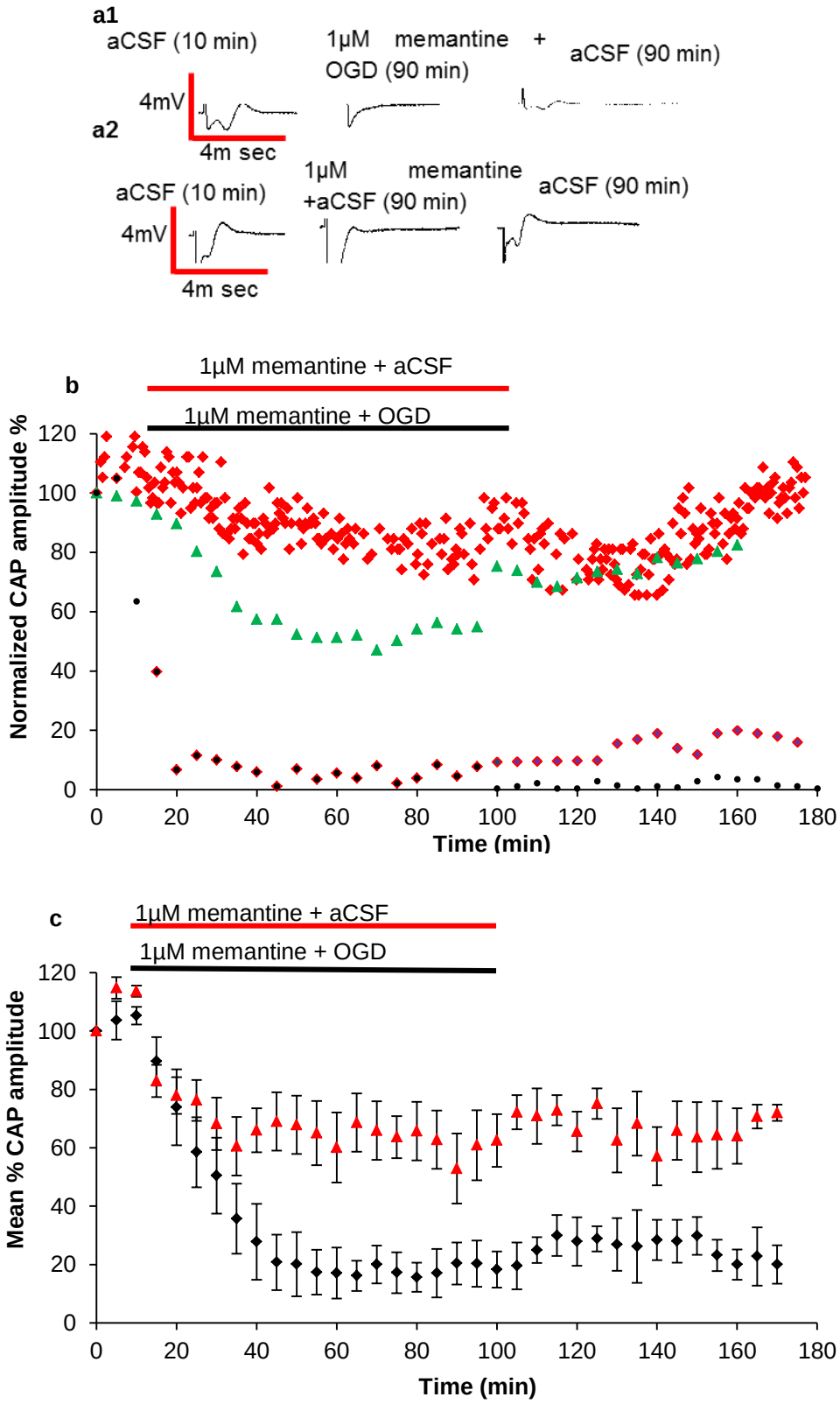


Figure 30- NMDA-R blocker memantine (1μM) evoked changes in the CAP amplitude during control conditions and OGD in P0P4 RONs.

a1 & a2, CAPs during normal conditions for 10 min, during exposure of P0 RON to OGD +1μM memantine and during perfusion with aCSF +1μM memantine for 90 min respectively, and after restoring the normal conditions for 90 min in both conditions. **b**, Plots data of individual experiments showing the effect of 1μM memantine during

normal aCSF (upper two plots), and during OGD (lower two plots) for 90 min period. **e,** Mean data showing two representative plots of CAPs amplitude in P0 RONs exposed to 90 min of aCSF + 1µM memantine (red), and 90 min of OGD + 1µM memantine (black). There was a gradual fall in the CAP during control conditions which frequently did not reach to zero, followed by no significant recovery after restoring the normal conditions. CAP is normalized to 100 % at zero time in both conditions, CAP at the end of 90 min period is (62.4 ± 9.0) during aCSF + 1µM memantine & (18.2 ± 6.2) during OGD + 1µM memantine, and at the end of the experiment is (70.8 ± 2.1) during aCSF + 1µM memantine & (19.9 ± 6.6) during OGD + 1µM memantine, n=6. Error bars are SEM.

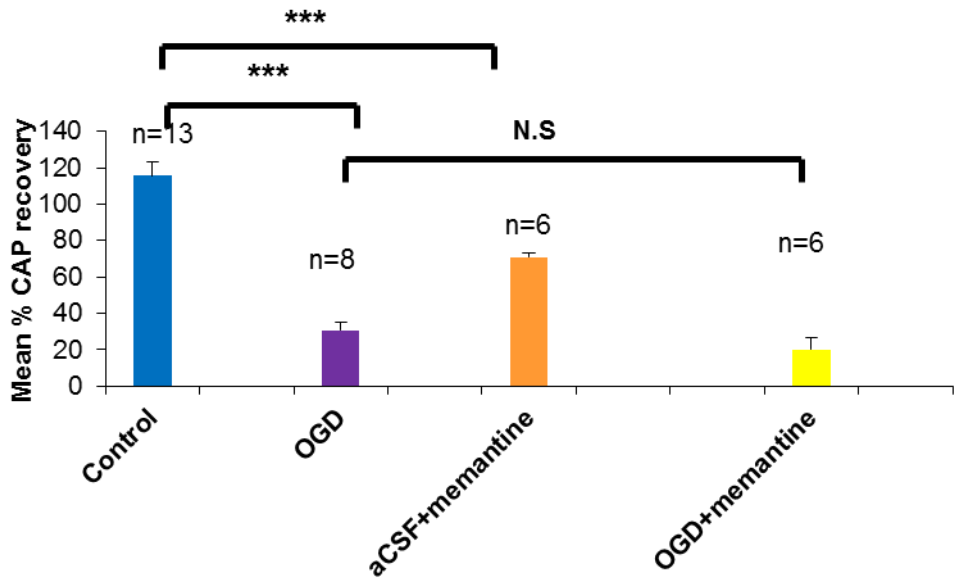


Figure 31- Histogram summarizing the recovery in the CAP amplitude in the presence of memantine in P0-P4 RONs.

The recovery in the CAP in case of control conditions co-perfused with 1µM memantine was significant in compare to the recovery in the CAP under control conditions alone. ***=p<0.0001. There is no significant comparison in the CAP amplitude recovery between OGD alone and OGD + 1µM memantine. Error bars are SEM.

Application of kynurenic acid during OGD and control

The blocking of ionotropic GluRs with the non-selective antagonist kynurenic acid (100µM) evoked a rise in the CAP amplitude in P0 RONs during aCSF. The effect is variable: kynurenic acid (100µM) during 90 min OGD produced a decline in the CAP amplitude to 28.8 ± 4.4% of the initial value at the end of OGD, (n=7), and to 32.7 ± 4.1% of the initial value, after re-perfusion with normal conditions. *As a result, Kynurenic acid did not protect P0 nerves from OGD-induced injury.* When kynurenic acid was co-applied with a 90 min period of aCSF, the CAP amplitude was 105.6 ± 3.5% of the initial value, (n=7) after 30 min exposure, and reversed after re-perfusion with normal conditions to 89.3 ± 9.9% of the initial value; ($P<0.0001$ vs the initial value); (*see Fig 32 & 33). Kynurenic acid is not toxic to the pre-myelinated optic nerves.*

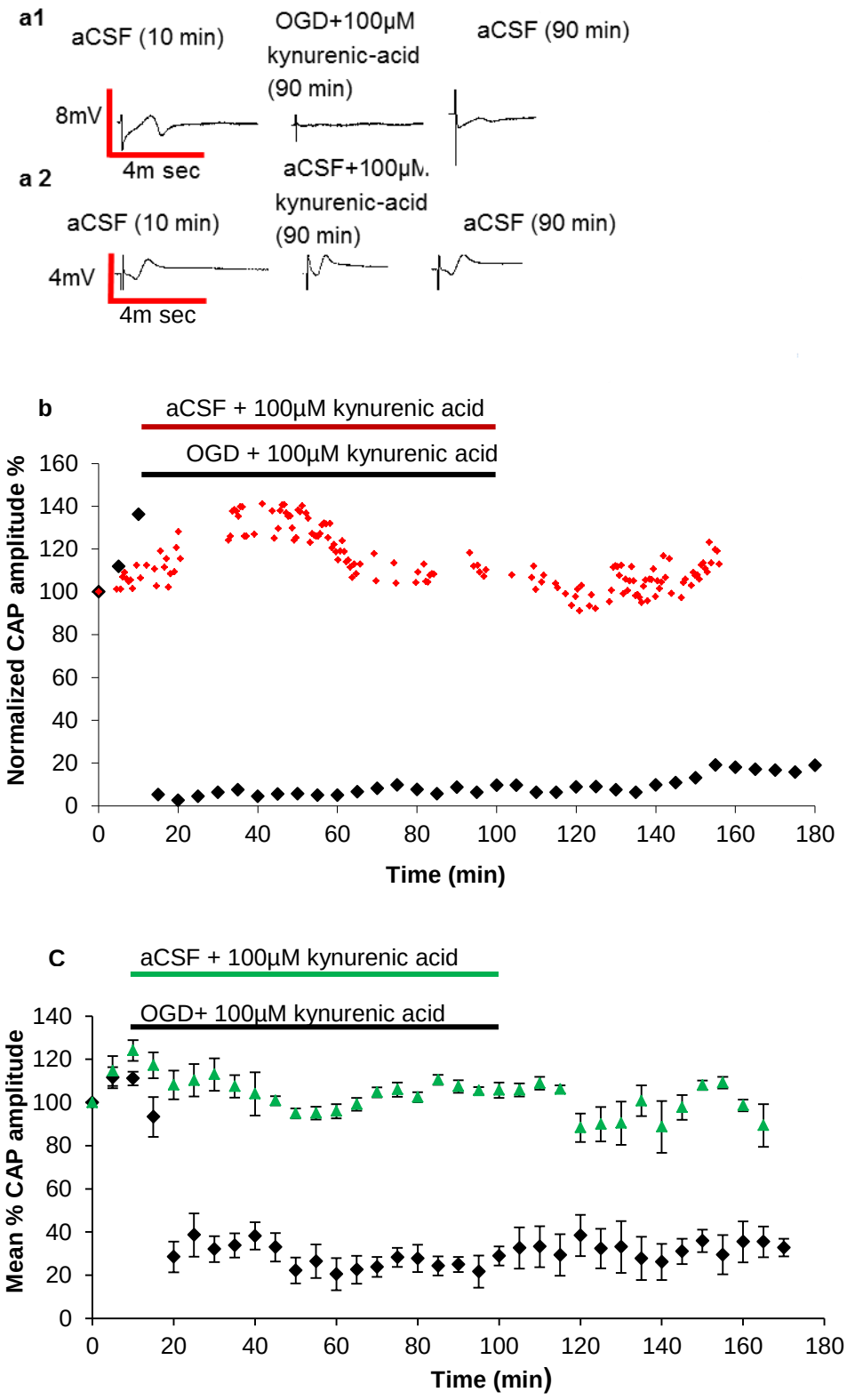


Figure 32- The iGluR blocker kynurenic acid (100µM) evoked very transient changes in the CAP amplitude during control conditions and gradual decline during 90 min OGD in P0-P4 RONs.

a1& **a 2,** representative CAPs recorded during normal conditions for 10 min, during OGD co-perfused with 100µM kynurenic acid for 90 min and during aCSF +100µM kynurenic acid for 90 min, and following restoration of normal conditions for 90 min in both conditions, respectively. **b**, Data of single experiment plotted against time, showing the

changes in the CAP amplitude during 90 min aCSF+100µM Kynurenic acid (red), and during 90 min OGD+ 100 µM Kynurenic acid (black). **c**, Mean data showing the mild changes in the CAPs amplitude during aCSF+100 µM Kynurenic acid (green), n=7, and the large decline in the CAPs amplitude during 90 min OGD+ 100µM Kynurenic acid (black), n=7. CAP is normalized to 100 % at zero time in both conditions, CAP at the end of 90 min period is (105.6 ± 3.5) during aCSF + 100µM Kynurenic acid & (28.8± 4.4) during OGD + 100µM Kynurenic acid, and at the end of the experiment is (89.3 ± 9.9) during aCSF + 100µM Kynurenic acid & (32.7± 4.1) during OGD + 100µM Kynurenic acid. Error bars are SEM.

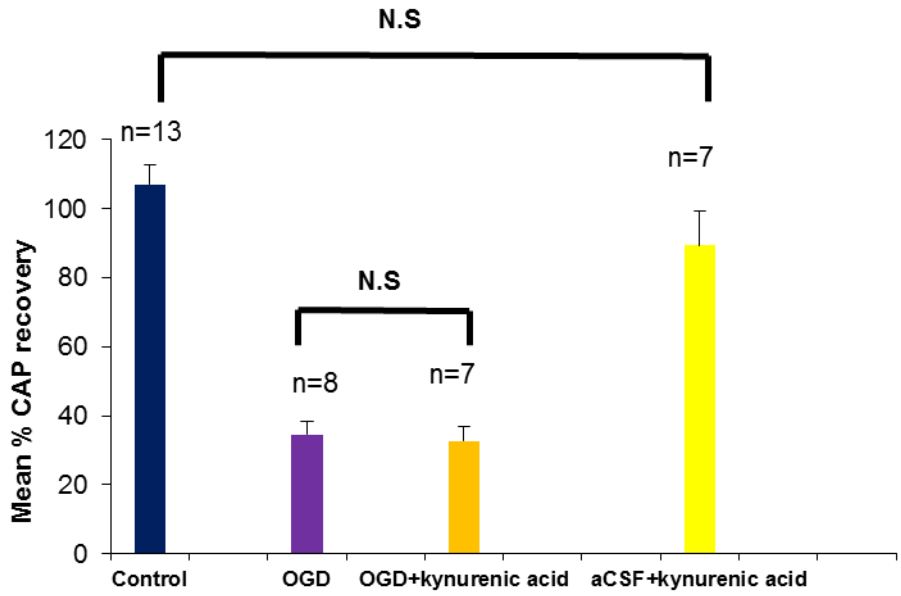


Figure 33- Histogram illustrating the comparison between control conditions and OGD with and without application of 100µM Kynurenic acid in P0-P4 RONs.

CAPs recovery in case of aCSF + Kynurenic acid (100µM) had no significant comparison to control conditions. There was no significant comparison in the CAP amplitude recovery between OGD alone and OGD+100µM Kynurenic acid. Error bars are SEM.

Kainic acid (30μM) + aCSF

The application of the excitotoxin kainic acid (30μM) to P0-P4 RONs had no permanent significant effect upon the CAP, which was also true for P10 RONs (Alix and Fern 2009). Kainic acid is a specific agonist for the kainate receptors which mimic the effect of glutamate. Kainate was added to aCSF for 90 min, and the CAP recovery following restoration of normal conditions was 111.6 ± 0.05% of the initial value, (n=5); *(see Fig 34 & 35). Kainate receptors possibly do not play an important role during OGD-induced injury in pre-myelinated optic nerve axons.*

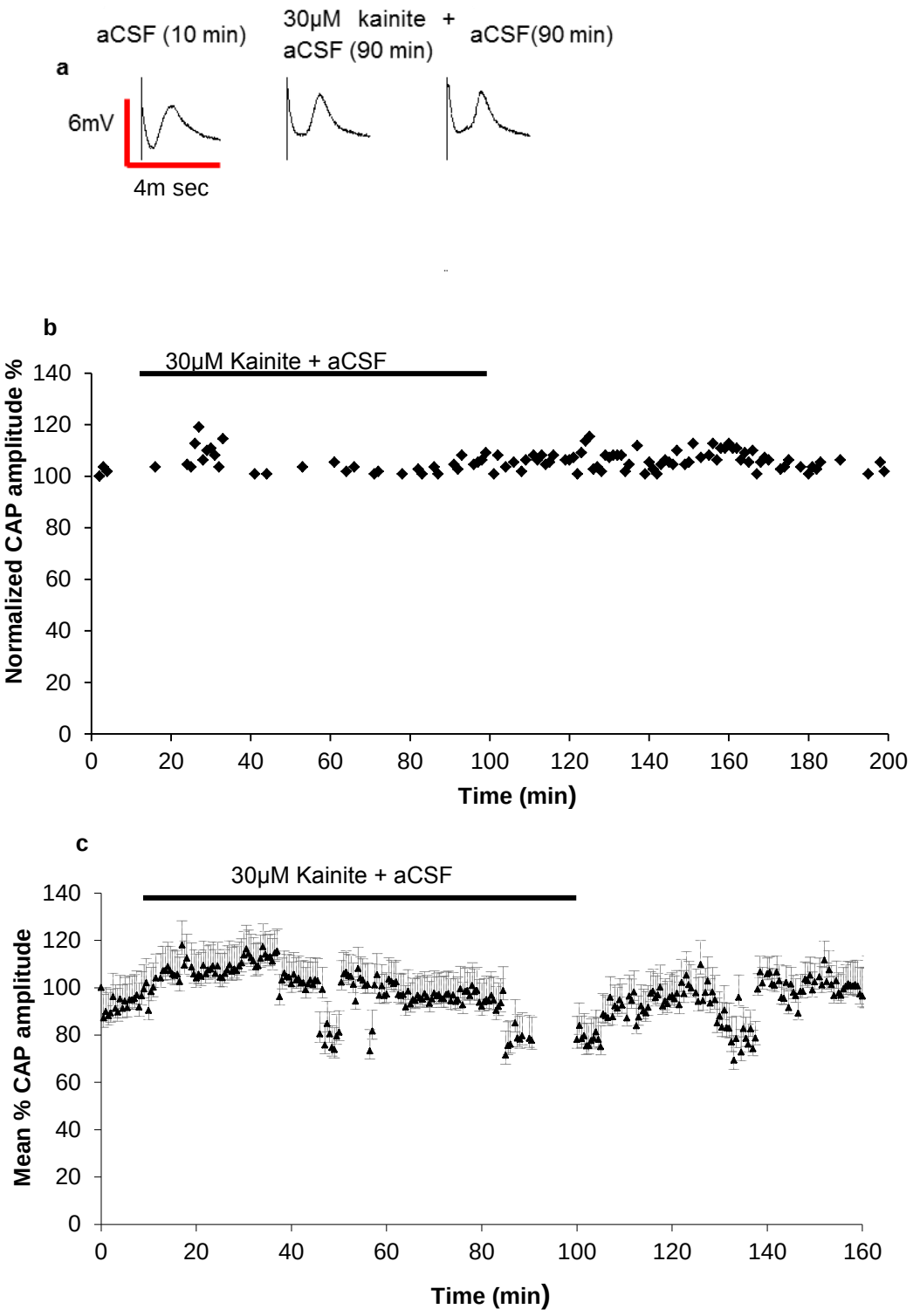


Figure 34- The excitotoxin Kainate (30μM) produced only transient changes in excitability of P0-P4 RONs.

a, A representative CAP recorded from a single experiment during normal conditions for 10 min, during acute exposure to 90 min (aCSF + 30μM Kainate), and after restoring the normal conditions. **b**, Data plot showing that while 90 min perfusion with 30μM Kainate during normal condition had very transient effect on the CAP amplitude. **c**, CAP normalized to 100 % at zero time, CAP at 100 min is (78.08 ± 3.6), and at 165 min is (111.6 ± 0.05), n=5. Error bars are SEM.

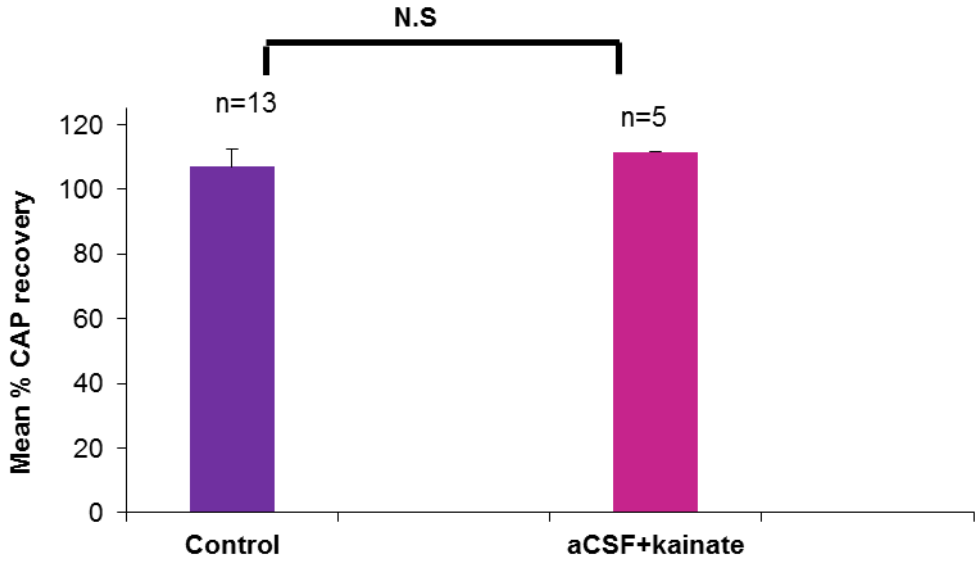


Figure 35- Histogram summary of Kainate data in P0-P4 RONs.

The CAP recovery in case of Kainate (30μM) during 90 min aCSF had non- significant comparison to the CAP recovery during control conditions. Error bars are SEM.

Effects of neurotransmitter blockers other than glutamate during OGD in P0-P4 RONs

A number of neurotransmitter blockers other than GluR blockers have been shown to have actions upon central white matter injury during neonatal development. The significance of the expression of neurotransmitter receptors other than glutamate in developing white matter during injury has not been completely examined, and previous studies have concentrated upon brief periods of receptor activation and later points in development. For this work, the injury capacity of a standard 90-min period of both OGD and aCSF co-perfused with a $GABA_A$-R antagonist, a GlyR antagonist, AD-R antagonist and a nACh-R antagonist were examined. The results reveal the potential role of neurotransmitters other than glutamate-mediated injury in developing white matter in early points of development.

A- $GABA_A$-R antagonist (100µM picrotoxin) during OGD and control

When OGD was performed in the presence of 100µM picrotoxin, the CAP fell to 39.3 ± 5.7% of the initial value at the end of OGD and recovered significantly following re-perfusion with normal conditions to 72.1 ± 3.6% of the initial value, (n=7; P<0.0001 vs OGD); *(see Fig 36 & 38).* Application of OGD was preceded by 10 min aCSF co-perfused with 100µM picrotoxin, and the CAP increased up to 117 ± 0.04% of the initial value. Optic nerves were exposed to aCSF co-applied with 100µM picrotoxin for 30 min period after

OGD and before re-introduction of normal conditions, during this time, the CAP increased to 80.01 ± 3.1% of the initial value; *(see Fig 36 c, d, e& g).*

The CAP gradually increased within the first 20-30 min during aCSF co-perfused with 100µM picrotoxin and reached 148.8 ± 14.2% of the initial value, then declined to 75.7 ± 3.7% of the initial value at the end of 90 min of perfusate. After this, the CAP dropped gradually following restoration of normal conditions to 46.9 ± 12.5% of the initial value, (n=6; P<0.0001 vs control). Another regime was performed during aCSF co-applied with 100µM picrotoxin; P0-P4 RONs were exposed to the perfusate for 180 min without re-instruction of normal conditions. A continuous perfusion with 100µM picrotoxin produced a gradual increase in the CAP amplitude to 195.7 ± 5.5% of the initial value at the end of the experiment, (n=5; P<0.0001 vs control); *(see Fig 37& 38).* The result shows that $GABA_A$-R block both increased CAP area under control conditions, and protected the RON from OGD-induced injury. *This indicates the protective role of the $GABA_A$-R antagonist against ischaemic injury in pre-myelinated axons.*

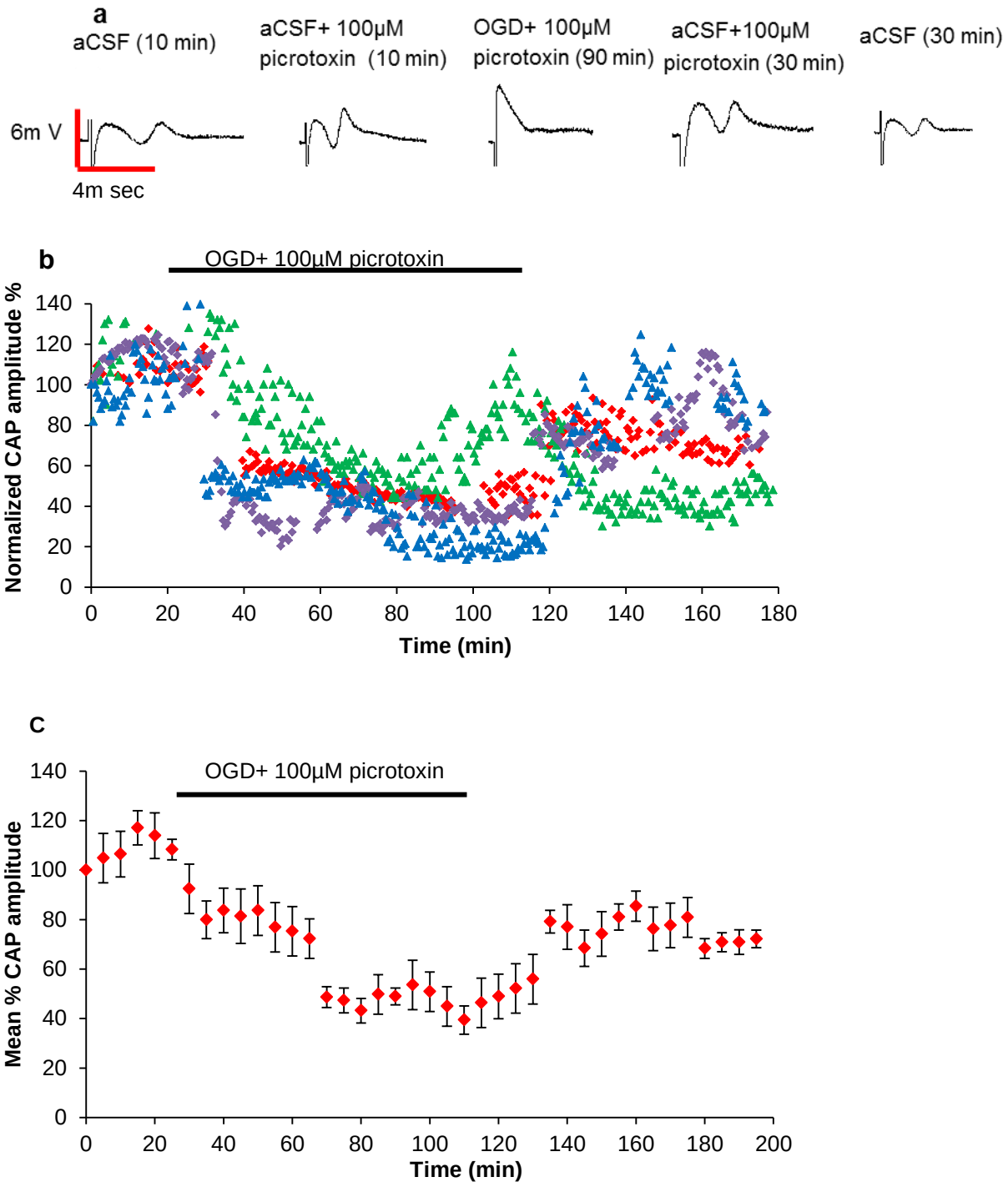
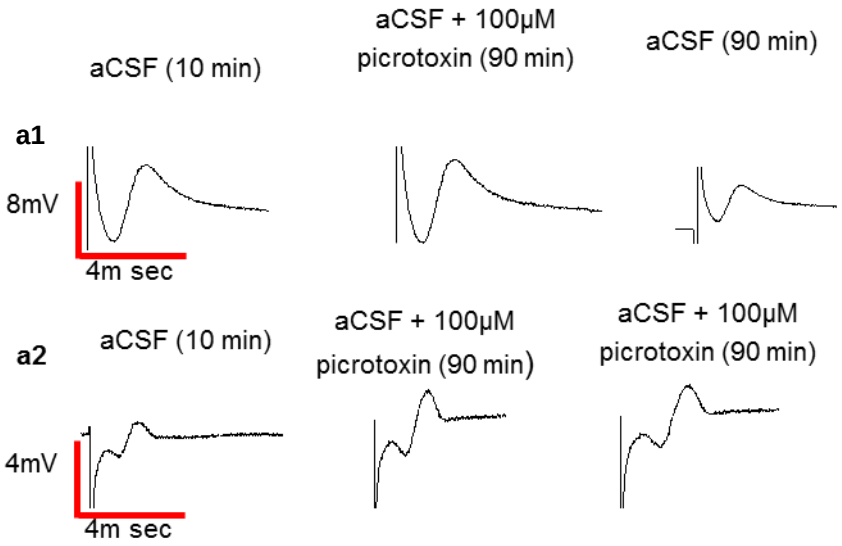
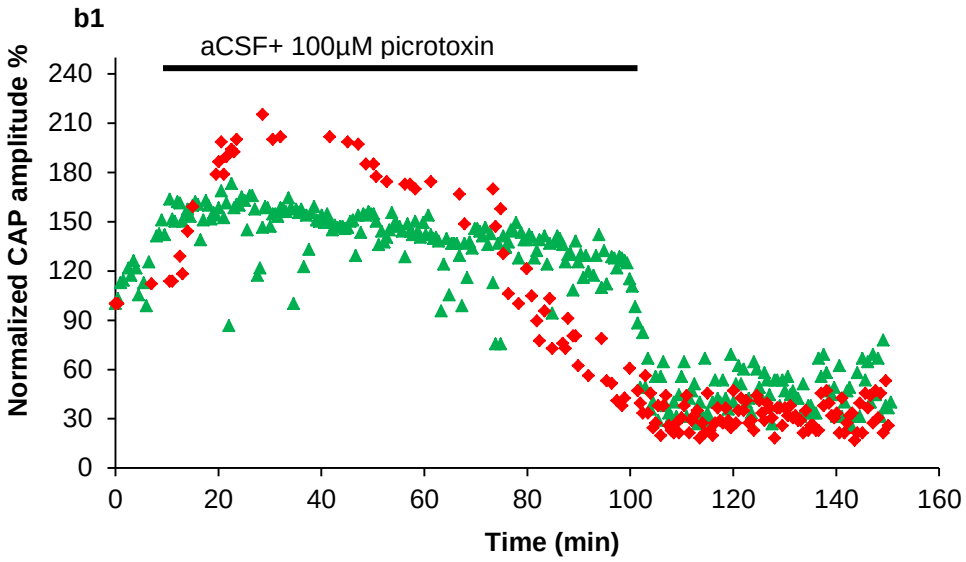
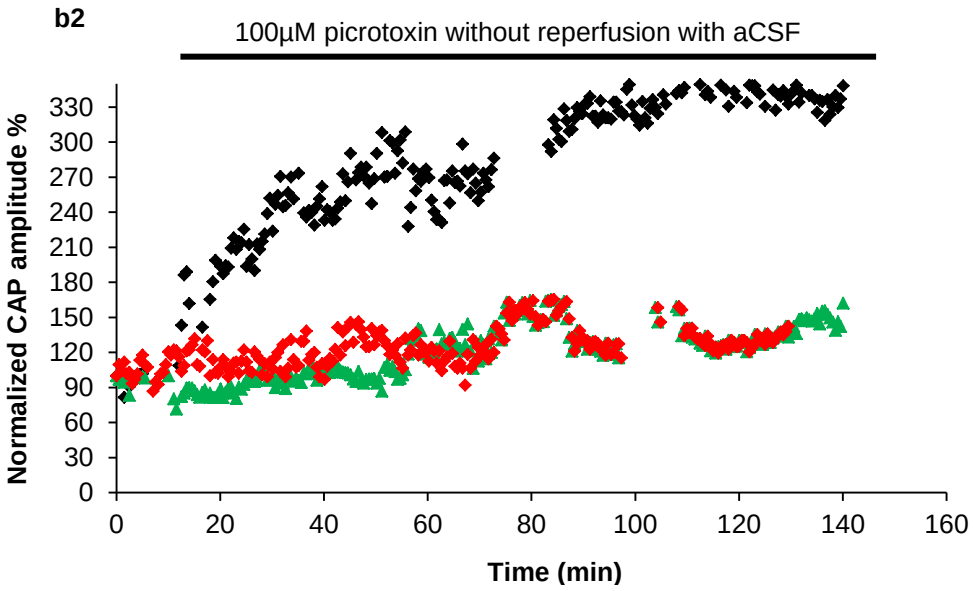


Figure 36- Application of $GABA_A$-R antagonist picrotoxin (100µM) during 90 min OGD evoked a partial decline in the CAP in a reversible fashion in P0-P4 RONs.

a, A representative CAP during normal conditions for 10 min, during the first 10 min exposure to aCSF+100µM picrotoxin, during exposure to OGD+ 100µM picrotoxin for 90 min, during re-perfusion with aCSF+100µM picrotoxin for 30 min and during re-instruction of normal conditions for 30 min. **b**, Plots of four individual experiments against time. **c,** Mean data showing the changes in the CAPs before, during OGD-co-applied with 100µM picrotoxin, and after restoring the normal conditions, n=7. CAP is normalized to 100% at zero time. CAP at the end of OGD is (39.3± 5.7); CAP after restoring the normal conditions is (72.1± 3.6). Error bars are SEM.


aCSF (10 min)
aCSF + 100μM picrotoxin (90 min)
aCSF (90 min)
a1
8mV
4m sec
a2
aCSF (10 min)
aCSF + 100μM picrotoxin (90 min)
aCSF + 100μM picrotoxin (90 min)
4mV
4m sec


b1
aCSF+ 100μM picrotoxin
Normalized CAP amplitude %
240
210
180
150
120
90
60
30
0
0
20
40
60
80
100
120
140
160
Time (min)


b2
100μM picrotoxin without reperfusion with aCSF
Normalized CAP amplitude %
330
300
270
240
210
180
150
120
90
60
30
0
0
20
40
60
80
100
120
140
160
Time (min)

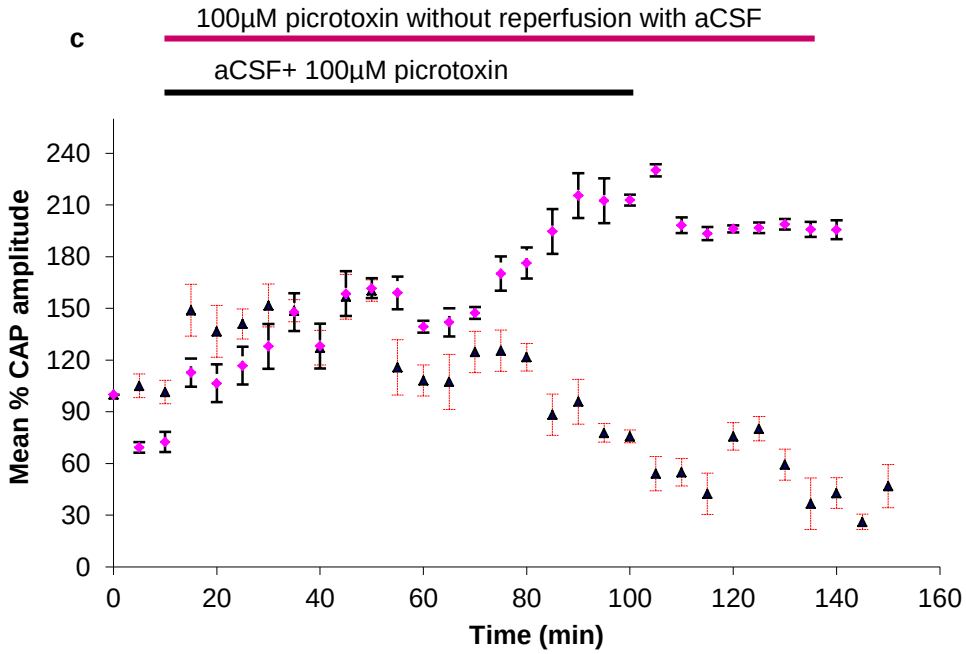


Figure 37- Persistent exposure to $GABA_A$-R antagonist picrotoxin (100µM) block the irreversible decline in CAP in P0-P4 RONs.

a1, CAPs recorded before, during, and after 90 min of perfusion with aCSF+100µM picrotoxin. **a2**, representative CAPs during normal conditions and 180 min of continuous perfusion with 100µM picrotoxin. **b1**, plots of three experiments showed the changes in the CAP during exposure to 90 min of aCSF+100µM picrotoxin and after reperfusion with normal conditions. **b2**, Plots of three experiments showed the changes in the CAP during continuous exposure to aCSF+100µM picrotoxin. **c**, Mean plot showing the irreversible decline in the CAPs after restoring the normal conditions (black plots, n=6), an effect that is blocked by continuous exposure to aCSF+100µM picrotoxin (pink plots, n=5). CAP is normalized to 100% at zero time, CAP at the end of 90 min exposure to aCSF+100µM picrotoxin is (75.7± 3.7), and after reperfusion with normal conditions is (46.9± 12.5), CAP during continuous perfusion with aCSF+100µM picrotoxin is (195.7± 5.5). Error bars are SEM.

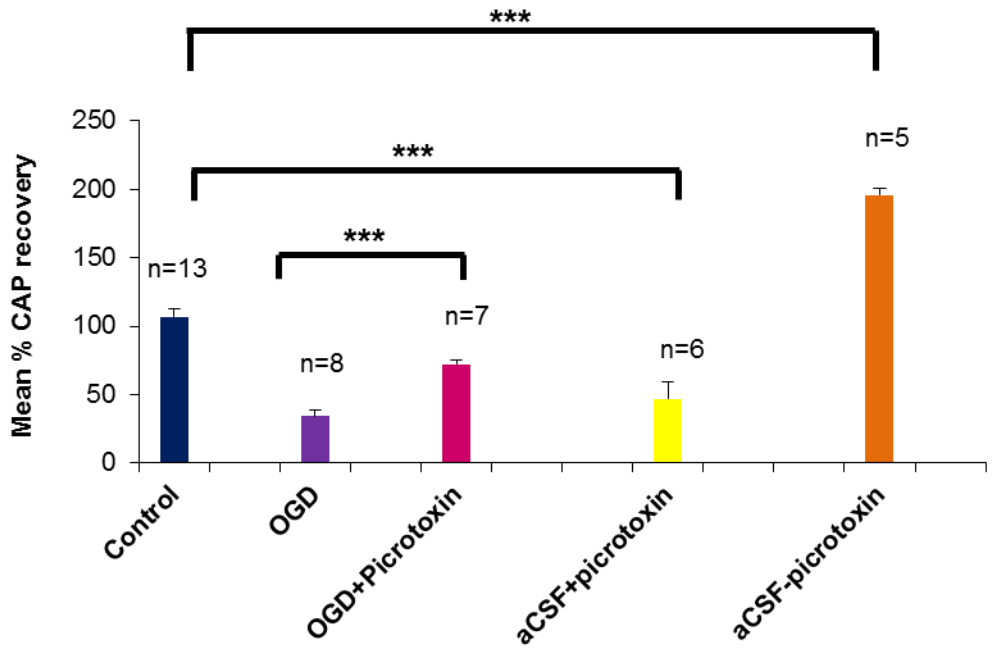
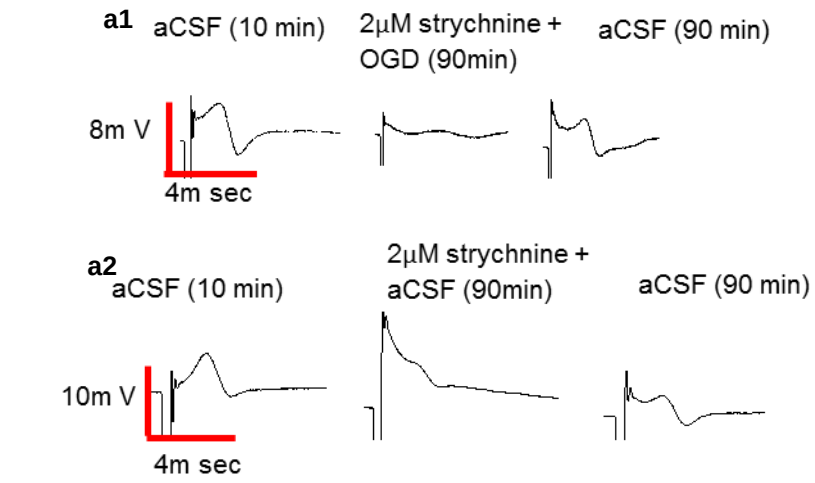


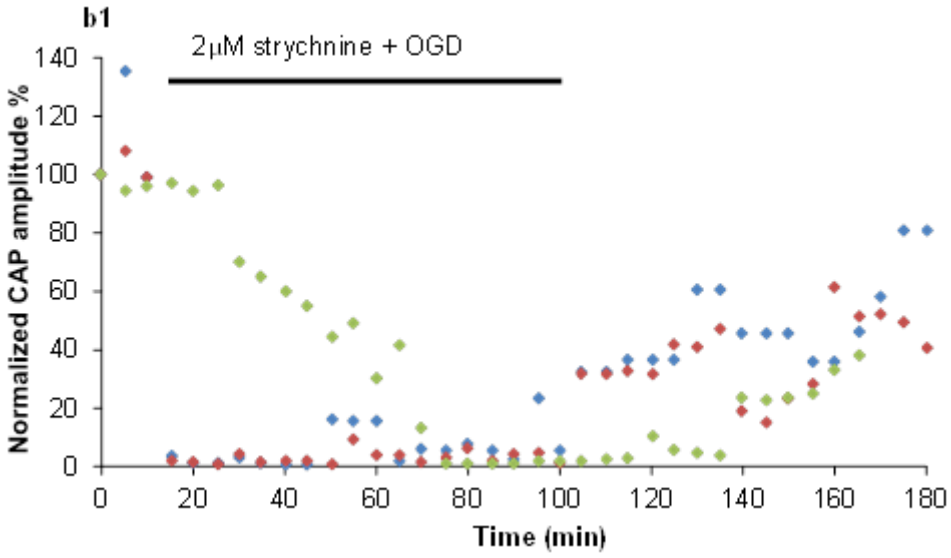
Figure 38- Summary of changes in the CAP amplitude of P0-P4 RONs during exposure to $GABA_A$-R antagonist in P0-P4 RONs.

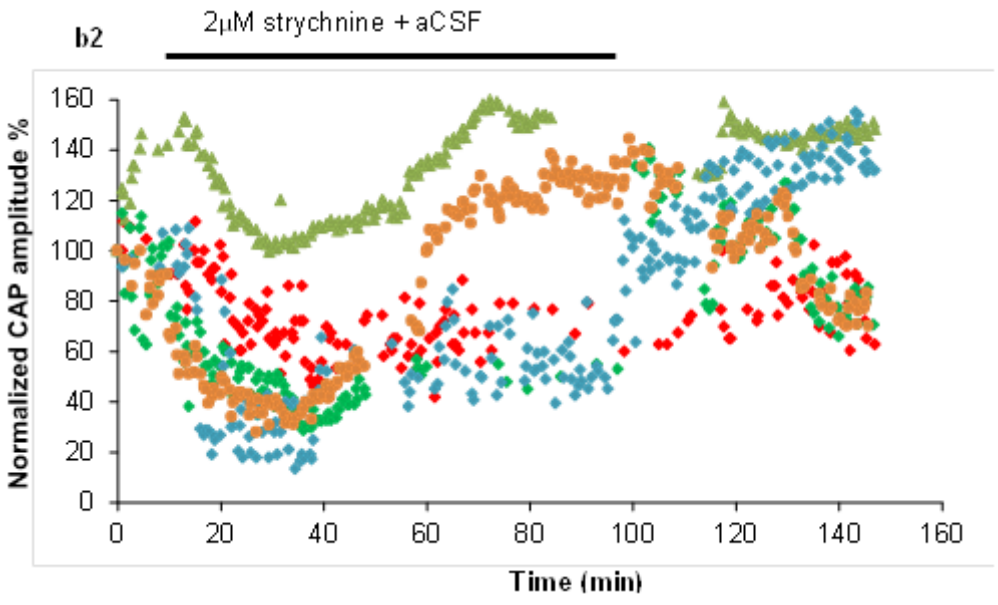
Histogram summarized the protective effect of $GABA_A$-R antagonist in control conditions and OGD. It showed a large recovery in the CAPs in both situations (OGD- & aCSF- picrotoxin (100µM) without re-perfusion with normal conditions). ***=p<0.0001

β- GlyR antagonist (2μM strychnine) during OGD and control

Co-application of strychnine during the 90-min period of OGD caused the CAP amplitude to decrease to 61.6 ± 13.0% of the initial value at the end of OGD, and it significantly recovered to 70.7 ± 6.6% of the initial value following restoration of normal conditions (n=7; P<0.0001 vs OGD); *(see Figs 39& 40).* During the application of strychnine to normal conditions, the CAP amplitude fell to 90.7 ± 7.3% of the initial value by the end of 90 min, and reversed after washout of strychnine to 98.7 ± 4.1% of the initial value, (n=7); *(see Figs 39 & 40). This result showed that, GlyR antagonist was protective against OGD-induced injury in pre-myelinated optic nerves and was not toxic under normoxic conditions.*


a1
aCSF (10 min)
2μM strychnine + OGD (90min)
aCSF (90 min)
8m V
4m sec
a2
aCSF (10 min)
2μM strychnine + aCSF (90min)
aCSF (90 min)
10m V
4m sec


b1
2μM strychnine + OGD
Normalized CAP amplitude %
Time (min)


b2
2μM strychnine + aCSF
Normalized CAP amplitude %
Time (min)

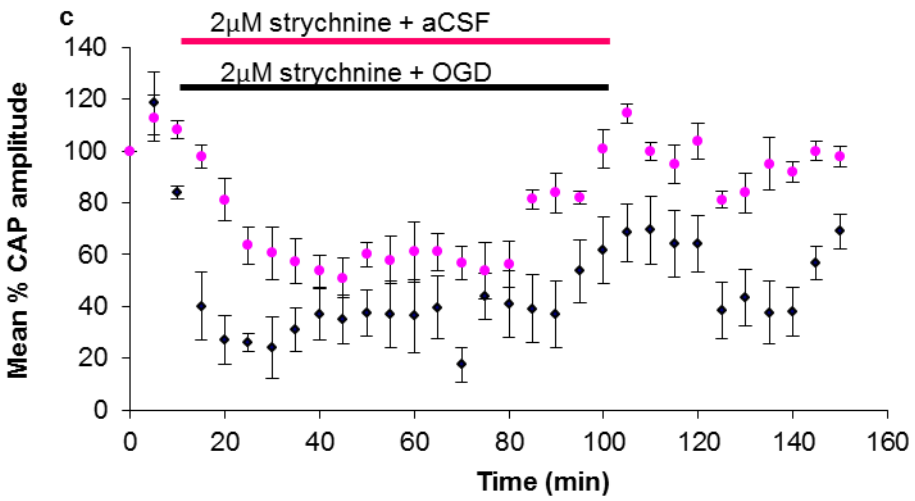


Figure 39- GlyR antagonist strychnine (2μM) has a protective effect during OGD in P0-P4 RONs.

a1, CAPs recorded from a single experiment during normal conditions, during acute exposure of RONs to 2μM strychnine for 90 min during OGD and following restoration of normal conditions for 90 min. **a2,** CAP recorded from a single experiment during normal conditions for 10 min, aCSF+ 2μM strychnine, and following restoration of normal conditions. **b1**, plots of three experiments during exposure to 2μM strychnine+OGD. **b2**, Plots of fife experiments during exposure to 2μM strychnine+ aCSF. Mean data showing the protective effect of strychnine during OGD and aCSF. CAPs during exposure to 2μM strychnine did not reached zero in aCSF (pink) or OGD (black), with large recovery after restoration of normal conditions. CAP is normalized to 100 % at zero time in both conditions. CAP at the end of OGD IS (61.6±13.0), and at the end of 90 min 2μM strychnine+ aCSF is (90.7± 7.3), CAP after restoration of normal conditions following 2μM strychnine+ OGD is (70.7± 6.6) and following 2μM strychnine+ aCSF is (98.7± 4.1), n=7. Error bars are SEM.

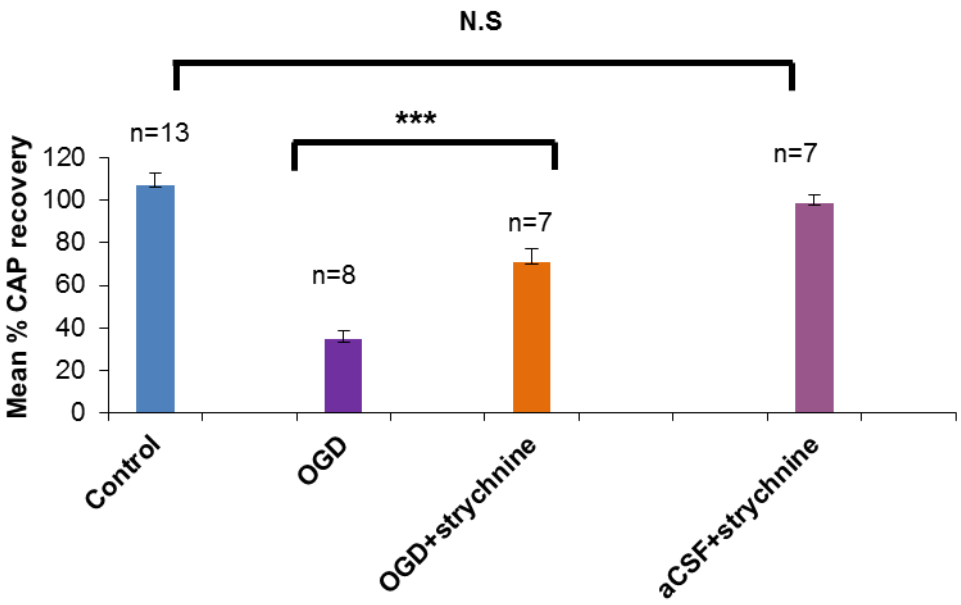


Figure 40- Histogram summarizing the reversible effects of GlyR antagonist upon the CAP recording during control conditions and OGD in P0-P4 RONs.

There was no significant comparison in the CAP recovery between aCSF + 2μM strychnine, and the control.

CAP recovery was highly significant in case of OGD + 2μM strychnine in compare to OGD alone. ***=p<0.0001. Error bars are SEM.

C- Combined AR antagonists (10µM propranolol hydrochloride [non-selective β-blocker]) and (10µM phentolamine hydrochloride [reversible and non-selective α-blocker])

The co-application of these combined antagonists during a 90-min period of OGD resulted in a profound fall in the CAP amplitude to 15.0 ± 4.5% of the initial value at the end of OGD and recovered insignificantly following restoration of normal conditions to 26.9 ± 8.8% of the initial value, (n=7; P<0.0001 vs the initial value); *(see Figs 41 & 42).* The recovery in the CAP during OGD alone was higher than the recovery during OGD co-perfused with both blockers, (P<0.01); (*see Figs 42).*

Perfusion of the nerve with aCSF containing 10µM propranolol + 10µM phentolamine produced a transient changes in the CAP amplitude to 94.2 ± 10.0% of the initial value at the end of 90 min, and to 73.4 ± 10.9% of the initial value following restoration of normal conditions, (n=6; P<0.01 vs control); *(see Figs 41 & 42). Although the combined antagonists are not toxic to the nerves they do not protect against OGD-induced injury in developing optic nerve axons.*

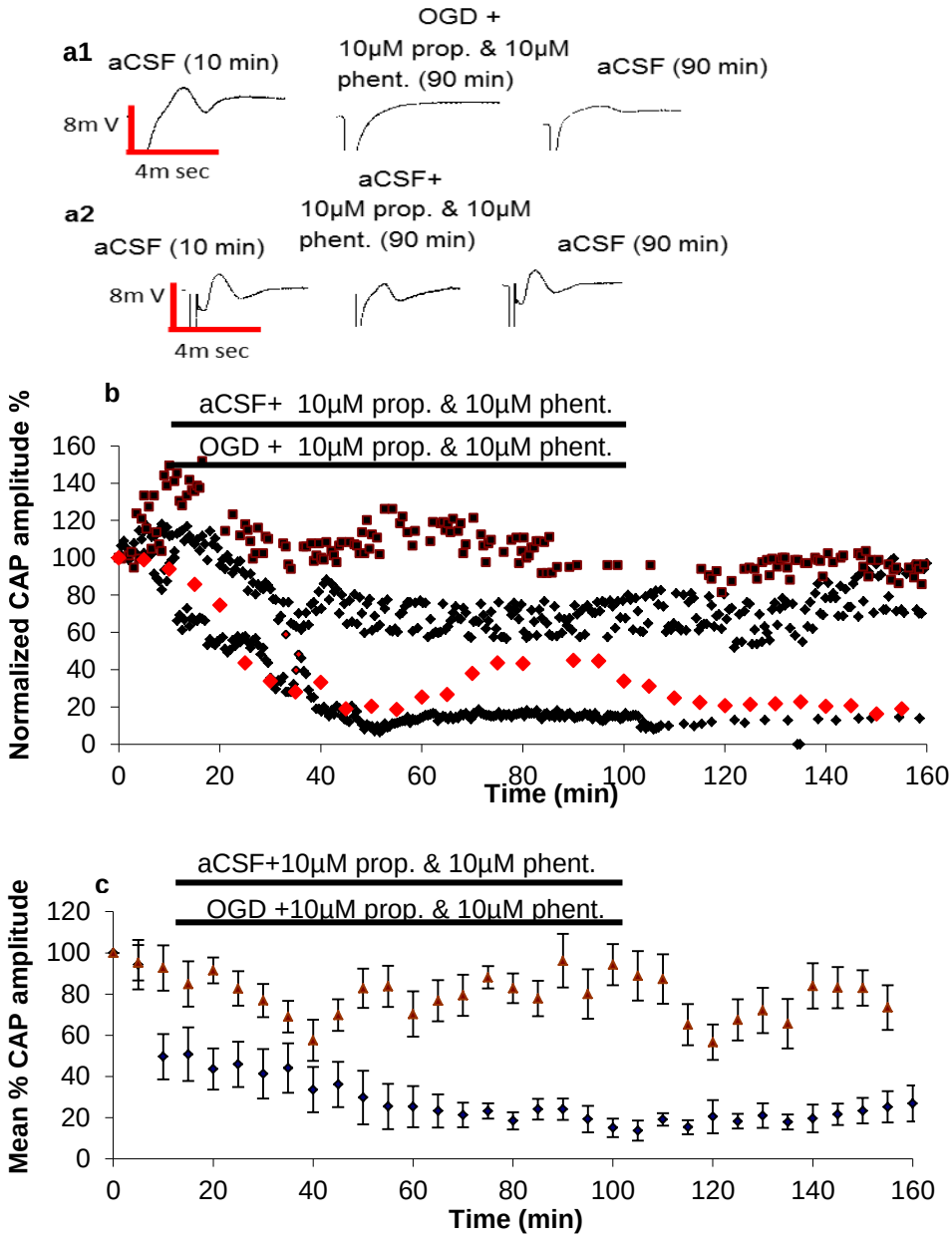


Figure 41- Combined AR blockers propranolol (10μM) and phentolamine (10μM) have non-reversible effect upon the CAP recovery during OGD in P0-P4 RONs.

a1, CAPs recorded before, during and after exposure to 90 min of OGD co-perfused with 10μM propranolol &10μM phentolamine, respectively. **a2**, CAP recorded before, during and after exposure to 90 min aCSF + 10μM propranolol &10μM phentolamine, respectively. **d**, plots against time during aCSF co-perfused with 10μM propranolol &10μM phentolamine (upper two plots) and OGD co-perfused with 10μM propranolol &10μM phentolamine (lower two plots). **e**, Mean data showing the changes in the CAPs during aCSF and OGD co-perfused with both blockers. There was a transient effect of aCSF co-perfused with both blockers upon the CAPs (red), n=6. Irreversible recovery in the CAPs during OGD co-perfused with combined blockers (black), n=7. CAP is normalized to 100 % at zero time, CAP is (15.0± 4.5) at the end of OGD co-perfused with combined blockers and (94.2± 10.0) at the end of aCSF co-perfused with combined blockers. CAP recovered to (26.9± 8.8) during reperfusion with normal conditions following OGD and (73.4± 10.9) following aCSF. Error bars are SEM.

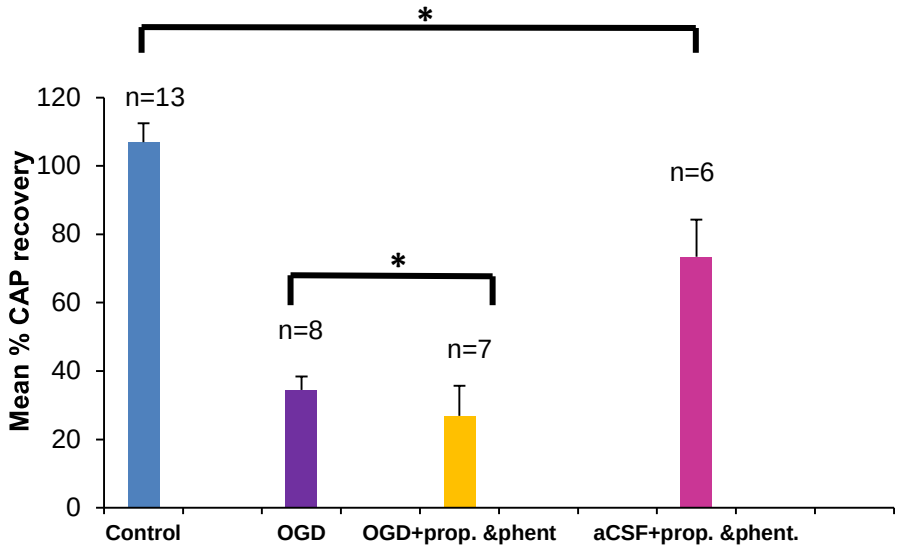


Figure 42- Histogram summarizing the mean recovery of the CAPs with and without co-application of combined AR blockers in P0P4 RONs.

The combined AR blockers did not protect the pre-myelinated optic nerve axons during OGD. The CAP recovery during aCSF co-perfused with both blockers was not highly significant in compare to the CAP recovery during normal conditions. The recovery in the CAP during OGD alone was higher than the recovery in the CAP during OGD+ both blockers. *=$p<0.01$. Error bars are SEM.

D- nACh-R antagonist (10µM mecamylamine) during control and OGD:

Acute exposure of RONs to 90 min OGD co-applied with 10µM mecamylamine evoked a decline in the CAP to 40.1 ± 10.0% of the initial value at the end of 90 min, which then recovered to 46.0 ± 10.8% of the initial value following restoration of normal conditions, (n=6; P<0.001 vs OGD), *(see Figs 43 & 44).* Perfusion of P0-P4 RONs with aCSF containing 10µM mecamylamine produced a decline in the CAP to 71.7 ± 13.25% of the initial value at the end of 90 min of perfusate, which then showed a significant recovery to 95.9 ± 7.12% of the initial value on restoration of normal conditions; (n=6), *(see Figs 43 & 44). nACh-R antagonist was not toxic to the developing optic nerves and produced a very mild but significant protection against OGD-induced injury.*

90 min OGD co-perfused with a GABA-R or GlyR blockade suppressed the pre-myelinated optic nerve CAP in a reversible manner. An AD-R blockade had no effect on the recovery following OGD. While a nACh-R blockade had a small but significant effect. These results show that over-stimulation of neurotransmitter receptors other than glutamate can create widespread damage to neonatal white matter; consequently, antagonising some of these receptors such as GABA and glycine is potentially protective, a phenomenon that may be clinically significant.

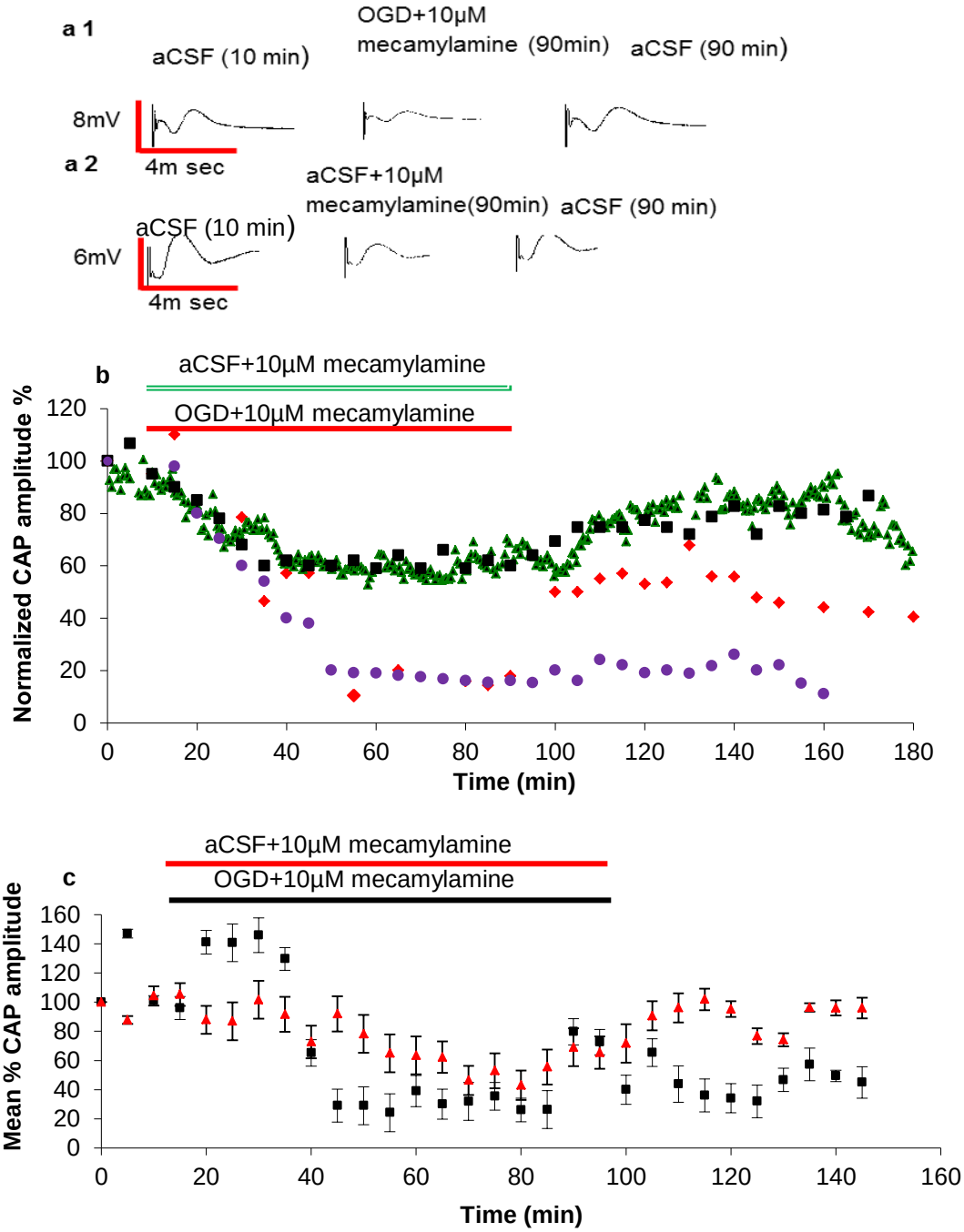


Figure 43- nACh-R blocker mecamylamine (10µM) has a reversible effect upon CAP in P0P4 RONs.

a1, A representative CAP recorded during normal conditions for 10 min, during exposure to OGD+10µM mecamylamine of 90 min and during restoration of normal conditions for 90 min. **a2**, A representative CAP during normal conditions, during exposure to 90 min aCSF+10µM mecamylamine and during restoration of normal conditions for 90 min. **d**, plots of four experiments d against time showing the changes in the CAPs during 90 min of aCSF+10µM mecamylamine (upper two plots) and OGD+10µM mecamylamine (lower two plots). e, Mean data showing the reversible effect of nACh-R blocker mecamylamine during 90 min aCSF (red), n=6, and OGD (black), n=6. CAP is normalized to 100 % at zero time, CAP is (40.1± 10.0) at the end of OGD +10µM mecamylamine and it is (71.7± 13.25) at the end of aCSF+10µM mecamylamine, CAP is (46.0± 10.8) after restoration of normal conditions following OGD +10µM mecamylamine and it is (95.9± 7.12) following aCSF+10µM mecamylamine. Error bars are SEM.

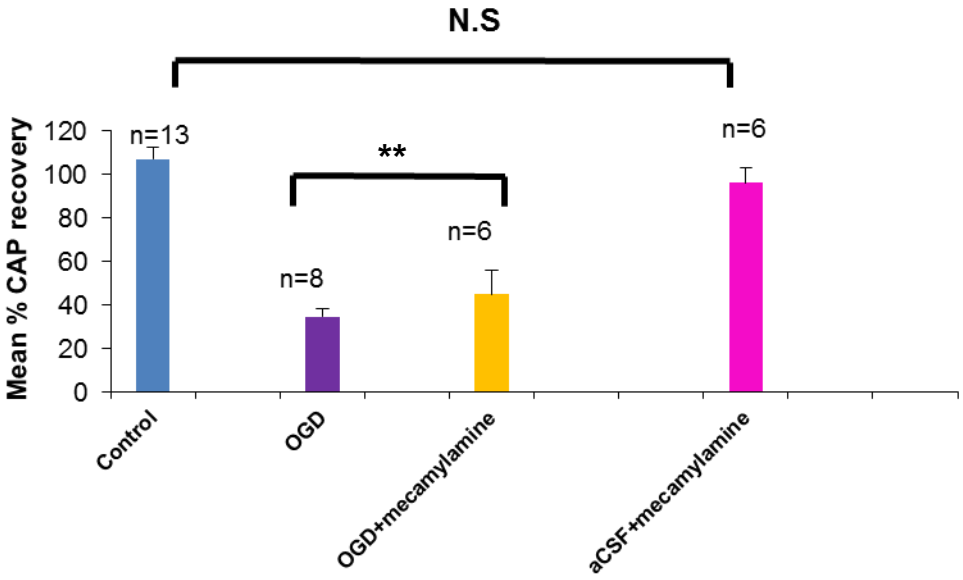


Figure 44- Histogram Summarizing the changes in the CAPs recovery in the presence of nACh-R blocker in PO-P4 RONs.

The comparable recovery in the CAPs during OGD alone and OGD+ 10µM Mecamylamine was significant. **=p<0.001.

There was no significant comparison between the CAP in case of control aCSF and the CAP in case of aCSF+10µM mecamylamine. Error bars are SEM.

The ionic features in P0-P4 RONs are novel

Stys *et al.* (1990) showed that ischaemic injury of myelinated optic nerve axons was critically dependent upon the presence of extracellular Ca^{2+}. In 1991 he found that two extracellular ions were essential for the development of permanent loss of function during ischaemia in myelinated axons: Ca^{2+} and Na^{+}. Therefore, the effect of these two ions during 90 min OGD in pre-myelinated axons was investigated. *This produced the interesting finding that removal of either* Ca^{2+} *or* Na^{+} *from the extracellular space protected the pre-myelinated optic nerve during OGD.* In the zero-Ca^{2+} experiments, $CaCl_2$ was omitted from the aCSF and (50µM) EGTA was added, and in the zero-Na^{+} experiments, Na^{+} was omitted and choline or $NMDG^{+}$ was added. In zero-Ca^{2+} OGD experiments, nerves were first exposed to 10 min normal conditions, followed by 10 min zero-Ca^{2+} aCSF. This was followed by 90 min OGD in zero-Ca^{2+}, and then the nerves were re-perfused with zero-Ca^{2+} aCSF for 20 min before re-instatement of normal conditions for 70 min. When Ca^{2+} was removed from the extracellular space during 90 min OGD, the CAP fell to 50.4 ± 10.01% of the initial value at the end of OGD. Afterward, the CAP recovered to 74.3 ± 1.3% of the initial value, (n=6; P< 0.0001 vs OGD) following restoration of normal conditions; *(see Figs 45 & 47).* Note that under control conditions, zero-Ca^{2+} evoked a transient decline in the CAP after an initial increase within the first 10 min to 67.4 ± 7.8% of the initial value, and recovered following restoration of normal conditions to 92.8 ± 2.01% of the initial value,(n=6); *(see Figs 46 & 47).* In a separate set of experiments in which Ca^{2+} is not removed from the perfusate, removing Na^{+} from the perfusion has a similar protective effect. As a result, the CAP

declined at the end of 90 min OGD to 45.1 ± 4.03% of the initial value, and the recovery in the CAP following restoration of normal conditions was enhanced by zero-Na^+ conditions to 74.3 ± 2.0% of the initial value, (n=6; P<0.0001 vs OGD); *(see Fig 48 & 50).* Note that under control conditions, zero-Na^+ evoked a large decline in the CAP to 20.9 ± 1.12% of the initial value, and recovered to 75.4 ± 5.2% of the initial value, (n=6) following restoration of normal aCSF, *(see Fig 49 & 50).* The dependence of ischaemic injury upon both Ca^{2+} and Na^+ indicates that these ions are potentially toxic to cell viability by causing permanent loss to axonal function (Siesjo, 1986; Choi, 1994). *It is therefore clear that, extracellular* Ca^{2+} *and* Na^+ *are essential for the development of permanent loss of function during OGD in pre-myelinated axons.*

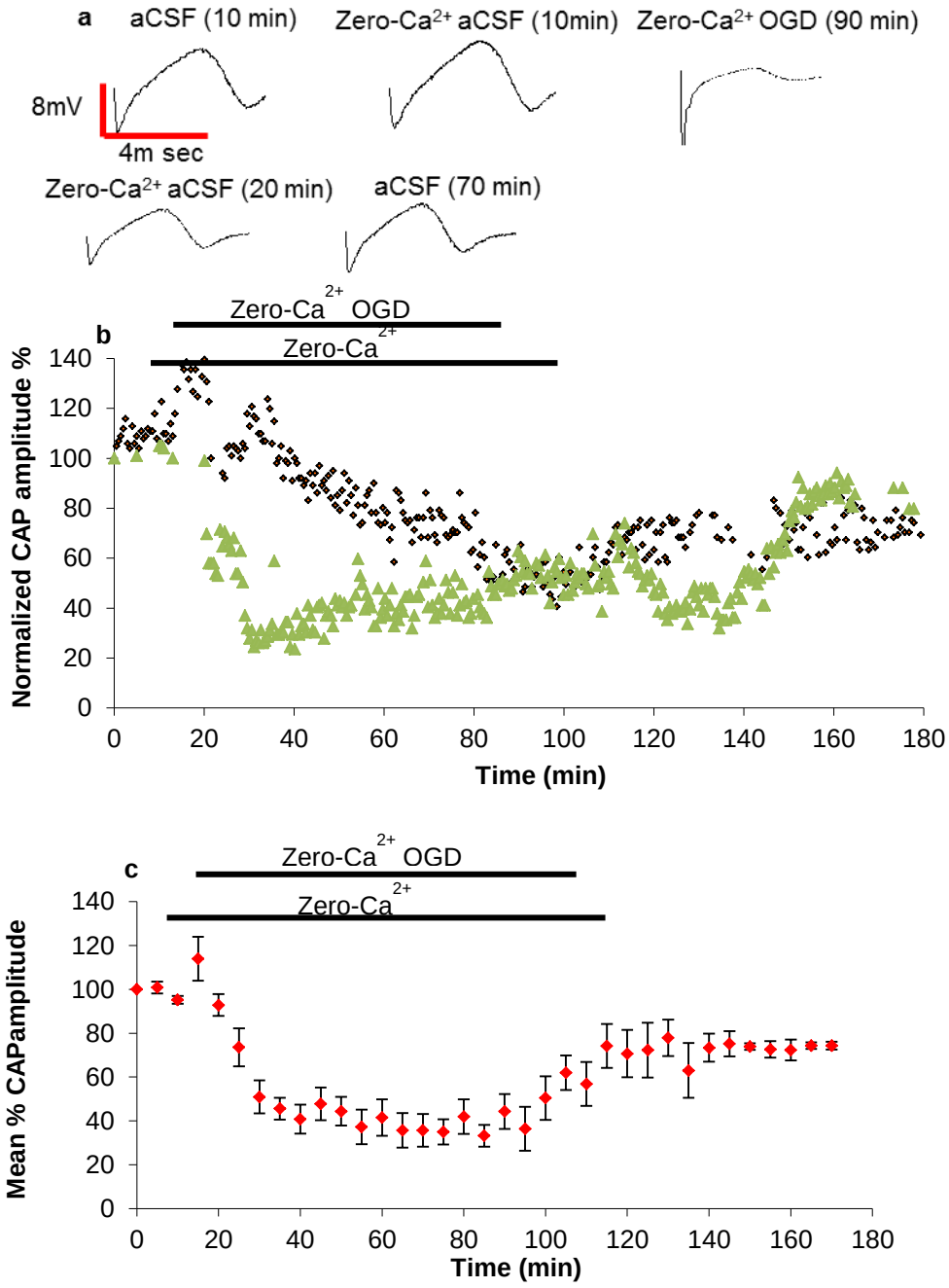


Figure 45- Exposure to 90 min OGD after initiating zero-Ca^{2+} condition for 10 min resulted in similar changes in the CAP amplitude of zero-Ca^{2+} control.

a, representative CAPs during 10 min normal conditions, 10 min of zero-Ca^{2+} aCSF, 90 min zero-Ca^{2+} OGD, during reperfusion of the nerve with zero-Ca^{2+} aCSF for 20 min period and during re-introduction of normal conditions for 70 min, respectively. b, plots of two individual experiments against time. c, Mean data plotted showing the protective effect of Ca^{2+} removal during 90 min OGD in pre-myelinated optic nerves. CAP is normalized to 100 % at zero time, CAP at the end of zero-Ca^{2+} OGD is (50.4 ± 10.01), and at the end of the experiment after re-introduction of normal conditions is (74.3 ± 1.3), n=6. Error bars are SEM.

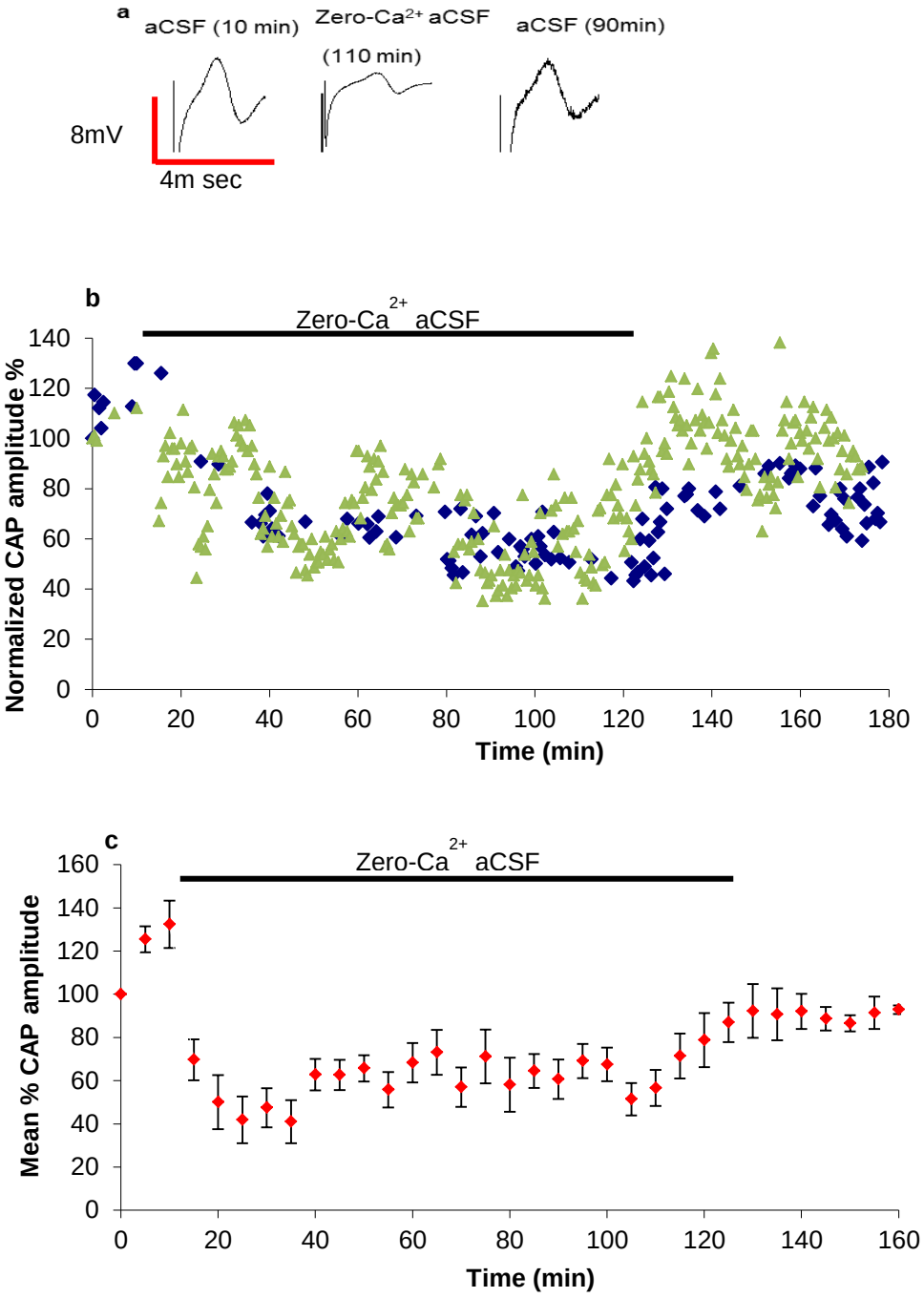


Figure 46- Removal of Ca^{2+} from control conditions evokes a transient decline in the CAP in P0-P4 RONs.

a, representative CAPs recorded during normal conditions, during zero-Ca^{2+} aCSF for 110 min, and during re-introduction of normal conditions for 90 min. b, Plots of two individual experiments against time. c, Mean data plotted against time showed that removal of Ca^{2+} from the perfusion evoked a transient decline in the CAP followed by a large recovery after re-institution of normal conditions, n=6. CAP at zero time is normalized to 100 %, CAP at the end of zero-Ca^{2+} aCSF is (67.4 ± 7.8), and at the end of the experiment after re-instruction of normal conditions is (92.8 ± 2.01). Error bars are SEM.

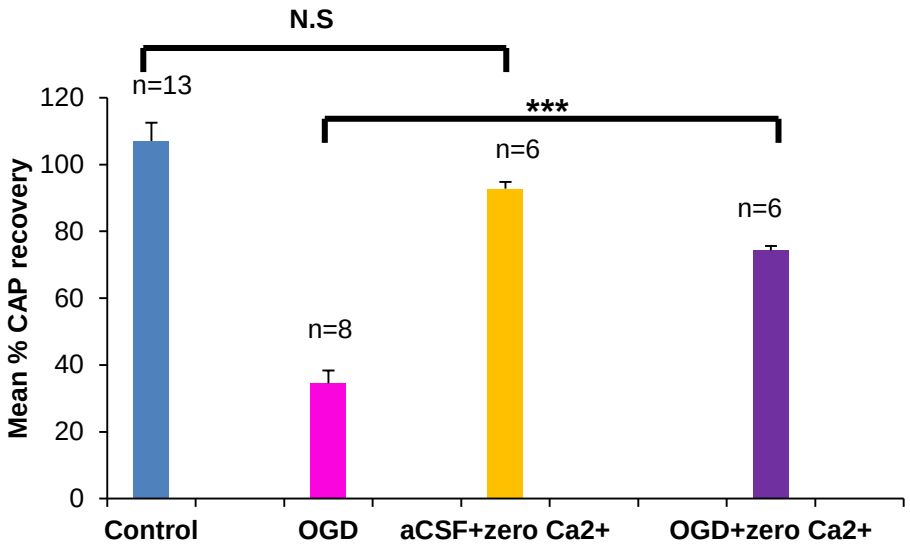


Figure 47- Histogram summarizing the effect of Ca^{2+} removal from the perfusate on the CAPs recovery in P0P4 RONs.

The recovery in the CAP following 90 min OGD is greatly enhanced by removal of Ca^{2+} from the perfusate. There was a significant recovery in the CAPs amplitude during zero-Ca^{2+} OGD in compare to OGD alone. ***=p<0.0001. There was no significant comparison in the CAP recovery between zero-Ca^{2+} aCSF and control. Error bars are SEM.

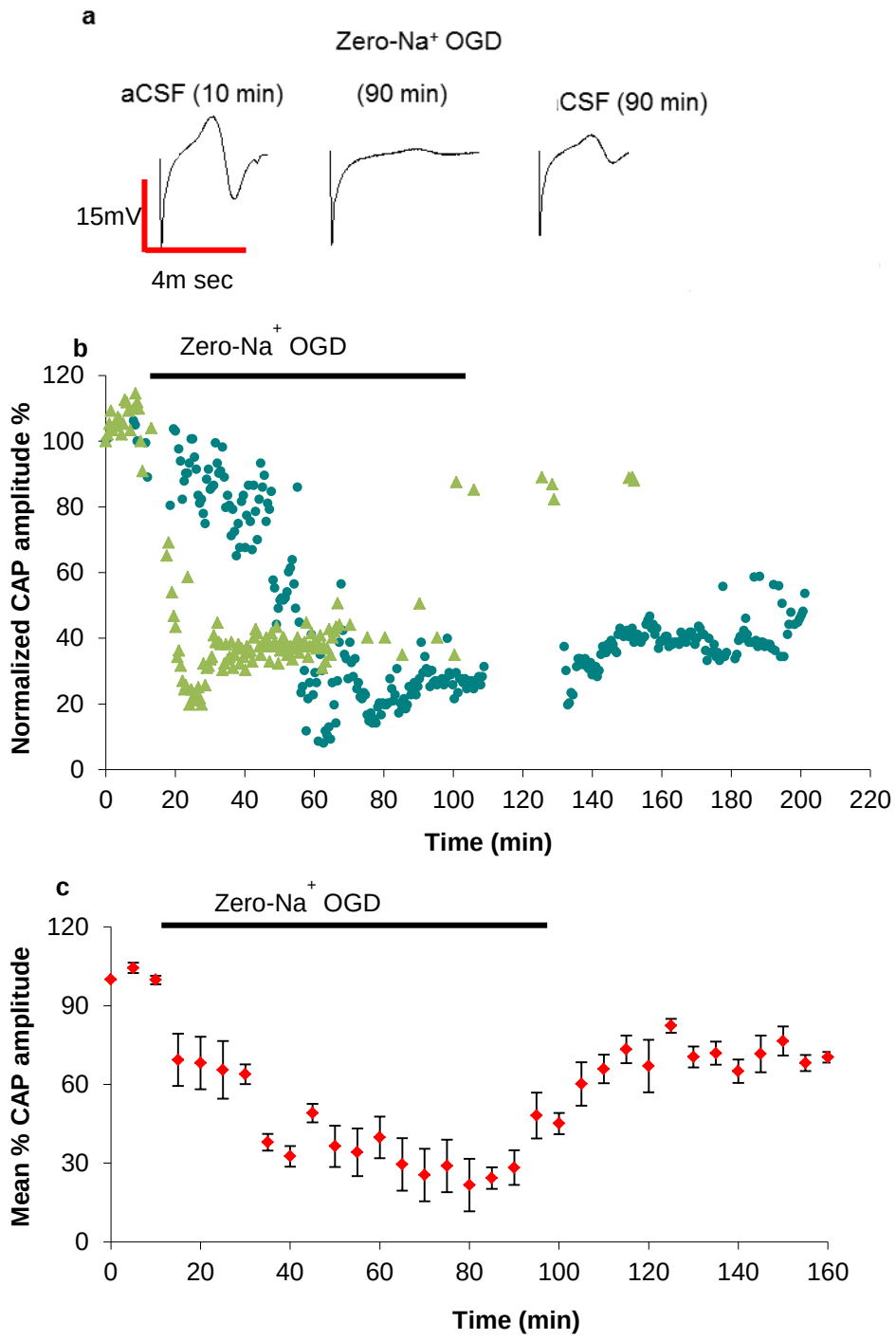


Figure 48- Removal of Na^+ during 90 min OGD was protective in P0-P4 RONs.

a, representative CAPs amplitude during normal conditions, during exposure of P0 RON to 90 min zero-Na^+ OGD, and after restoring the normal conditions. **b,** Plots of two individual experiments against time. **c,** Mean data showing the changes in CAPs amplitude during and after exposure to 90 min Zero-Na^+ OGD, n=6. CAP at zero time is normalized to 100%, CAP at the end of zero-Na^+ OGD is (45.1 ± 4.03), CAP at the end of the experiment after restoring of normal conditions is (70.3 ± 2.0). Error bars are SEM.

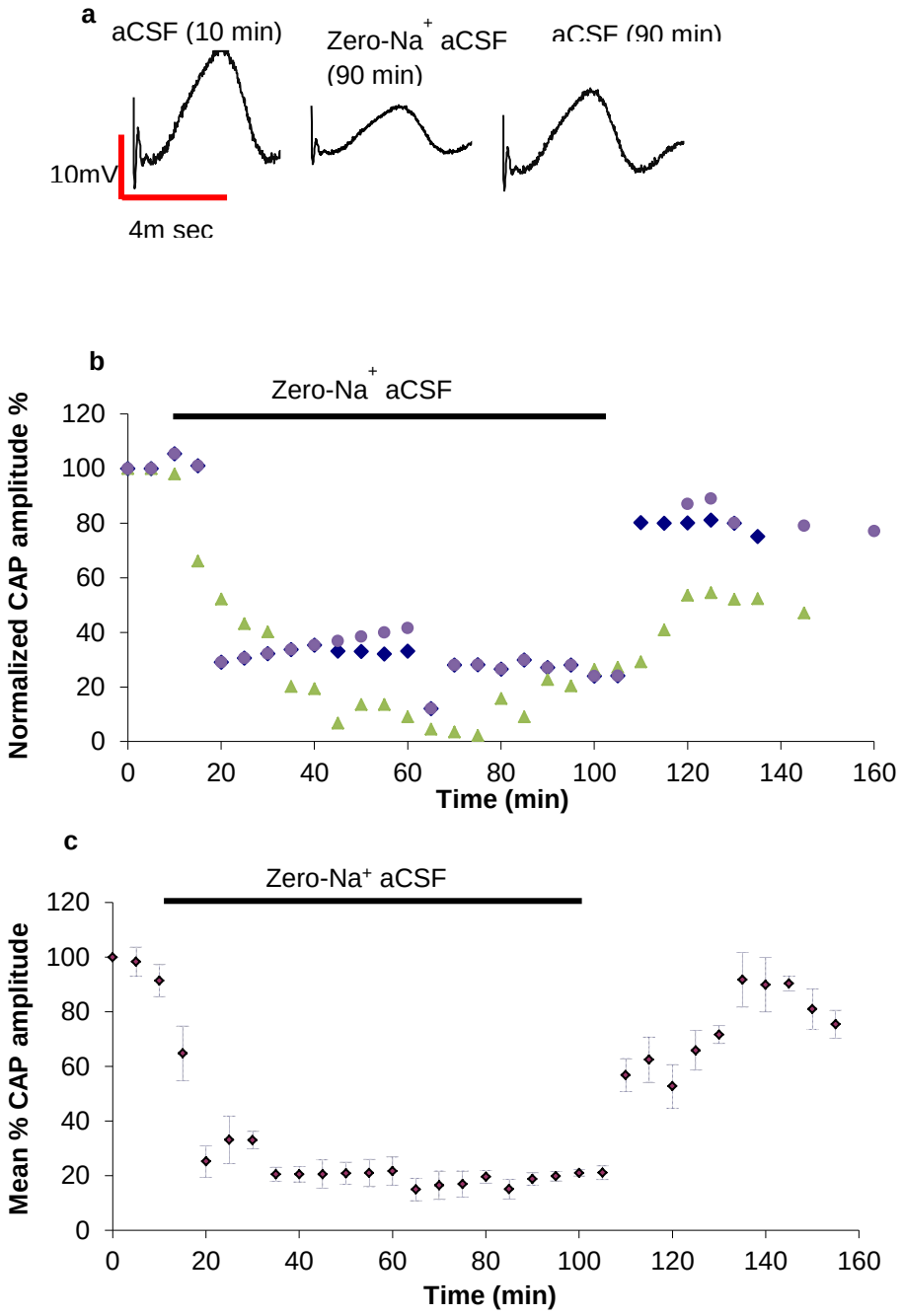


Figure 49- CAPs recorded under Zero-Na$^+$ aCSF in P0-P4 RONs.

a, representative CAPs recorded during normal conditions for 10 min, during exposure to 90 min zero-Na$^+$ aCSF, and after restoring the normal conditions normal conditions. **b,** Three individual data plotted against time. **e,** Mean data showing large reversible effect of zero-Na$^+$ on the CAP amplitude, n=6. CAP at zero time is normalized to 100 %, CAP at the end of 90 min zero-Na$^+$ aCSF is (20.9 ± 1.12), and at the end of the experiment after restoring the normal conditions is (75.4 ± 5.2). Error bars are SEM.

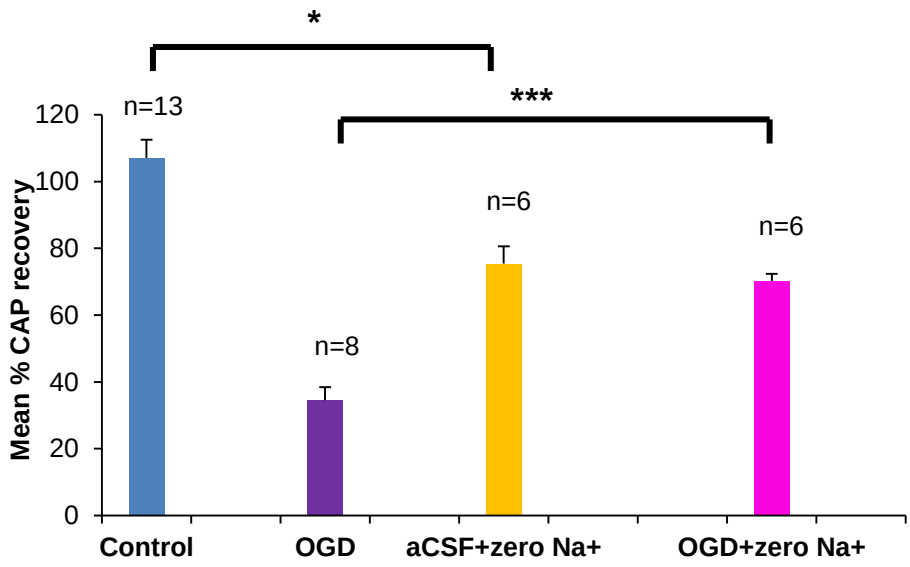


Figure 50- Histogram summarizing the effect of zero-Na+ conditions on the CAP recovery in P0P4 RONs.

The recovery in the CAPs following 90 min of OGD is greatly enhanced by removal of Na+ from the perfusate. Removal of Na+ from the solutions exhibited a significant recovery in the CAPs in compare to OGD alone. ***=p<0.0001. There is a significant comparison in the recovery of the CAP during zero Na^+ aCSF in compare to control conditions. *=p<0.01. Error bars are SEM.

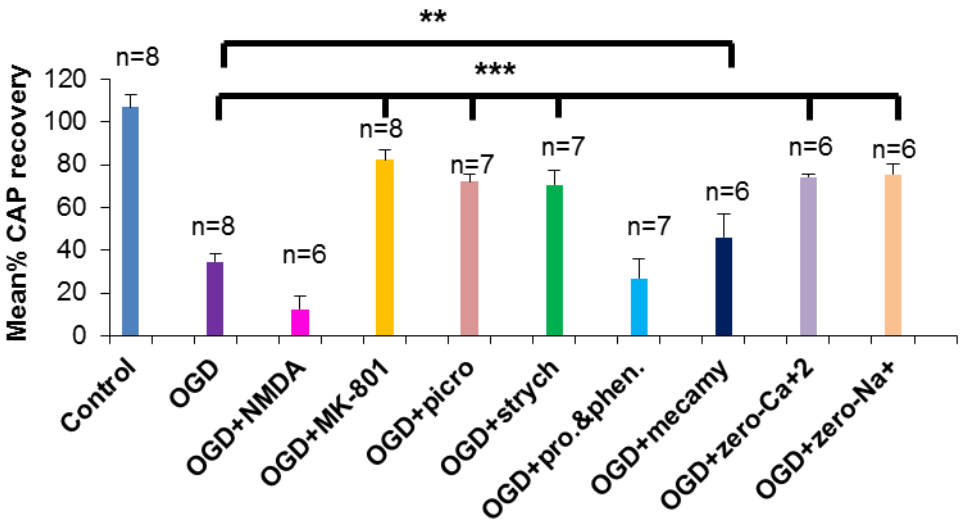


Figure 51- Summary of Mean % CAPs recovery in P0P4 RONs.

Histogram showed the mean recovery in the CAPs following exposure to the standard period (90 min) of OGD and OGD + drug treatments. The significance in the recovery in the CAPs was in case of NMDA-R blockade, $GABA_A$-R blockade, and GlyR blockade. Also removal of both Na^+ and Ca^{2+} from the solution produced significant recovery in the CAPs in compare to OGD. $^{***}P<0.0001$.

The recovery in the CAPs in case of nACh-R blockade was also significant. $^{**}P<0.001$. Error bars are SEM.

Discussion

In this chapter, the sensitivity of pre-myelinated central white matter to the withdrawal of oxygen and glucose at 37°C in isolated P0-P4 RON axons was assessed by electrophysiological recording of CAPs. The effect of GluRs and other neurotransmitter receptor blockade was tested during a standard period of 90 min normal aCSF or OGD. CAPs recorded from P0-P4 RONs were stable and biphasic in their shape, which makes the measurement of the CAP area problematic (Foster *et al.,* 1982). In addition, long-duration stimulus pulses (between 100-600 μs) are required to elicit supra-maximal responses in neonatal RONs, and the CAP amplitude was used instead of the CAP area (Fern *et al.,* 1998).

As seen in Chapter III, OGD for 60 min resulted in a significant injury in the completely myelinated adult rodent optic nerve, which is illustrated by using isolated adult RONs and MONs and assessed by the loss of the evoked CAPs with low levels of recovery. In contrast, optic nerves at ages P0-P4 showed no loss of function during 60 min OGD and the CAP was unchanged during the whole period of OGD, thus demonstrating that they are highly resistant to a 60-min period of ischaemia. It is clear from this result that there is a significant energy reserve in neonatal nerves relative to their energy requirement.

Optic nerves from this age group, when exposed to a longer period of OGD (90 min), showed a significant loss in the CAPs at the end of OGD exposure with partial recovery after re-perfusion with normal conditions. The resistance of the neonatal central white matter to energy deprivation is thought to be a consequence of the low metabolic rate of the immature CNS (Duffy *et al.,* 1975; Hansen, 1985; Thurston and McDougal, 1969). In this age group all axons are pre-myelinated; some axons begin to acquire a single layer of myelin from age P6-P7, and the proportion of axons that acquires a single layer of myelin increases from a few% at P6 to 85% at P28 – these become ensheathed completely by adulthood (Foster *et al.,* 1982; Skoff *et al.,* 1976). Therefore, metabolic activity is associated with the anabolic process of myelination and the degree of energy sensitivity (Azzerelli *et al.,* 1980; Chugani *et al.,* 1991; Davison and Dobbing, 1966; Kennedy *et al.,* 1972; Rice *et al.,* 1981). My results in Chapters III and IV showed that pre-myelinated axons have a high resistance to energy deprivation and the rate at which the CAP is lost during energy deprivation is presumably related to the metabolic rate of the nerve. Nerves with a high metabolic rate will use energy reserves more rapidly, and so become rapidly depleted with a corresponding rapid loss of the CAP and low recovery as seen in Chapter III in RONs and MONs. In contrast, P0-P4 RONs exhibited a degree of resistance to energy deprivation due to low metabolic rate as seen in this result. However, this age group (P0-P4) matches the time of augmented glycogen deposition in the CNS (Kohle and Vannucci, 1977), and it is possible that any axonal energy stores in the form of glycogen might contribute to the heightened tolerance of pre-myelinated white matter to

ischaemic injury in the CNS (Fern *et al.,* 1998). Glycogen is the largest energy reserve in the CNS (Lowry *et al.,* 1964), and the glycogen is restricted to the astrocytes in adult white matter and provides metabolic support of the axons in the white matter; and the glial support of neurons in other preparations have been well recognized (Poitry-Yamate *et al.,* 1995; Tsacopoulos et al., 1994). Axons in the neonatal CNS can tolerate glucose withdrawal because they have access to the larger glycogen energy reserve (Fern *et al.,* 1998). The increased glycogen that is present in the glia in the neonatal CNS cannot explain the tolerance of neonatal white matter to OGD because the lactate and pyruvate, the energy substrates transferred from glia to neurons, cannot be metabolized in the absence of oxygen (Fern *et al.,* 1998). Astrocyte glycogen breaks down to lactate that is transferred to the extracellular space and then taken up by neurons or axons as an aerobic fuel (Brown *et al.,* 2004). It is not known, however, whether neonatal white matter axons may contain a glycogen energy reserve that could act to support function during OGD.

In addition to high energy reserves and low energy requirement in neonatal RONs, there are other factors that might explain the OGD tolerance at this point of development. Waxman *et al.* (1989) showed that pre-myelinated optic nerves of a diameter 0.2 µm have low densities of Na^+-channels. However, even a low density of Na^+-channels can support action potential conduction, and would be expected to depolarize the small calibre fibres to threshold because of high input resistance of these fibres (Waxman *et al.,* 1989). A low Na^+-channel density in these pre-myelinated axons might relate to low Na^+ ion influx and weakly promote the reversal of Na^+/K^+

exchange during energy deprivation. Alix *et al.* (2012) showed low levels of voltage-gated Ca^{2+}-channels (VGCCs) expression at P0-P4 RONs which may render them less sensitive to ischaemic injury, whereas P8-P10 RONs express clusters of functional VGCCs as they prepare for myelination (Alix *et al.,* 2008). In Optic nerves at P8-P10, undergoing initial radial expansion, ischaemic injury was mediated by Ca^{2+}-influx through VGCCs whereas P0-P4 RONs the injury was mediated predominantly by GluRs (Alix *et al.,* 2012).

The role of NMDA-R agonists and antagonists in OGD-induced injury in neonates

The contribution of NMDA-Rs-mediated excitotoxicity during energy deprivation has not previously been examined in immature central axons (Alix and Fern, 2009; Alix *et al.,* 2012). NMDA-Rs have recently been demonstrated in both adult and neonatal oligodendrocytes, whereas non-NMDA-Rs were discovered in precursor oligodendrocyte cells some time ago and were shown to correspond with the window of susceptibility to OGD-induced injury (Follett *et al.,* 2000; Deng *et al.,* 2003). NMDA-Rs expressed on the oligodendrocytes exhibit a weak voltage-dependent Mg^{2+} block, which causes these receptors to permit a large influx of Ca^{2+} during ischaemia where there is a glutamate release and membrane depolarisation (Karadottir *et al.,* 2005). However, Alix and Fern (2009) showed that NMDA-R have a role in central white matter injury using P10 RON. Following on from these studies, I investigated whether or not the mechanism of central white matter injury in pre-myelinated optic nerves is mediated by NMDA-Rs. It was found that activation of NMDA-Rs exacerbated injury in optic nerves at this stage of

during energy deprivation. When P0-P4 RONs were exposed to 90 min OGD in the presence of NMDA-R agonists (NMDA + glycine), injury levels were increased compared to OGD alone and over-activation of NMDA-Rs during energy deprivation led to a fall in the CAP to zero with low recovery after restoration of normal conditions. Therefore, NMDA-type GluRs in pre-myelinated optic nerves aggravate OGD-induced injury, which implicates NMDA-Rs in excitotoxic injury in neonatal white matter disorders.

This OGD-induced injury in pre-myelinated optic nerves is significantly reduced by co-application of NMDA-R antagonist MK-801. During normal aCSF, blockade of NMDA-Rs with MK-801 for 90 min led to a decline in the CAP which failed to recover completely after restoration of normal conditions. Over-activation of NMDA-Rs with exogenous administration of NMDA + glycine for 90 min also led to a decline in the CAP which failed to recover completely after washout. The results of the current study showed that MK-801 was highly protective against OGD-induced injury in P0-4 RON while it was also toxic to the nerves under normoxic conditions. NMDA-Rs contribute to OGD-induced injury in pre-myelinated optic nerves and that NMDA-R activation can be toxic under normoxic conditions. In contrast to MK-801, the protective effect of memantine was small and non-significant on pre-myelinated optic nerves during OGD-induced injury. When P0-P4 RONs were exposed to 90 min OGD in the presence of memantine, the CAP declined at the end of 90 min with low levels of recovery after restoration of normal conditions. This might be because of its rapid off-rate binding kinetics (Chen *et al.,* 1992; Chen and Lipton, 2006). Therefore an increased concentration of memantine might become protective against OGD-induced

injury in neonatal white matter. During normal aCSF, perfusing with memantine for 90 min caused a gradual decline in the CAP. The recovery levels following memantine wash-out were significantly less than those found in aCSF alone and indicate toxicity from acute NMDA-R blockade at this age. Memantine is less toxic and clinically well tolerated than MK-801 (Chen *et al.,* 1992; Chen and Lipton, 2006). These results show that both NMDA-Rs over activation and NMDA-Rs block were acutely toxic at this point in white matter development. It is therefore clear that MK-801 is highly protective, while memantine is not protective against OGD-induced injury in pre-myelinated optic nerves; therefore, the non-competitive NMDA-R antagonist MK-801 may provide an effective pharmacological prevention of PVL in premature infants.

As a broad-spectrum antagonist of excitatory amino acid receptors, kynurenic acid can antagonise the glycine site of NMDA-Rs as well as neuronal nACh-Rα-$_7$ -containing nicotinic receptors (Moroni *et al.,* 2012). Lugo-Huitron *et al.* (2011) hypothesised that kynurenic acid may have direct antioxidant properties and may be able to inhibit oxidative stress because of free radical scavenging activity. Accordingly the antagonistic effect of kynurenic acid during OGD-induced injury in pre-myelinated RONs was tested. When P0-P4 RONs were exposed to 90 min OGD co-perfused with kynurenic acid, the CAP fell significantly at the end of OGD and exhibited a small recovery after restoration of normal conditions that was not significantly different from that seen in OGD alone. Kynurenic acid was applied during 90 min aCSF, resulting in a significant increase of the CAPs within the first 30 min which recovered following the washing off of kynurenic acid. This

increase in the CAP during the 1st few min of the experiment is possibly a result of membrane depolarization due to an increase in the extracellular K^+ and conduction block (Baker and Bostock, 1989; Bergmans and Michaux, 1970). The administration of kynurenic acid had no protective effect during OGD-induced injury in immature white matter, while it was not toxic under normoxic conditions. This result might indicate that the glycine site of NMDA-R does not play an important role in excitotoxicity mediated by the over-activation of the NMDA-R during OGD-induced injury in the neonatal brain. This is consistent with the result of a study by Fatokun *et al.* (2008) on brain ischaemia and mitochondrial dysfunction. This study reported resistance of mitochondrial inhibitors to blockade by kynurenic acid. They found that kynurenic acid failed to prevent mitochondrial poisons (3-NPA or KCN) induced toxicity; therefore raising the possibility that activation of the glycine site may not be required for the induction of neurotoxicity following mitochondrial disruption (Fatokun *et al.,* 2008). Therefore, my results and others suggest that activation of NMDA-Rs during ischaemia is independent of the glycine site or might occur distal to the site of action of kynurenic acid (Fatokun *et al.,* 2008).

Matute (1998) showed that activation of KA-Rs in oligodendrocytes by the excitotoxin kainaic acid induced toxic damage to the adult optic nerve. Exposure of the pre-myelinated RONs to a long period of kainaic acid (a specific agonist for the kainate receptors) produced only small and transient changes in the CAPs, which is also true for P10 RONs (Alix and Fern 2009). In a comparison between the effects of NMDA-R agonists (NMDA + glycine) and KA-R agonist (kainate) during a 90-min period of normal aCSF, NMDA

caused a decline in the CAP which was not reversed after restoration of normal conditions, whereas kainate caused very mild changes in the CAP with complete recovery following restoration of normal conditions. This result might indicate that ischaemic injury in neonatal white matter is not mediated by the over-activation of kainate receptors. McCarran and Goldberg (2007) showed that in murine white matter at P3 and P7, activation of AMPA/KA-Rs does not contribute to ischaemic axonal injury (McCarran and Goldberg, 2007). According to these results, the excessive stimulation of NMDA-GluRs might be the major mechanism in OGD-mediated injury in developing white matter at this age group which coincides with a risky period of PVL. Therefore, it is reasonable to speculate that specific NMDA-R antagonists alone or in combination could be used as an effective treatment for neonatal white matter ischaemic injury.

The role of neurotransmitter receptor blockades other than glutamate during OGD-induced injury in neonates

Although the excitatory amino acids are thought to play an important role in the pathogenesis of central white matter ischaemia, less attention has been paid to the role of the inhibitory amino acids (Hutchinson *et al.,* 2002). Several experimental studies have shown elevated levels of glutamate and aspartate accompanied by an increase in the concentration of GABA and glycine in the CSF of patients with head injuries (Palmer *et al.,* 1994), and in the extracellular space during white matter ischaemia by using micro-dialysis measurements (Shimada *et al*., 1993; Richards *et al.,* 1993). The concentration of GABA during normal conditions is <0.002 µM - 0.02 µM, and

increases during ischaemic conditions in correlation with an increase in the concentration of glutamate; this indicates that brain ischaemia is associated with an increase in both excitatory and inhibitory amino acids (Hutchinson *et al.,* 2002). The increase in GABA concentration might be due to one of two mechanisms: firstly an increase in glutamate that leads to neuronal damage and consequently to an elevation in GABA, which plays a role in the pathogenesis of ischaemic injury; or secondly, the increased production and release of GABA in an attempt to counteract the excitatory amino acid glutamate as an endogenous protective mechanism (Westerink and DeVries, 1989). Studies show that acetylcholine and adrenergic agonists are also released into the extracellular space of the CNS during ischaemia (e.g., Richards *et al.,* 1993; Uchihashi *et al.,* 1998; Yamamuro *et al.,* 1996). Constantinou and Fern, (2009) studied the role of these four neurotransmitter receptor systems: $GABA_A$, glycine, adrenergic and nACh, on developing white matter during OGD but used P7-P12 RONs. In the current study, P0-P4 RONs were used to examine the effects of acute exposure to receptor blockers for these receptors that have known actions on central white matter. The injury capacity of a standard 90-min period of GABA, glycine, adrenergic, and nACh blockade during OGD and normal aCSF upon P0-P4 RONs was tested. During 90 min OGD, co-application of the $GABA_A$-R antagonist picrotoxin produced a decline in the CAP by the end of OGD with significant recovery after restoration of normal conditions. The data showed that picrotoxin is highly protective against OGD-induced injury in pre-myelinated optic nerves. During normal aCSF, perfusion with picrotoxin resulted in a CAP increase during the first 20-30 min, and then a decline to the end of the

experiment after re-institution of normal conditions. Application of picrotoxin during normal aCSF without removal of picrotoxin from the perfusate led to a gradual increase in the CAP at the end of the experiment. These results showed that $GABA_A$-R antagonist both protected the pre-myelinated optic nerves from OGD-induced injury and increased CAP amplitude under normoxic conditions, indicating a protective role of $GABA_A$-R antagonist against ischaemia in developing brain. The presence of endogenous GABA in the neonatal optic nerves has been reported by several studies (Lake, 1992; Ochi *et al.,* 1993; Sakatani *et al.,* 1992); and the activation of $GABA_A$-Rs produces a partial block in the neonatal optic nerve (Sakatani *et al.,* 1991; Sakatani *et al.,* 1992), which is caused by extracellular K^+ accumulation and axonal depolarisation (Sakatani *et al.,* 1994; Simmonds, 1983).

Several studies have demonstrated the neurotoxic effects of glycine mediated by NMDA-Rs (Patel *et al.,* 1990). Moreover, ischaemic conditions elicit an increase in the extracellular concentration of glycine (Baker *et al.,* 1991; Oda *et al.,* 2007), which has been shown to contribute to the ischaemic injury mediated by NMDA-Rs (Oda *et al.,* 2007). When P0-P4 RONs were exposed to 90 min OGD in the presence of the glycine-receptor antagonist strychnine, the CAP fell at the end of OGD and recovered after restoration of normal conditions. The data showed that strychnine was protective against OGD-induced injury in pre-myelinated optic nerves. During 90 min normal aCSF, strychnine produced a transient changes in the CAP which is recovered to normal after re-institution of normal conditions. The present results show that the blocking of glycine receptors potentially produces a neuroprotection against OGD-induced injury in developing white matter and

that glycine therefore may be toxic independently of any actions at NMDARs. It is therefore clear that beside to the protective effect against OGD-induced injury, strychnine is not toxic to P0-P4 RONs during normoxic conditions.

A similar effect was seen in neonatal RONs (P7-P15) during 80 min perfusion with GABA and glycine (Constantinou and Fern, 2009). Both $GABA_A$ and GlyR antagonists produced a significant recovery in the CAPs of the neonatal RONs after washout, which indicates the significant role of these receptors in neonatal white matter injury at this critical age and the protective effect of these antagonists against OGD-induced injury. In contrast, combined-adrenoceptor and nACh-R activation did not significantly effect recovery in the CAP post OGD. When P0-P4 RONs were exposed to 90 min OGD in the presence of combined blockade of ARs with propranolol hydrochloride + phentolamine hydrochloride there was a significant fall in the CAP at the end of OGD with low recovery after a restoration of normal conditions. Administration of combined blockers during normal aCSF produced a mild decline in the CAP by the end of 90 min which recovered significantly after washout of the antagonists. Although combined antagonists are not protective against OGD-induced injury in developing optic nerves they also are not toxic to the nerves. The α- and β-AR had no effect on the CAP recovery during OGD and they are not toxic to the nerves under normoxic conditions. Tonic AR activation is required for nerve viability, while the over-activation of these receptors is also damaging (Constantinou and Fern, 2009). Over-activation of α-AR can lead to glial injury following oxidative stress, although this occurs via delayed mechanisms (Khorchid *et al.,* 2002). While β_1-AR activation causes Ca^{2+}-dependent axonal

depolarisation in neonatal RONs but this does not involve changes in the extracellular K^+ (Honmou and Young, 1995).

Application of the nACh-R blockader mecamylamine during 90 min OGD evoked a decline in the CAP by the end of OGD with recovery after restoration of normal conditions. Whereas application of mecamylamine during normal aCSF produced a small decline in the CAP at the end of 90 min of perfusion which recovered significantly after restoration of normal conditions. The data showed that the nACh-R antagonist was not toxic to pre-myelinated optic nerves, and in addition to that it produced a small degree of protection against OGD-induced injury. Activation of nACh-R produced a partial decline in the CAPs in P10 RONs, and the axonal depolarisation was not dependent on the Ca^{2+} influx into the axons that caused by receptor activation (Constantinou and Fern, 2009). These authors found that nACh-R activation leads to Ca^{2+} elevation in both astrocytes and oligodendroglia, whereas α-ARs were recognised to have the same effect at least in astrocytes (Constantinou and Fern, 2009). However, there is a good relationship between neonatal white matter injury and prolonged activation of neurotransmitter receptors such as nACh-Rs and α-ARs that are capable of elevating Ca^{2+} in glia (Constantinou and Fern, 2009).

In summary, 90 min OGD co-perfused with a GABA or GlyR blockade suppressed the pre-myelinated optic nerve CAP in a reversible manner. Adrenoceptor blockade for the same period did not protect the pre-myelinated optic nerve axons from OGD-induced injury, while a nAChR blockade had a small but significant effect. I conclude that the over-activation of neurotransmitter receptors other than glutamate such as GABA and

glycine receptors in this age group can create widespread damage in pre-myelinated white matter during OGD; consequently, antagonising some of these receptors is potentially protective. nACh-Rs and ARs are not involved in OGD-induced injury in pre-myelinated white matter, a phenomenon that may be clinically significant.

The effect of Ca^{2+} and Na^{+} removal during OGD and normal conditions in pre-myelinated optic nerves

In 1992, Stys *et al*., found that Ca^{2+} and Na^{+} were the two extracellular ions that were essential for the development of permanent loss of function during anoxia in adult RON. The effect of the removal of either Ca^{2+} or Na^{+} from the extracellular space in P0-P4 RONs during both OGD and normal aCSF was investigated. It was found that removal of either Ca^{2+} or Na^{+} from the extracellular space protected the pre-myelinated axons during OGD at this age group. The removal of Ca^{2+} from the extracellular space during 90 min OGD produced a partial fall of the CAP which recovered significantly after restoration of normal conditions. Zero-Ca^{2+} aCSF for 90 min evoked a transient decline in the CAP after an initial increase within the first 10 min, and then exhibited very partial ablation with significant full recovery after restoration of normal conditions. Recovery of the CAP occurred with the removal of Ca^{2+} during both OGD and normal aCSF, indicating that pre-myelinated axons might be directly damaged by accumulation of intracellular Ca^{2+}. The levels of Ca^{2+} increase in the postnatal period, and Ca^{2+}-channels, have been shown to contribute to injury in white matter (Lorenzon and Foehring, 1995; Fern *et al.,* 1995). The axons of

developing white matter are the focus of injury in various diseases that attack immature white matter including PVL, the main pathology associated with cerebral palsy (Back and Rivkees, 2004). PVL is thought to be partially ischaemic in origin (Alix *et al.,* 2008), since ischaemic axonal injury is mainly Ca^{2+} -dependent (Stys *et al.,* 1992; Fern *et al.,* 1995), Ca^{2+}-channels may play a major role for Ca^{2+} influx into developing white matter (Alix *et al.,* 2008). Ionic mechanisms in adult RONs that mediate toxic Ca^{2+} overload during anoxia include non-inactivating Na^{+}-channels (Stys *et al.,* 1993), voltage-gated Ca^{2+}-channels (Fern *et al.,* 1995), reverse Na^{+}/Ca^{2+} exchanger (Stys *et al.,* 1992), and activation of L-type voltage-gated Ca^{2+}-channels (Brown et al., 2001). However, these channels might have a role in exacerbating Ca^{2+} overload during OGD in pre-myelinated optic nerves. Removing Na^{+} from the solution in the presence of Ca^{2+} has a similar protective effect on the CAP recovery. Zero-Na^{+} OGD produced a decline in the CAP with recovery after restoration of normal conditions. These results suggest the fundamental role of the ionic basis in the pathogenesis of pre-myelinated axonal injury. During energy deprivation, CNS axons can no longer make ATP and the ATP-dependent Na^{+}/K^{+} exchange fails, causing moderate Na^{+} influx through non-inactivating voltage-gated Na^{+}-channels and subsequent disruption of axon ion homeostasis and loss of excitability (Fern *et al.,* 1998; Ransom *et al.,* 1990; Stys *et al.,* 1992). Although voltage-gated Na^{+}-channels are expressed somewhat equally along the non-myelinated axons (Westenbroek *et al.,* 1989), they accumulate at high density at the AIS (Catterall, 1981; Wollner and Catterall, 1986). The density of Na^{+}-channels is < $2\mu m^{2}$ in the neonatal optic nerve axons and increases with age until P25, then decreases in

adulthood (Xia and Haddad, 1994). These Na^+-channels result in persistent Na^+ currents which exacerbate white matter injury following OGD (Alzheimer *et al.,* 1993). However, influx of both ions through voltage-gated channels and Na^+/Ca^{2+} exchanger has a significant role in the injury processes (Fern and Ransom, 1997). My data suggests that injury at P0-4 is more similar to that in the adult, in contrast to Alix *et al.* (2008) found that developing central axons (P10) express functional voltage-gated Ca^{2+}-channels (L-type and P/Q type. These Ca^{2+}-channels represent a major pathway for ischaemic Ca^{2+} influx into developing white matter in P10 RONs (Alix *et al.,* 2008). Therefore, combined blockade of voltage gated channels or Na^+/Ca^{2+} exchanger with NMDA-R blockade may be of clinical significance in protecting neonatal brain from such injury.

This result raised questions about the potential role of Ca^{2+} ion in preserving the action potential conduction in the absence of Na^+ in P0-P4 RONs?

Na^+ is an important ion in inducing the action potential conduction in both myelinated and non-myelinated white matter and consequently removal of Na^+ render the nerves useless. But in early point of development removal of Na^+ from the perfusate did not stop the conduction of action potential. Axonal Ca^{2+}-channels modulate axonal excitability via Ca^{2+}-activated K^+-channels especially under conditions of large Ca^{2+}-influx during repetitive stimulation (Lev-Ram and Grinvald, 1986). It has been shown that axonal Ca^{2+}-transients regulate the frequency and speed of action potentials, possibly by activating Ca^{2+}-dependent K^+-channels (Callewaert et al., 1996).

A Ca^{2+}-dependent K^{+}-conductance has been reported in RON (Lev-Ram and Grinvald, 1986).

Do N-type or L-type Ca^{2+}-channels that regulate Ca^{2}+, play role in mediating axonal action potential conduction?

Chapter V

The effect of blockers upon the ultra-structural changes evoked by OGD:

Introduction

The axons of central white matter permit functional interactions between specialised brain regions (Barbour, 2001).These axons transmit the electrical activity throughout the white matter and translate it into the release of neurotransmitters at synapses (Barbour, 2001). The neurotransmitter glutamate is released from white matter in an activity-dependent manner which activates glutamate receptors that are expressed on the axons and glial cells of white matter (Karadottir *et al.,* 2005). Over-activation of these receptors during ischaemia can induce excitotoxicity and cause damage to the oligodendrocytes and their progenitors (Follett *et al.,* 2000). Glial precursor cells also express NG2-proteoglycan (NG-2(+) cells) in the corpus callosum; these cells express iGluRs which are activated during action potential and induce the release of glutamate from un-myelinated axons (Ziskin *et al.,* 2007). This signalling occurs through anatomical axo-glial synaptic junctions, which indicates that axons conduct action potentials and participate in the rapid signalling with a different group of glial progenitors (Fulton *et al.,* 1992; Ziskin *et al.,* 2007). Functional glutamatergic synapses onto NG-2(+) have been identified in various brain regions in immature and mature animals (Paukert and Bergles, 2006), while vesicular glutamate signalling between axons and neighbouring NG-2(+) cells has been recognized in the corpus callosum and immature RONs (Kukley *et al.,* 2007; Ziskin *et al.,* 2007). However, in this time of pre-myelination, axons express clusters of voltage-gated channels and various receptors that might be the cause of axonal sensitivity to OGD-induced injury (Alix, 2012). In contrast,

numerous studies have identified the role of pre-mature glia susceptibility to ischaemic injury in white matter. These premature oligodendrocytes populate the developing white matter during the period most at risk of PVL (Back *et al.,* 2001), and they are more sensitive to excitotoxic damage than mature oligodendrocytes (Follett *et al.,* 2000; Deng *et al.,* 2003). It is not obvious whether or not the injury of developing oligodendrocytes during OGD is mainly dependent on extracellular glutamate (Deng *et al.,* 2006). Ionotropic glutamatergic receptors are expressed by both astrocytes (Gallo and Ghiani, 2000; Verkhratsky and Kirchhoff, 2007) and oligodendroglia (Fern and Moller, 2000; Follett *et al.,* 2000; Gallo and Ghiani, 2000; Karadottir *et al.,* 2005; Micu *et al.,* 2006; Salter and Fern, 2005), where there appears to be polarized expression with NMDA-Rs largely found on the processes and non-NMDA-Rs on the somata (Salter and Fern, 2005). Glial cells express a number of receptors other than glutamate (Aoki, 1992; Belachew *et al.,* 1998; Rogers *et al.,* 2001; Wang and Lidow, 1997). Cells of the oligodendrocyte lineage can express functional adrenoceptor, nACh-Rs, GABA-A-Rs, and glycine receptors (Aoki, 1992; Belachew and Gallo, 2004; Belachew *et al.,* 1998; Belachew *et al.,* 2000; Cohen and Almazan, 1993; Gilbert *et al.,* 1984; Kirchhoff and Kettenmann, 1992; Oikawa *et al.,* 2005; Pastor *et al.,* 1995; Rogers *et al.,* 2001; Takeda *et al.,* 1995; Wang and Lidow, 1997). Astrocytes express a wide range of receptors, including nACh-Rs and adrenoceptors (Duffy and MacVicar, 1995; Mantyh *et al.,* 1995; Sharma and Vijayaraghavan, 2001). Astrocyte glycine receptor expression might be limited to cells in the spinal cord (Kirchhoff *et al.,* 1996; Pastor *et al.,* 1995), while GABA-A-R expression has been observed in neonatal optic nerve

astrocytes (Butt and Jennings, 1994). In the previous chapter, electrophysiological recordings, which was done on P0-P4 RONs, showed that axonal injury is largely mediated by glutamate through over-activation of NMDA-Rs, and there were a number of neurotransmitters other than glutamate that have effects on neonatal white matter. GABA or glycine was shown to have a reversible action on the neonatal RONs. These results are consistent with a previous study on P7-P15 RONs and revealed that neurotransmitters (non-glutamate)-mediated injury in developing white matter (Constantinou and Fern, 2009). This is the first study to show the effects of these neurotransmitters in P0 RONs during OGD.

In this chapter, electron microscopy was used to illustrate the ultra-structural changes to the axons as well as to the glia evoked by OGD, and the role of blockers in protecting the immature white matter structures. High power fields were collected blindly from cross-section ultra-micrographs. In addition, immuno-staining for the expression of the NR1 subunit of NMDA-Rs in neonatal white matter axons and glia was done.

Ultra-structural analysis of P0 RONs following a standard period of 90-min control, OGD or OGD + receptor antagonist

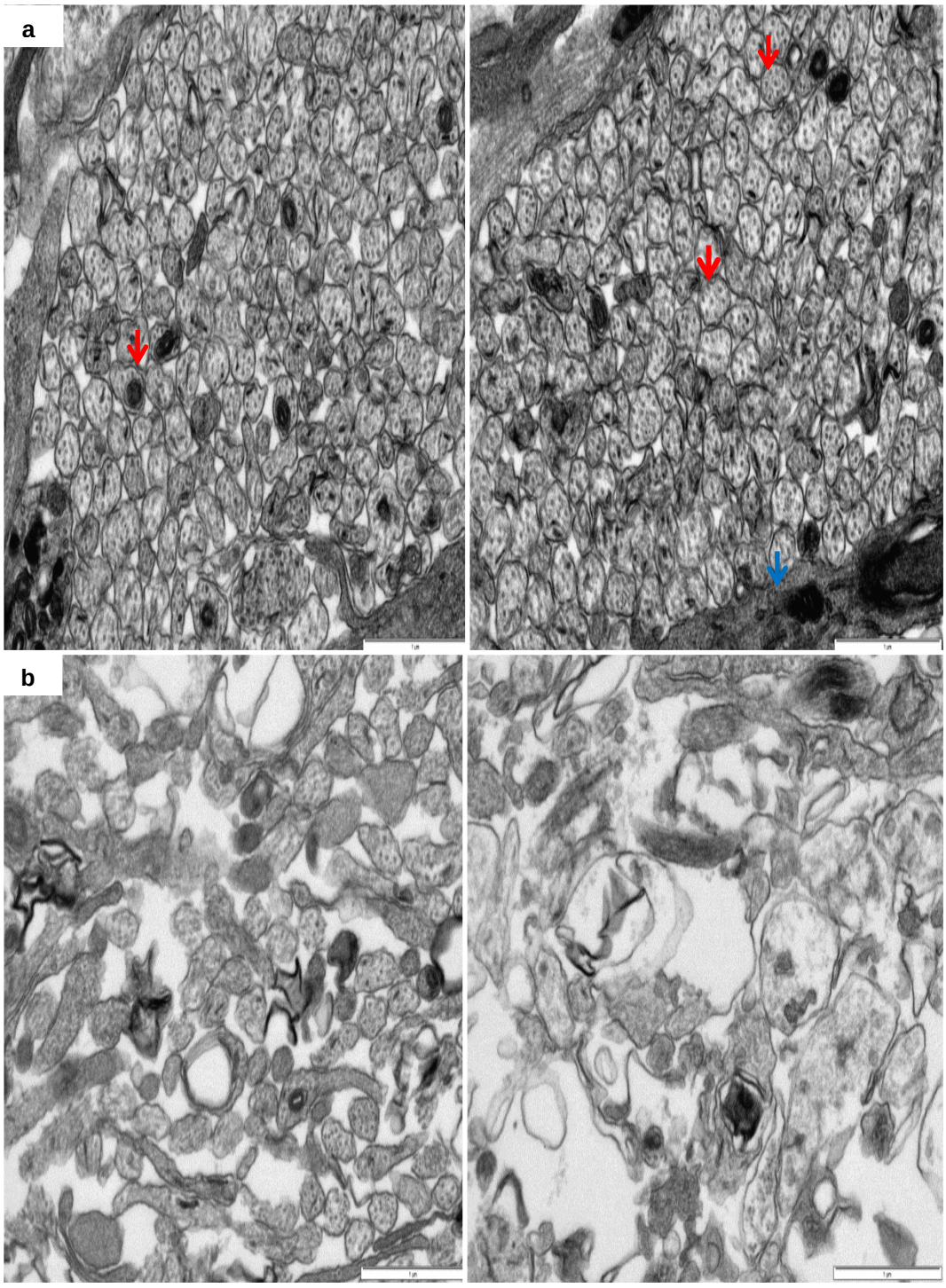
Controls

P0 RONs was analysed in cross-sections; axons were readily identified by the presence of axolemma, microtubules and a cylindrical structure. Axons were generally found in clusters embedded within fields of less electron-dense and fewer cylindrical processes that either contained few tubular or filamentous elements, or glial filaments, identifying them as astrocyte processes *(see Fig 52, a).* Astrocytes and occasional glioblasts populated the underlying intermediate zone where axons are observed in the cross-sections of the electron-microscope with an axon diameter of ≤ 500 nm *(see Fig 53, a).* Astrocytes contain obvious mitochondria, endoplasmic reticulum, Golgi apparatus and glycogen particles *(see Fig 53, a).* Blinded counting of axonal density showed a high number of identifiable axons in control nerves (mean= 11 ± 0.737axons/μ^2; n=1949 axons/13 sections form 3 nerves). Viability scoring showed that the majority of axons have a score of "3", which correlates to no pathology (mean= 2.9 ± 0.09; n=1949/12 sections). Viability scoring of glia in P0 RONs showed that the majority of glia also had a score of "3" (mean= 2.9 ± 0.05; n=24/16 sections).

OGD

Axon injury in nerves exposed to 90 min OGD was highly variable, with some axons showing axoplasmic and mitochondria swelling, the presence of flocculent debris in the axon cylinder, and loss of microtubules *(see Fig 52, b).* Glia injury during 90 min OGD was also significant. The cells appeared swollen and showed severely disrupted processes, and the membrane integrity was lost in many cases *(see Fig 53, b).* Blinded counting of axonal density showed a significantly lower number of identifiable axons following OGD (mean= 2.7 ± 0.74 axons/μ^2; n=300 axons/7 sections in 3 nerves; $P<0.0001$ vs control perfused RONs) *(see Fig 52 c & d).* Blinded axonal viability scores were significantly lower in identifiable axons following OGD; most scored 1.5 (mean= 1.7 ± 0.31; $P<0.0001$ vs control perfused RONs) *(see Fig 52 c & e).* Blinded assessment of astrocyte injury following OGD resulted in an extremely low score of “1” in many cells (mean= 1.0 ± .018; n=30/13 sections, 3 nerves; $P<0.001$ vs control perfused RONs) *(see Fig 53 a, b & c).*

Control & OGD



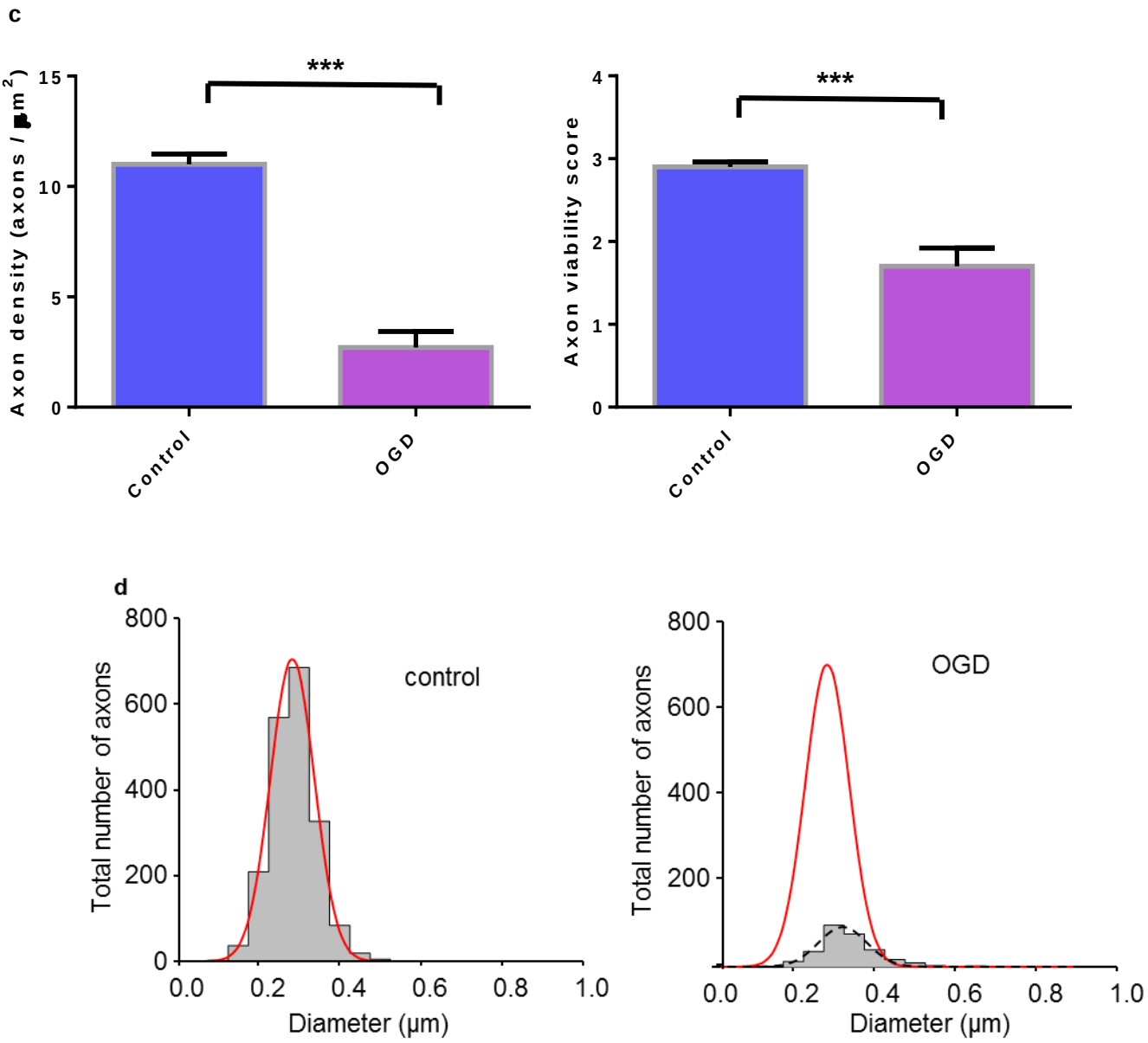
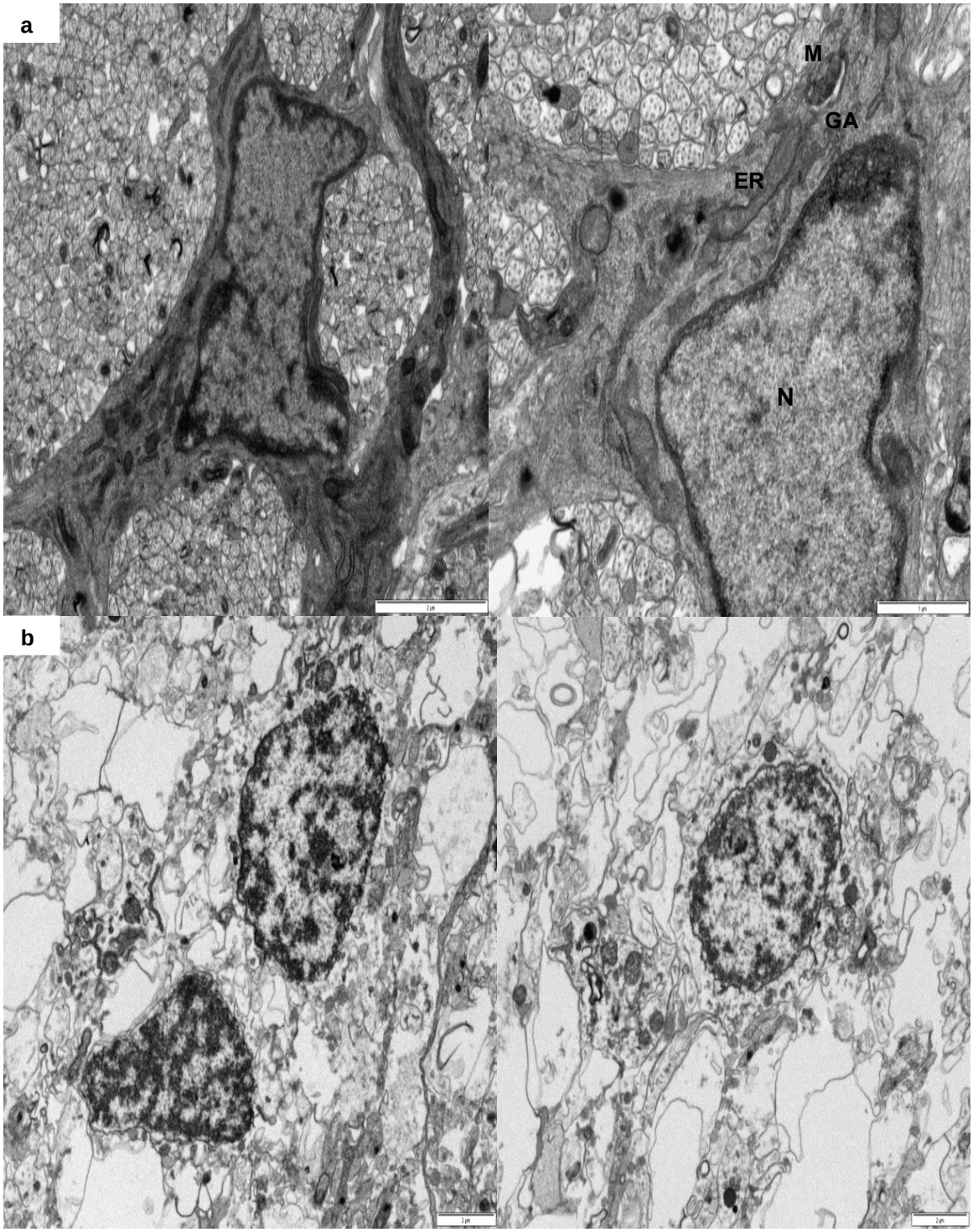


Figure 52- Pre-myelinated central axons following control conditions and OGD.

a, Normal axons (red arrows) with intact axolemma, neurofilaments (dark spots), microtubules and mitochondria. Astrocyte processes (blue arrow) are also seen in the image. **b,** OGD induced axonal injury: Axonal swelling with degeneration and loss of axolemma, loss of microtubules, swollen mitochondria and the cytoplasm filled with dendritic swelling. **c,** OGD induced significant damage to the axonal density and axonal viability score; $^{***}P < 0.0001$ vs control . In controls, the majority of axons had a score of **"3"**, correlating to no pathology. Blinded axon viability score were significantly lower in identifiable axons following OGD. Error bars represent SEM. **d,** Total number of axons during control (Gaussian equation is applied to the figure (**d**) (red curve is control and is added to the all figures below); area=98.8, mean of axonal diameter= 0.285, σ^2= 0.003), and OGD (black dots); area= 13.8, mean of axonal diameter=0.323, σ^2= 0.004) respectively. Scale bar = 1 μm.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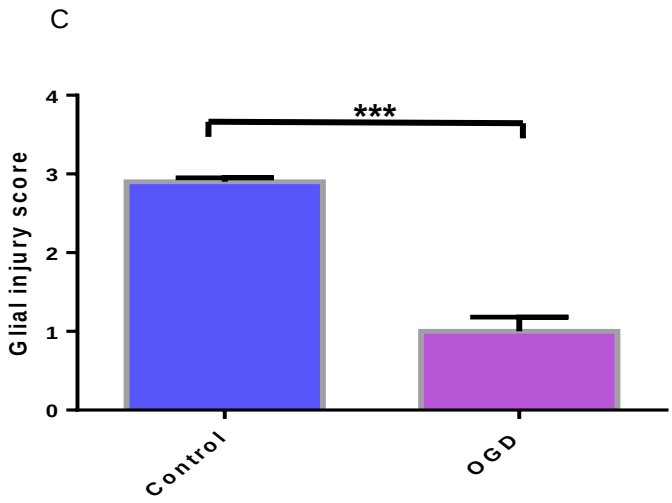


Figure 53- Glia of pre-myelinated RON during control and OGD.

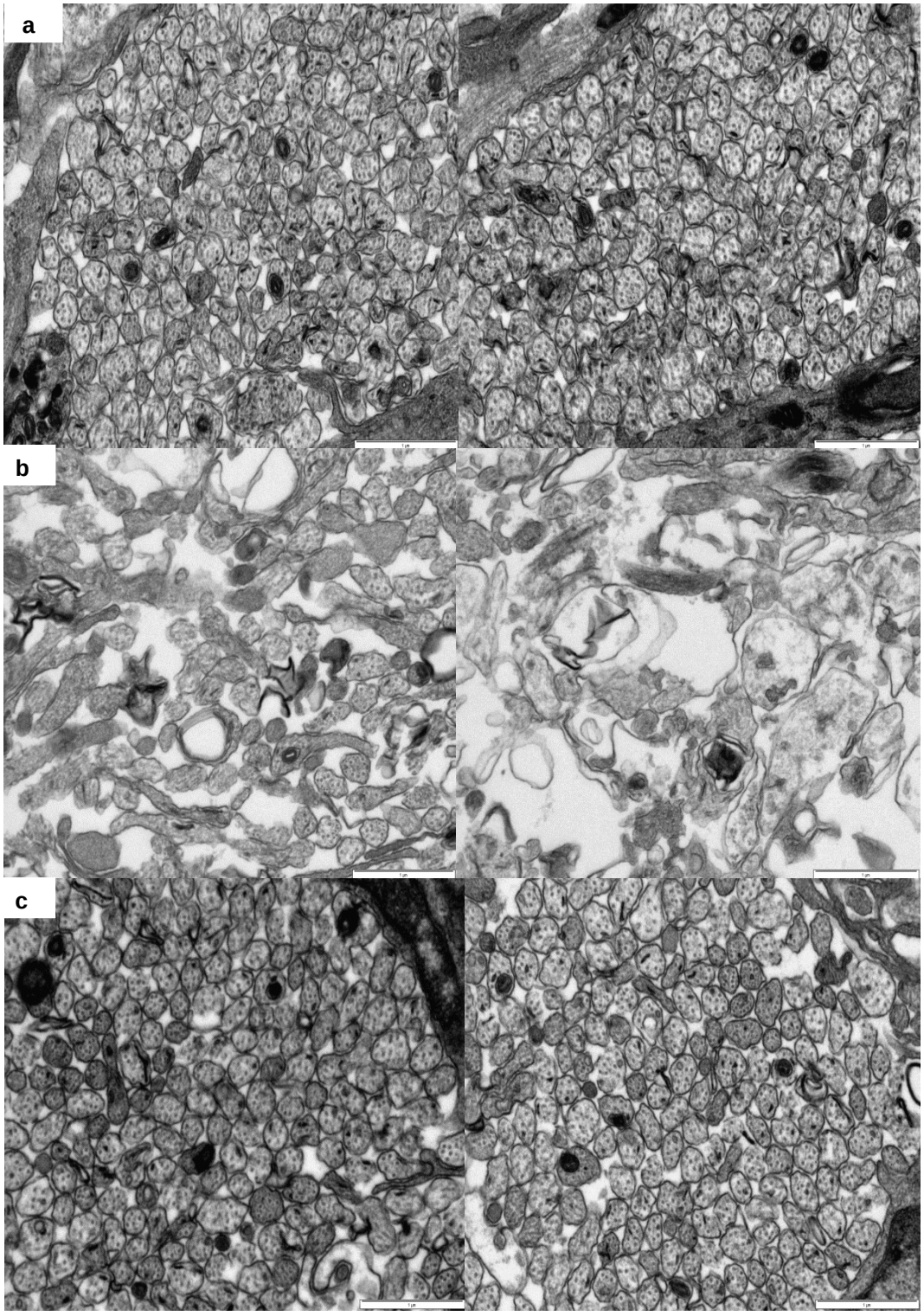
a, Normal astrocytes lined with cell membrane (red arrow); containing mitochondria (M), Golgi apparatus (GA), endoplasmic reticulum (ER), glycogen particles (GP) and nucleus (N). **b,** OGD induced glial injury: Astrocyte swelling with damage of membrane integrity (red arrow), organelles are in continuity with the expanded extracellular space, abnormal collections with altered tubule-vascular structures. **c,** Astrocytes show high levels of injury following OGD in compare to control; $^{***}P< 0.0001$. Error bars represent SEM. Scale bar = 2 µm.

OGD + 2µM strychnine

GlyR block is protective against OGD (90 min)-induced disruption of axons and glia structures. The normal structures of the axons and astrocytes were significantly preserved in post-OGD performed in the presence of 2µM strychnine *(see Fig 54 & 55).* The number of identifiable axons following OGD was low. This effect is significantly reduced by a GlyR block (mean= 7.75 ± 0.67 axons/μ^2; n=853/8 sections, 3 nerves; P<0.0001 vs OGD) *(see Fig 54, c, d & e).* Blinded axon viability scores were significantly lower in identifiable axons following OGD, an effect that it was prevented by the GlyR block (mean = 2.6 ± 0.02; P<0.0001 vs OGD) *(see Fig 54, d).* The distribution of axon viability scores in these three conditions (control, OGD & OGD + 2µM strychnine) shows that GlyR block restores a more normal pattern of viability *(see Fig 54, e).*

GlyR block also protected the normal structures of astrocytes. Blinded assessment of astrocyte injury post-OGD performed in the presence of 2µM strychnine resulted in a significantly improved score, in contrast to the injury scores of astrocytes post-OGD (mean= 3 ± 0.01; n=15/14 sections, 3 nerves; P<0.0001 vs OGD) *(see Fig 55, c & d).*

OGD + 2μM strychnine



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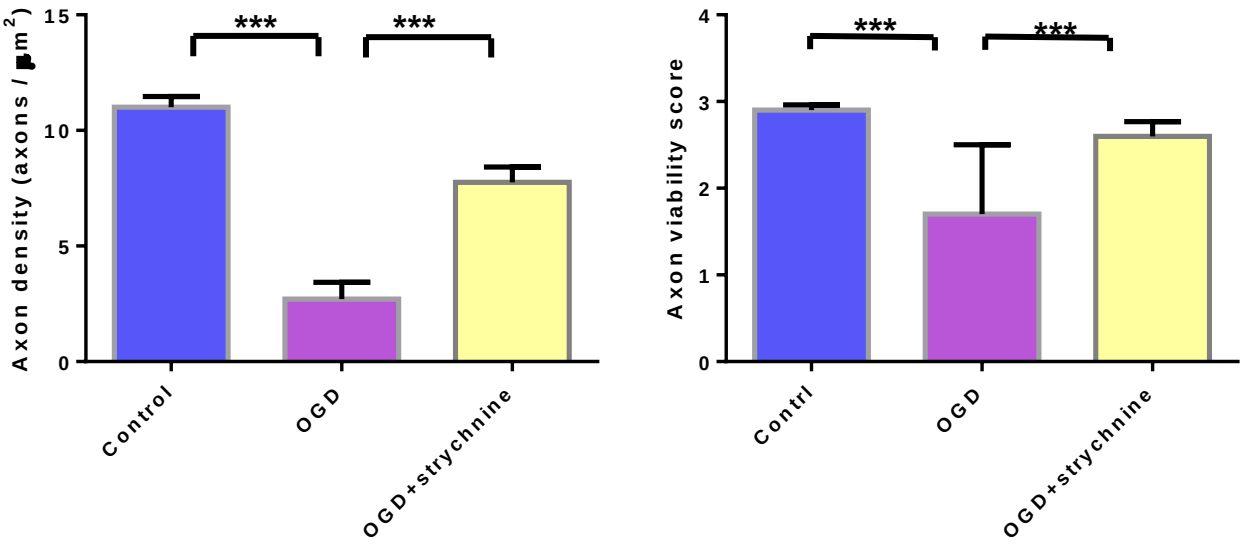

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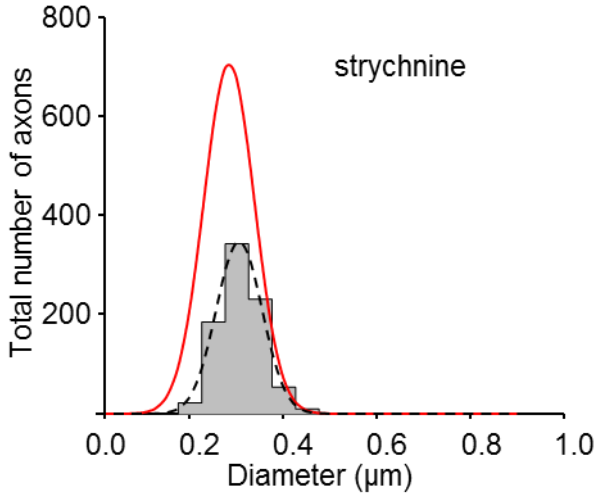
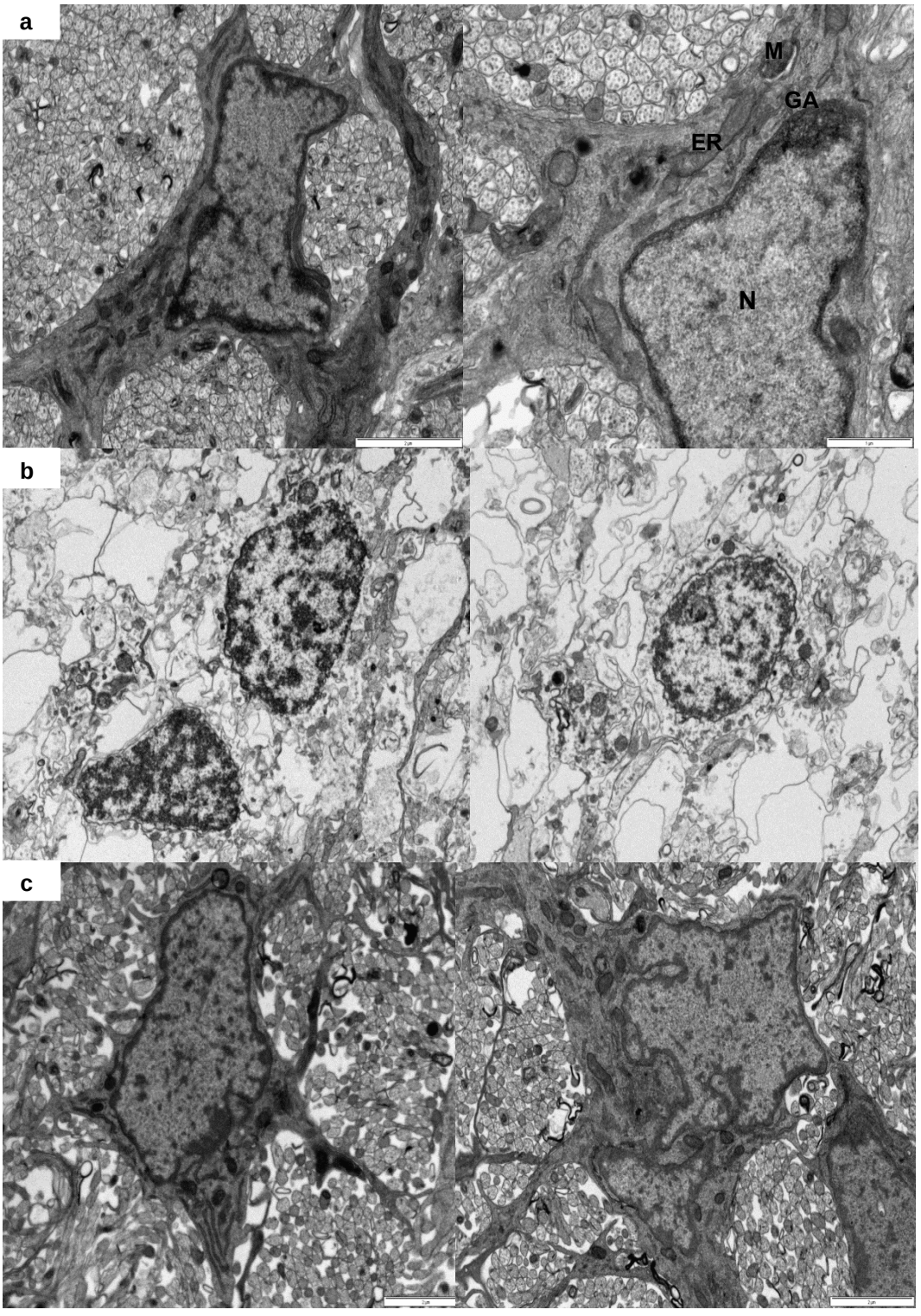


Figure 54- GlyR block strychnine (2µM) is protective against OGD- induced disruption of axon structure in P0 RONs.

a, Representative ultra-micrographs from control**. b,** OGD (90 min)-induced axonal injury. **c,** Representative ultra-micrographs from OGD + strychnine (90 min). d, Histogram summarizing the significance of OGD-induced axonal injury and the protective effect of strychnine; $^{***}P< 0.0001$. Error bars represent SEM. **e,** Total number of the axons during OGD co-applied with strychnine ((black dots); area =42.6, mean of axonal diameter =305, $6^2= 0.002$). Scale bar = 2 µm.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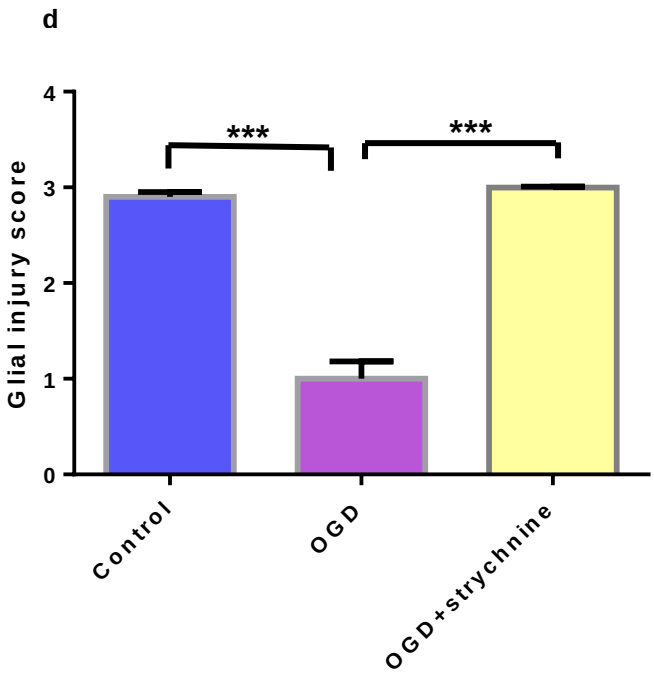


Figure 55- GlyR block strychnine (2μM) is protective against OGD-induced glial injury in P0 RONs.

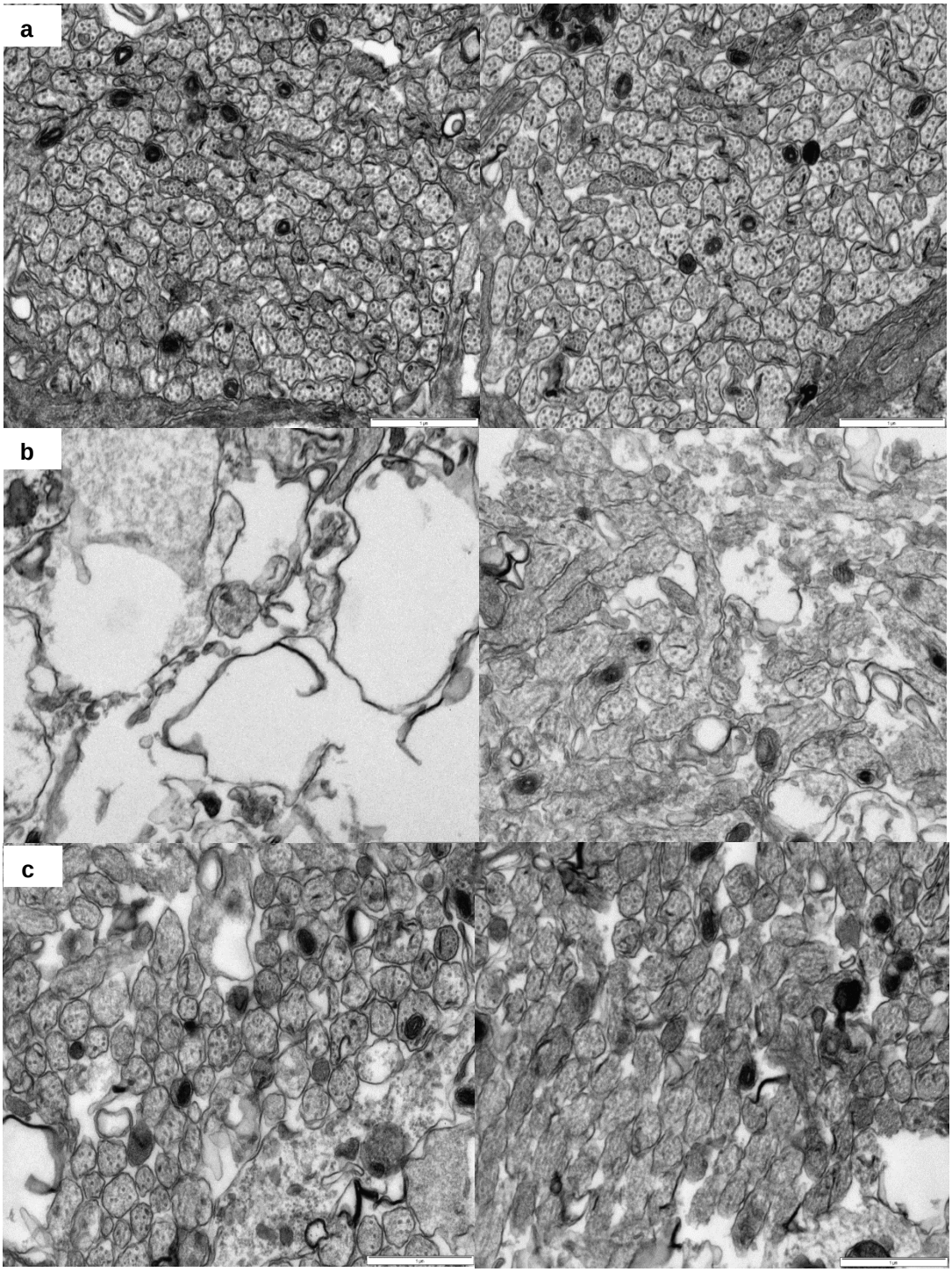
a, Representative ultra-micrographs from control**. b,** OGD (90 min)-induced glial injury. **c,** Representative ultra-micrographs from OGD + strychnine (90 min). **d,** Strychnine produced significant protective effect against OGD-induced glial injury; ***P< 0.0001. Error bars represent SEM. Scale bar = 2 μm.

OGD + 100µM picrotoxin

The number of identifiable axons following OGD was significantly increased by the $GABA_A$-R blocker picrotoxin (mean= 6.0 ± 1.19 axons/μ^2; n=300 axons/7 sections; P <0.0001 vs OGD). Blinded assessment of axonal injury post-OGD produced an extremely low score, an effect that was not highly significantly restored post-OGD in the presence of 100µM picrotoxin (mean= 1.9 ± 0.10; P<0.01 vs OGD) *(see Fig 56, c, d & e).*

A $GABA_A$-R block preserves the normal structures of astrocytes during exposure to OGD-induced injury. The astrocyte viability score post-OGD was restored post-OGD in the presence of 100µM picrotoxin (mean= 1.7 ± 0.20; n=30/13 sections; P<0.0001 vs OGD) *(see Fig. 57, c & d).*

OGD + 100μM picrotoxin



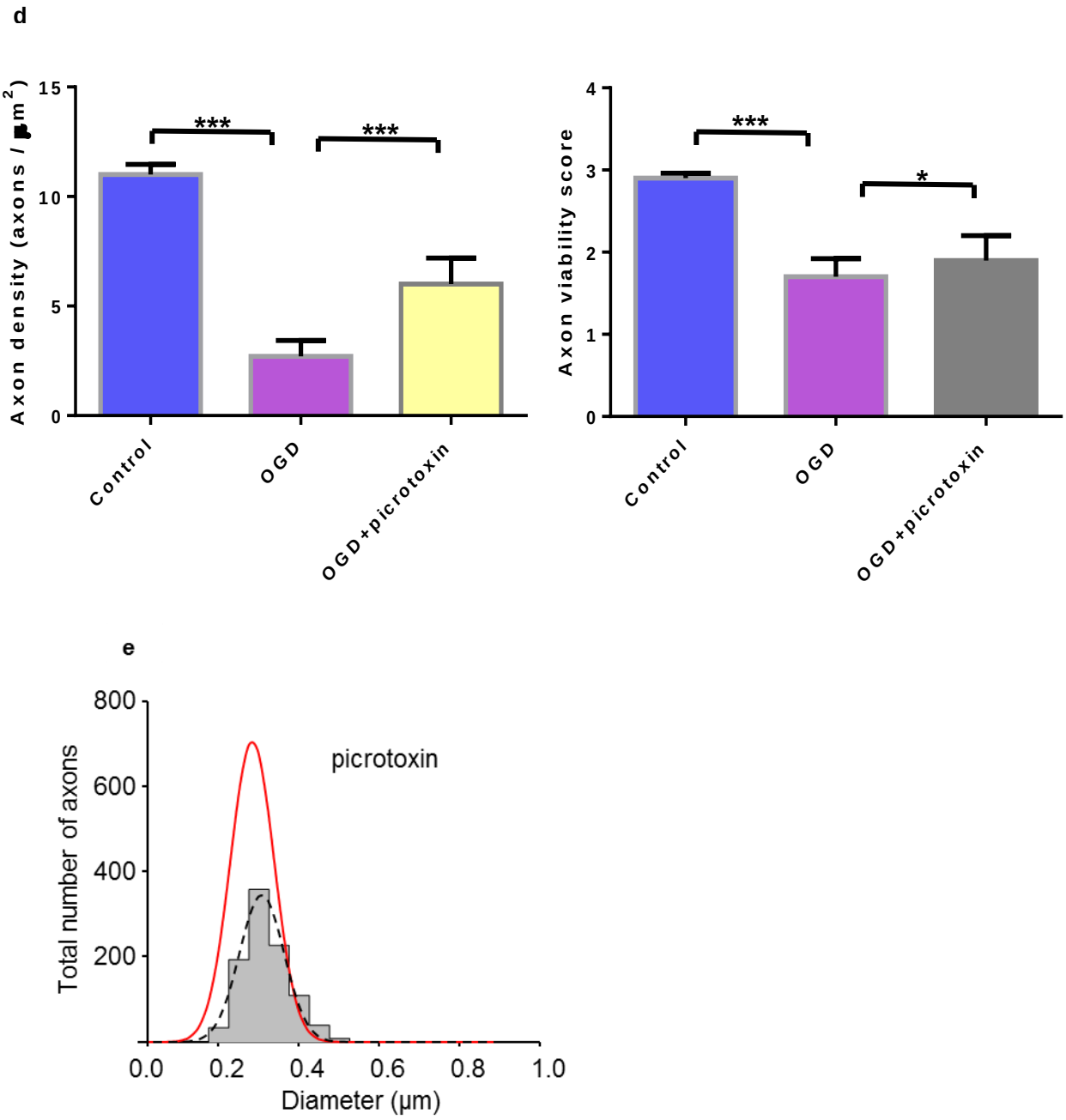
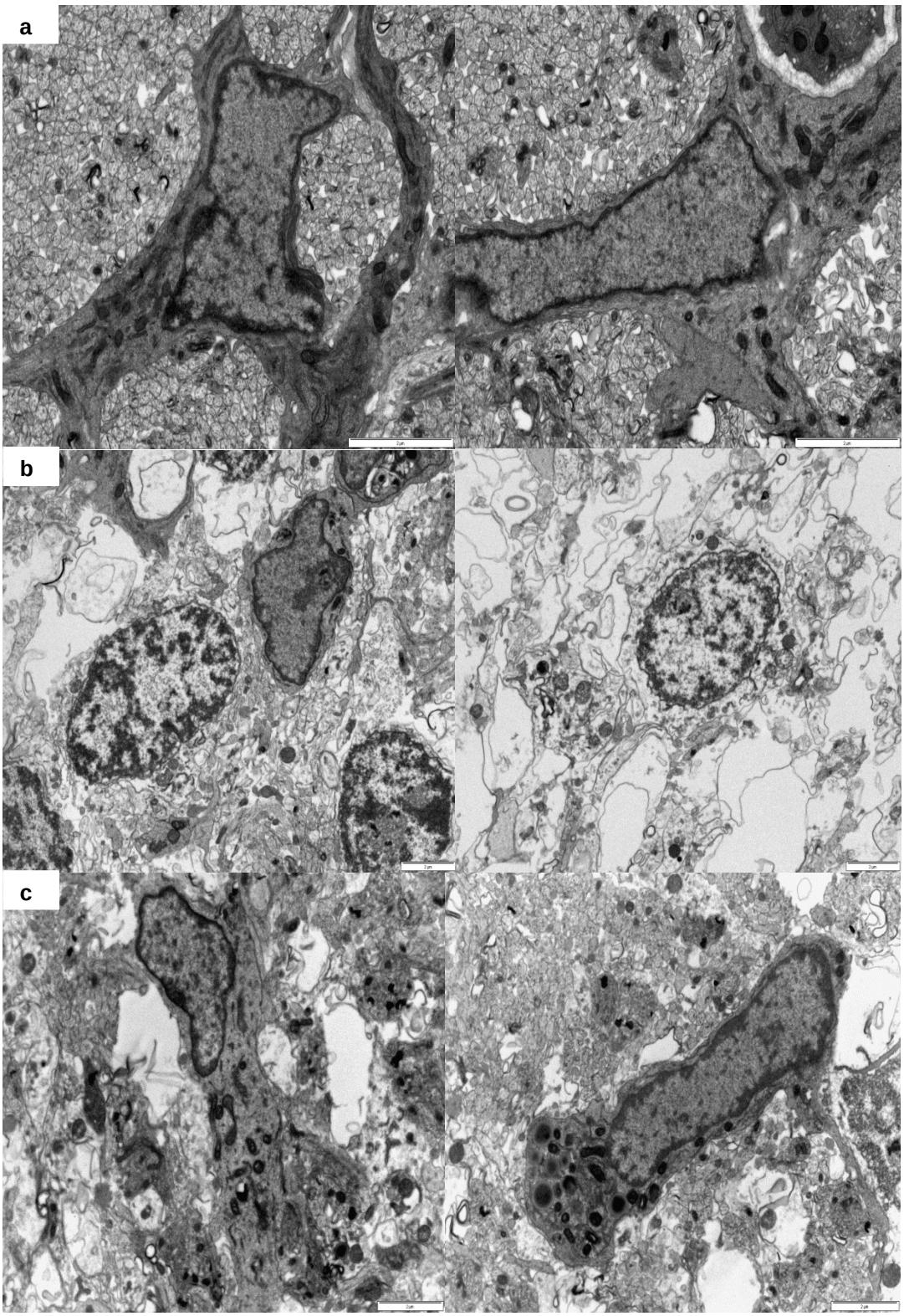


Figure 56- GABA-R block picrotoxin (100µM) is protective against OGD-induced axonal injury in P0 RONs.

a, Representative ultra-micrographs of axons from control. **b,** OGD (90 min)-induced axonal injury. **c,** Representative ultra-micrographs of axons from OGD + picrotoxin (90 min). **d,** Picrotoxin produced significant protection against OGD-induced axonal density damage and less significant effect of protection against OGD-induced axonal injury; $^{***}P<0.0001$ & $^{*}P<0.01$ respectively. Error bars represent SEM. **e,** Total number of the axons during OGD co-applied with strychnine (black dots); area= 47.7, mean of axonal diameter= 0.308, δ^2=0.003). Scale bar= 2 µm.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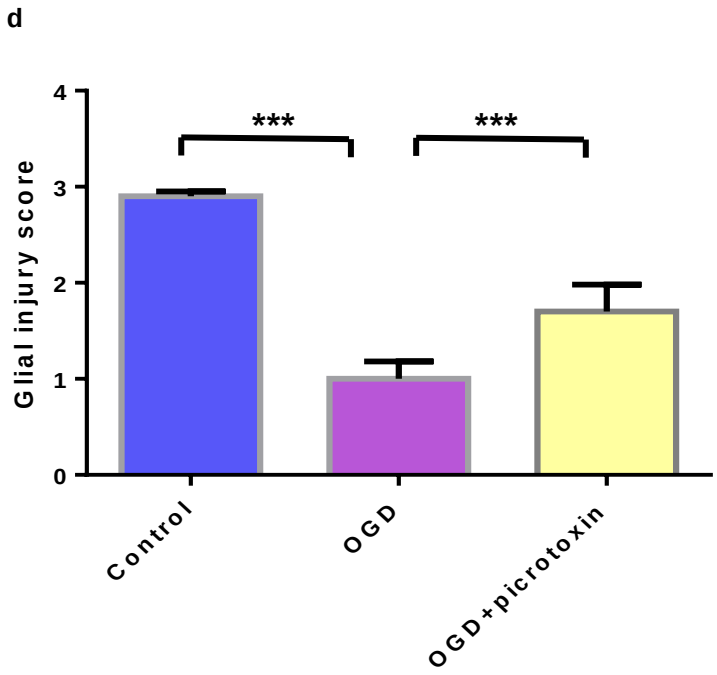


Figure 57- GABA-R block picrotoxin (100μM) is protective against OGD-induced glial injury in P0 RONs.

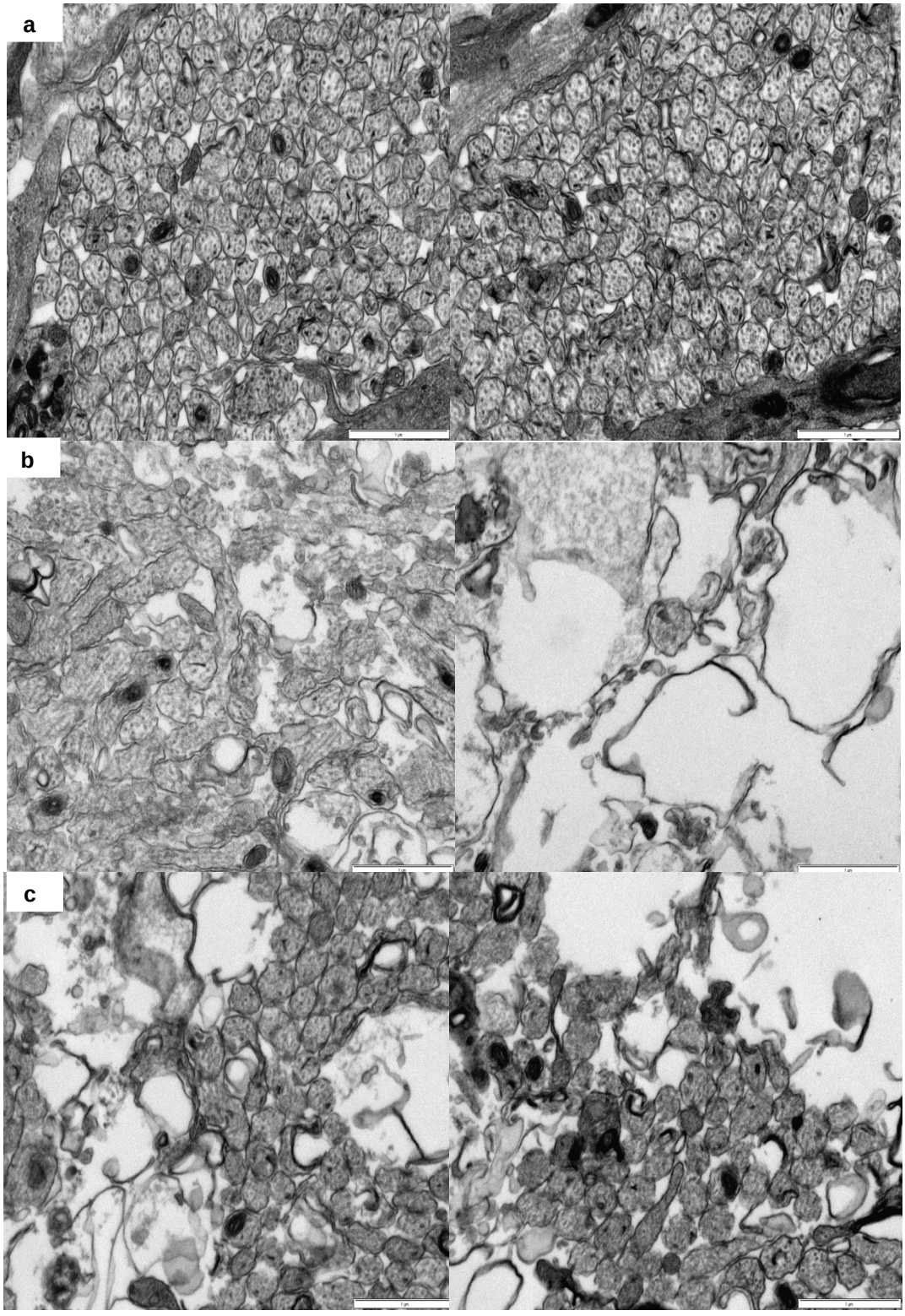
a, Representative normal glia from ultra-micrographs of glia during control. **b,** OGD (90 min)-induced glial injury. **c,** Representative ultra-micrographs from OGD + picrotoxin (90 min). **d,** Picrotoxin produced significant of protection against OGD-induced glial injury; $^{***}P< 0.0001$. Error bars represent SEM. Scale bar= 2 μm.

OGD + 10µM propranolol hydrochloride/10µM phentolamine hydrochloride

The number of identifiable axons following OGD performed in the presence of combined (α, and β)-AR block was (mean= 4.6 ± 0.146 axons/μ^2; P<0.001; n=917/13 sections) *(see Fig 58, c, d & e)*, in contrast to the identifiable axons following OGD. Blinded assessment of axonal injury scoring post-OGD performed in the presence of the combined-AR block was not significantly different from OGD alone (mean= 1.6 ± 0.07; n=9/13 sections) *(see Fig 58 c & d).*

The effect of the combined- AR block was not significant in protecting astrocytes from OGD-induced injury (mean= 1.3 ± 1.2; n=18/14 sections), these data were not significantly different to OGD (mean= 1.0 ± 1.09; n=30/13 sections) but showed a similar trend to the functional data *(see Fig 59 c & d).*

OGD + 10μM propranolol hydrochloride/10μM phentolamine hydrochloride



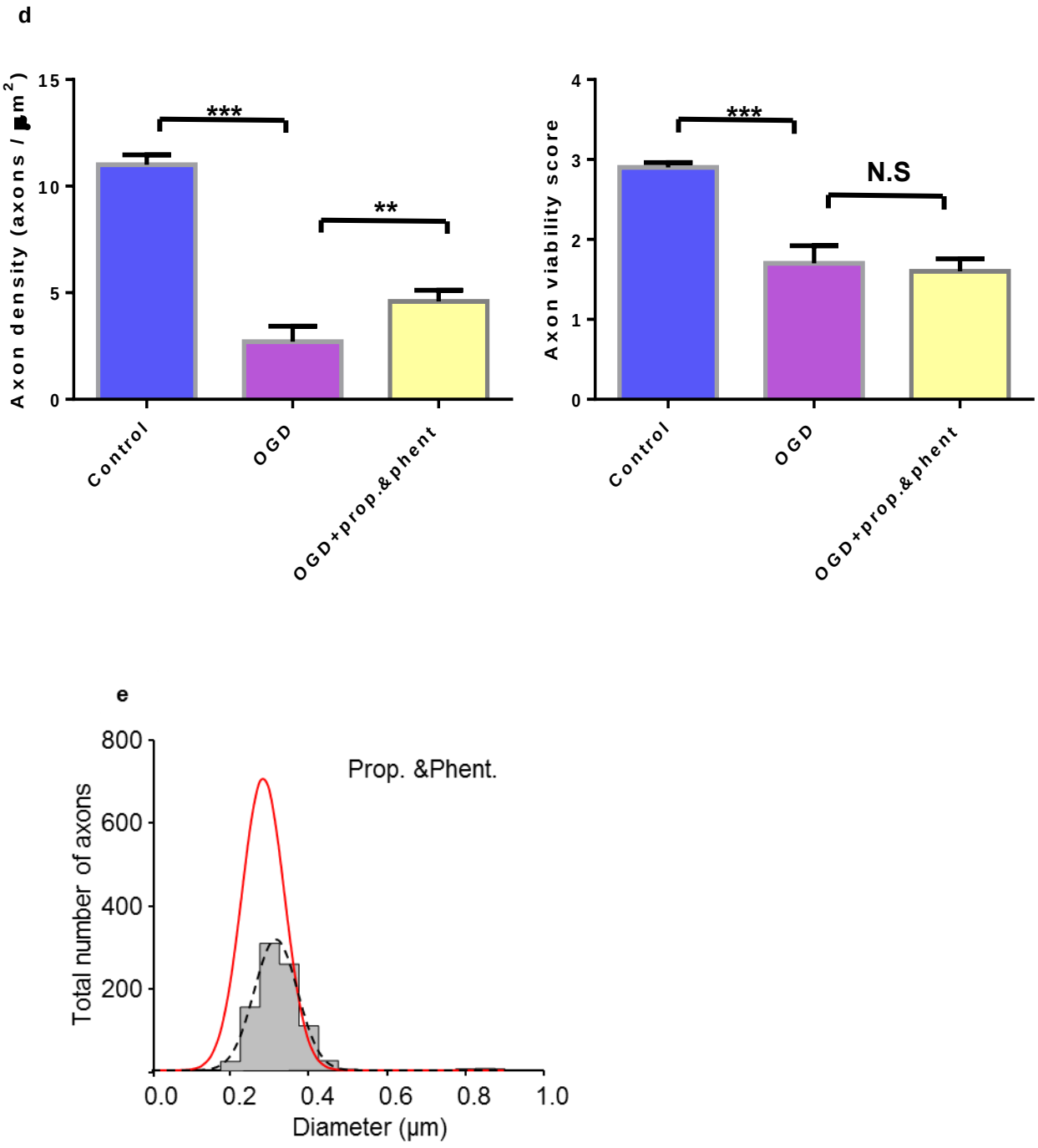
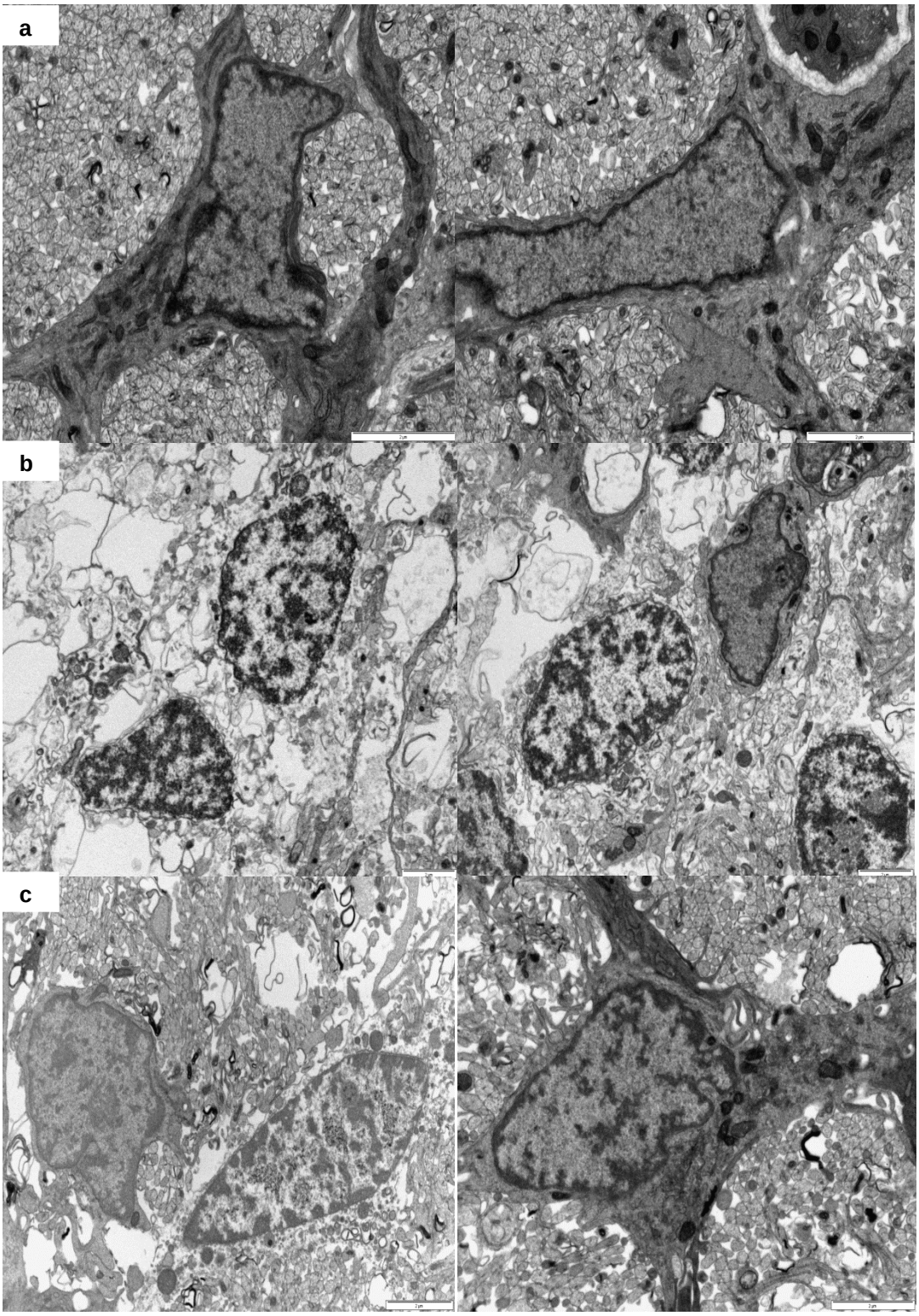


Figure 58- Combined AR block propranolol (10μM) & phentolamine (10μM) is not protective against OGD-induced axonal injury in P0 RONs.

a, Representative ultra-micrographs of axons from control. **b,** OGD (90 min)-induced axonal injury. **c,** Representative ultra-micrographs of axons from OGD + combined antagonists (90 min). **d,** Histograms showed that, combined antagonist produced some protection against OGD-induced damage to axonal density; **P<0.001; and non-significant effect against axonal viability score. Error bars represent SEM. **e,** Total number of the axons during OGD co-applied with combined antagonists (black dots); area= 49.8, mean of axonal diameter=0.325, 6^2= 0.003). Scale bar= 2 μm.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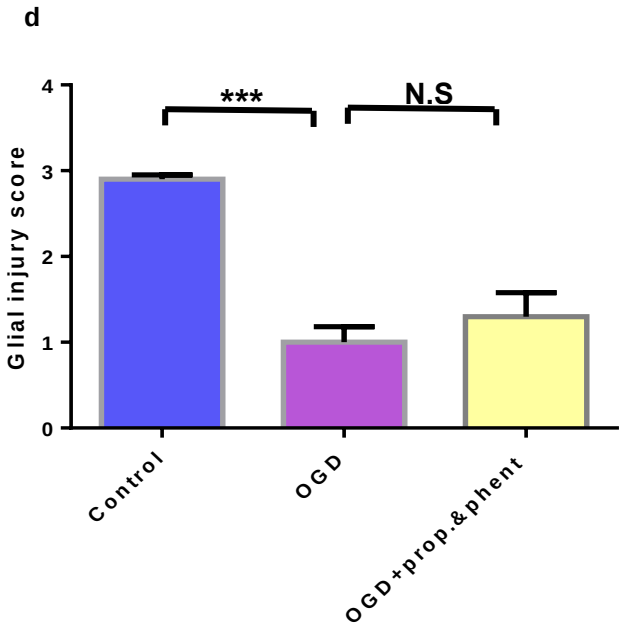


Figure 59- Combined AR block propranolol (10μM) & phentolamine (10μM) produced very mild protection against OGD-induced glial injury in P0 RONs.

a, Representative normal glia from ultra-micrographs of glia during control. **b,** OGD (90 min)-induced glial injury. **c,** Representative ultra-micrographs from OGD + combined (90 min). **d,** combined antagonists produced mild protection against OGD-induced glial injury. Error bars represent SEM. Scale bar= 2 μm.

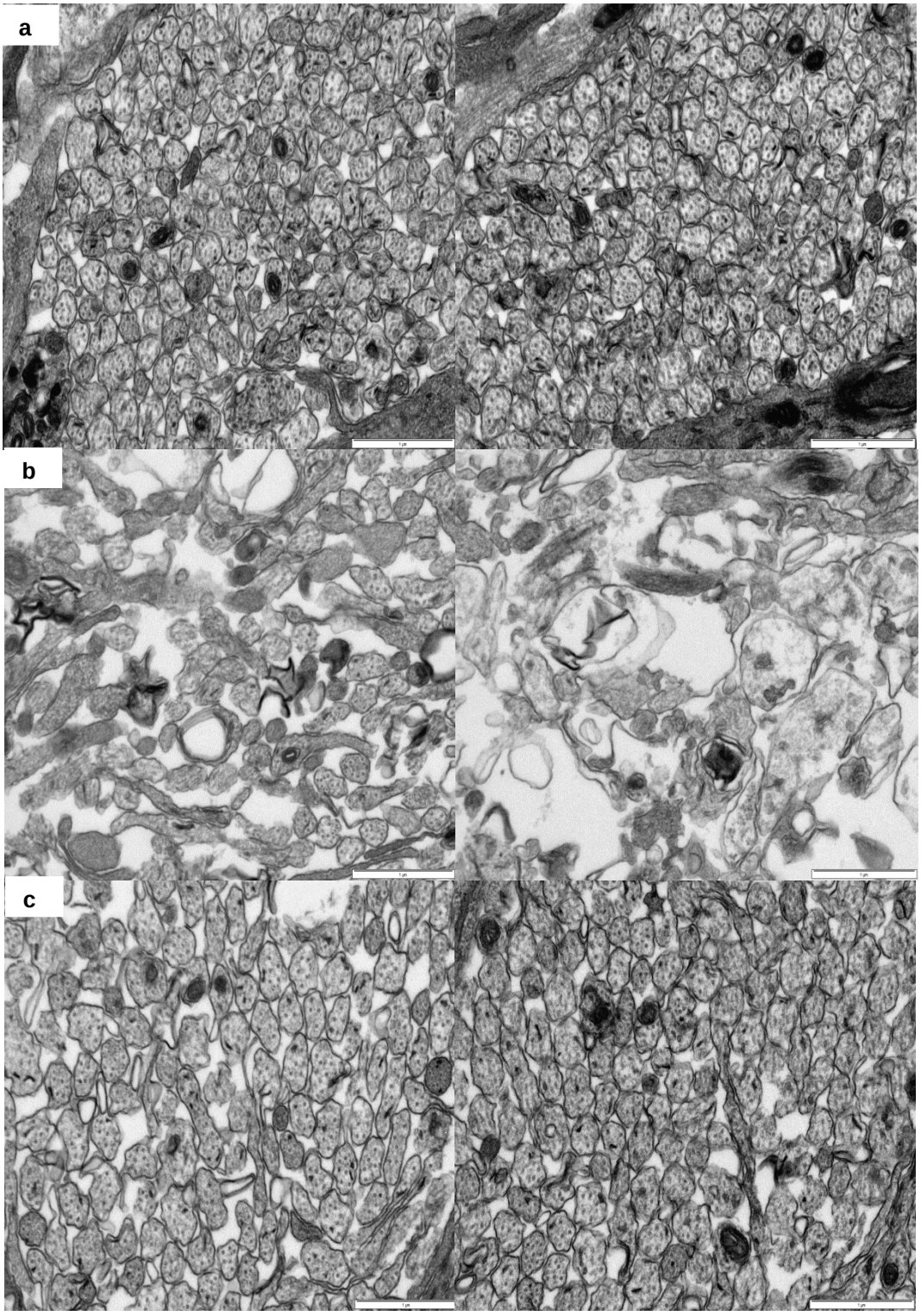
OGD + 10µM mecamylamine

nACh-R block was protective against the OGD (90 min)-induced disruption of axons and glia structures. The normal structures of the axons and astrocytes were significantly preserved in post-OGD performed in the presence of 10µM mecamylamine *(see Fig 60 & 61).* The number of identifiable axons following OGD performed in the presence of the nACh-R block was significantly higher in comparison to OGD alone (mean= 7 ± 2.6 axons/μ^2; n=1001/9 sections; P<0.0001 vs OGD). Blinded axon viability scores were significantly lower in identifiable axons following OGD, an effect that was prevented by the nACh-R block (mean= 2.5 ± 0.7; n=1001/9 sections; P<0.001 vs OGD); the distribution of axon viability shows that an nACh-R block restores a more normal pattern of viability *(see Fig 60, c, d & e).*

nACh-R block preserves the normal structures of astrocytes. Blinded assessment of astrocyte injury post-OGD performed in the presence of 10µM mecamylamine produced a significantly improved score in comparison to that of astrocytes injury post-OGD which improved only slightly (mean= 3 ± 0.0; n=11/9 sections; P<0.0001 vs OGD) *(see Fig 61, c & d).*

It was found that $GABA_A$, glycine and nACh- receptor blockade was highly protective against 90 min OGD-induced injury in both axons and glia in P0 RONs; (P<0.0001 vs OGD), *(see Fig 62, 63 & 64).*

OGD + 10μM mecamylamine



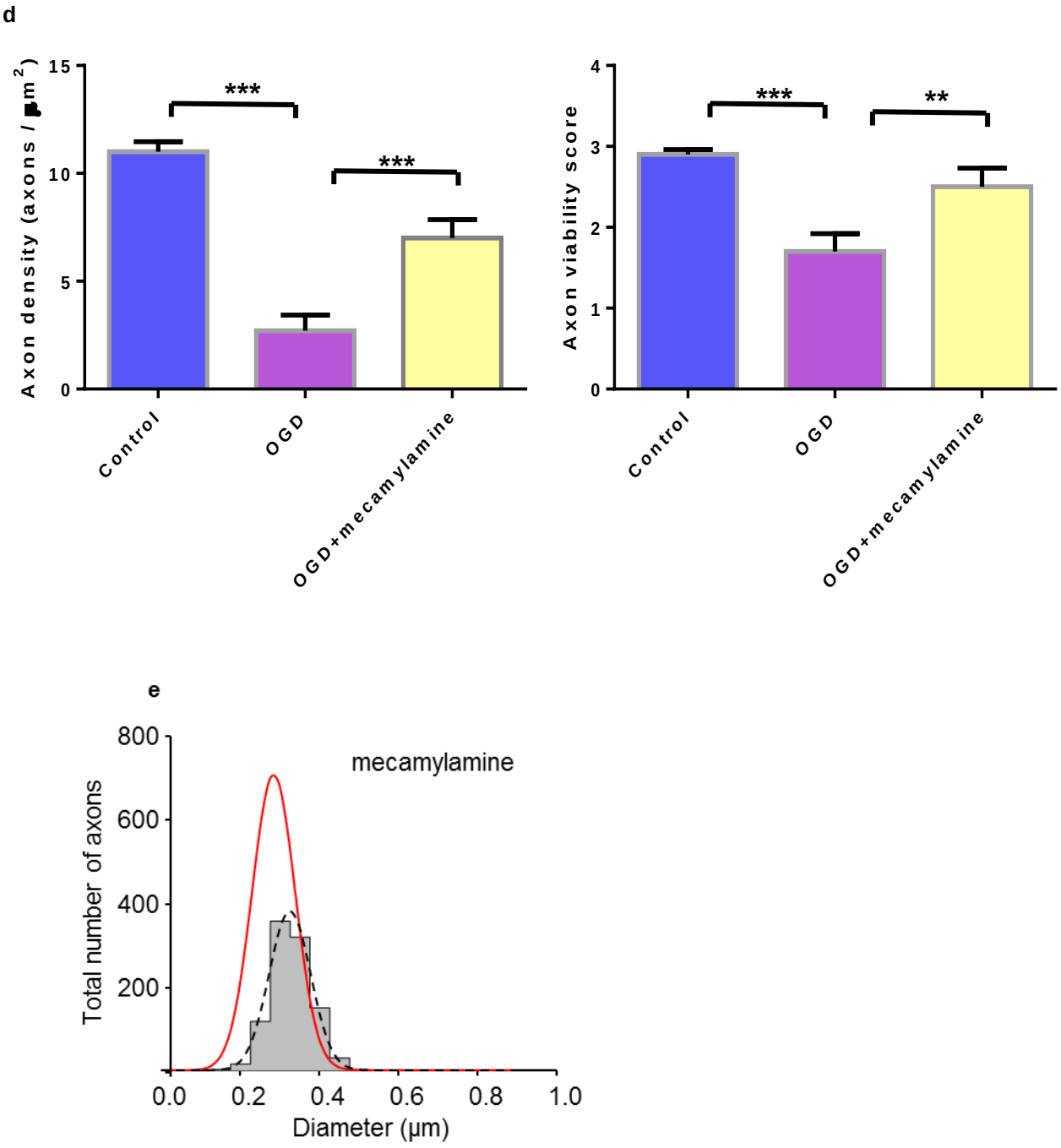
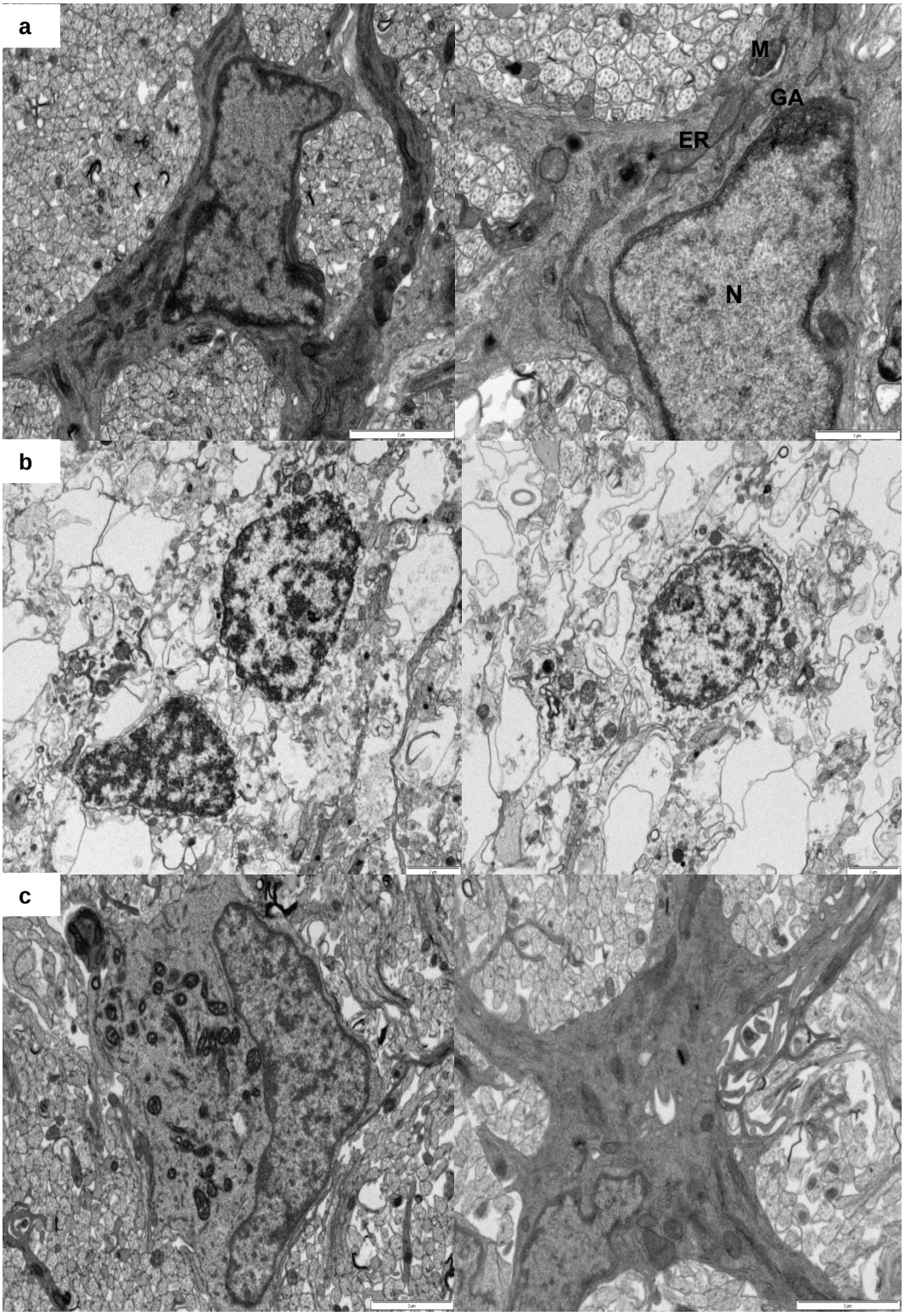


Figure 60- nACh-R block mecamylamine (10µM) is protective against OGD-induced axonal injury in P0 RONs.

a, Representative normal axons from control ultra-micrographs**. b,** OGD (90 min)-induced axonal injury.

c, Representative ultra-micrographs from OGD + mecamylamine (90 min). **d,** Histogram summarizing the significance of OGD-induced axonal injury and the protective effect of mecamylamine in restoring the identifiable axons and increasing the axons viability score. $^{***}P< 0.0001$ and $^{**}P<0.001$; respectively. Error bars represent SEM. **e,** Total number of the axons during OGD co-applied with mecamylamine (black dots); area= 49.8, mean of axonal diameter= 0.325, $6^2= 0.003$). Scale bar= 1µm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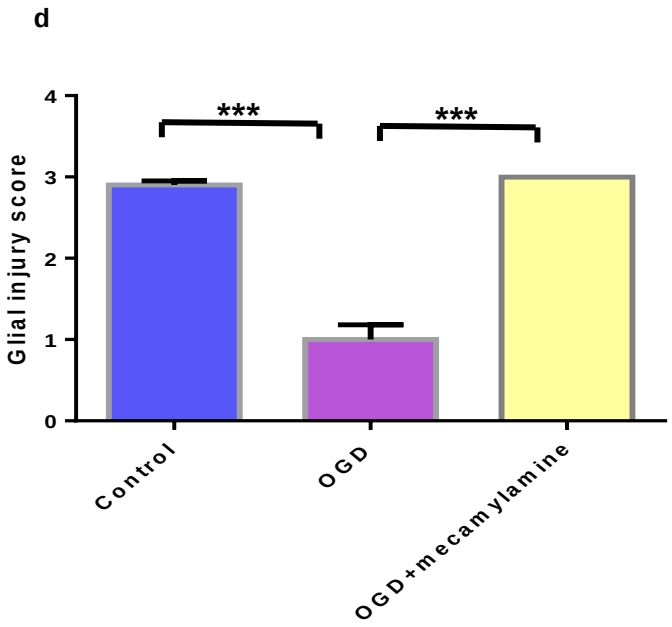

Figure 61- nACh-R block mecamylamine (10μM) is protective against OGD-induced glial injury in P0 RONs.

a, Representative normal glia from control ultra-micrographs**. b,** OGD (90 min)-induced glial injury. **c,** Representative ultra-micrographs from OGD + mecamylamine (90 min). **d,** Mecamylamine produced significant protective effect against OGD-induced glial injury; $^{***}P< 0.0001$. Error bars represent SEM. Scale bar= 2μm.

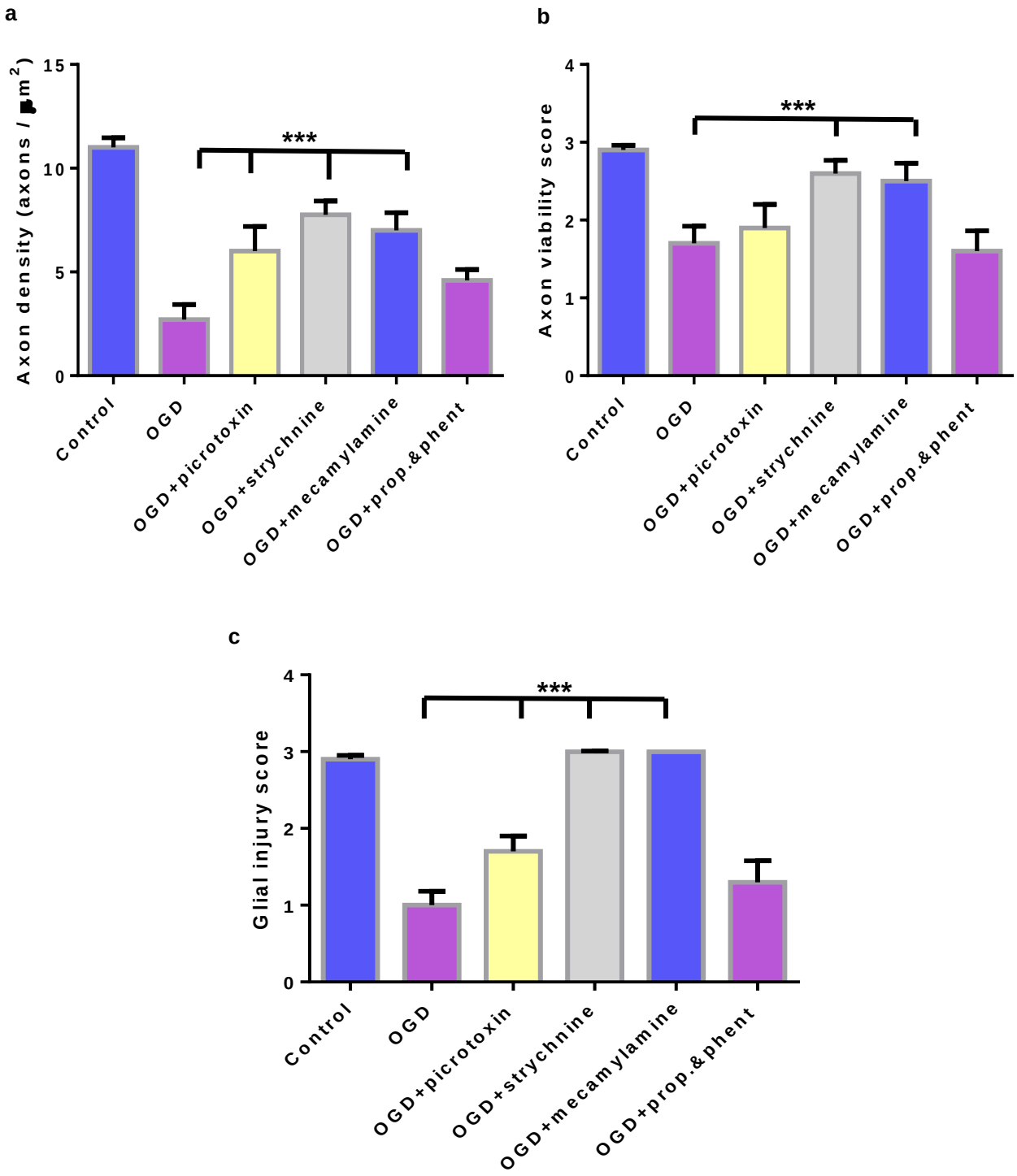

Figure 62- Histogram summarizing the effects of neurotransmitter blockade against standard period of (90 min) OGD-induced axonal and glial injury in P0 RONs.

a, It shows that, Gly, nACh and GABA receptors blockade are significantly protective against OGD-induced axonal damage. Strychnine, picrotoxin, and mecamylamine are significantly protective against OGD-induced axonal damage in P0 RONs; ***P<0.0001. **b,** Gly and nACh receptors blockade are significantly protective against OGD-induced axonal injury; ***P <0.0001. But GABA-R blockade has no significant effect on OGD-induced axonal injury. **c,** Gly, nACh and GABA receptors blockade are significantly protective against OGD-induced glial injury; ***P <0.0001. Error bars represent SEM.

Localisation of NR1 expression in P0 and P8 RONs

Immuno-staining protocol with mix mouse monoclonal NG2+ antibody showed the expression of NG2+ cells in P0 and P8 RONs *(see Fig 63).* O4 marker showed absence of O4 cells in P0 RONs *(see Fig 63).* The NR1 subunit of the NMDA-R co-localised primarily with axonal markers NF-H (+) in P0 RONs *(see Fig 65),* while few of the NR1 subunits were co-localised in the GFAP+ astrocytes of P0 RONs *(see Fig 64),* and they were not co-localised on the NG2+ cells of P0 RONs *(see Fig 66).* NR1subunits were co-localised on NG2+ cells in the P8 RONs *(see Fig 66)* and on the axonal marker NF-H (+) in P8 RONs *(see Fig 67).* Controls showed the absence of immuno-staining when the NR1 primary antibody was omitted from the protocol. This part of work was done in collaboration with a Masters student (Narasimha Beeraka).

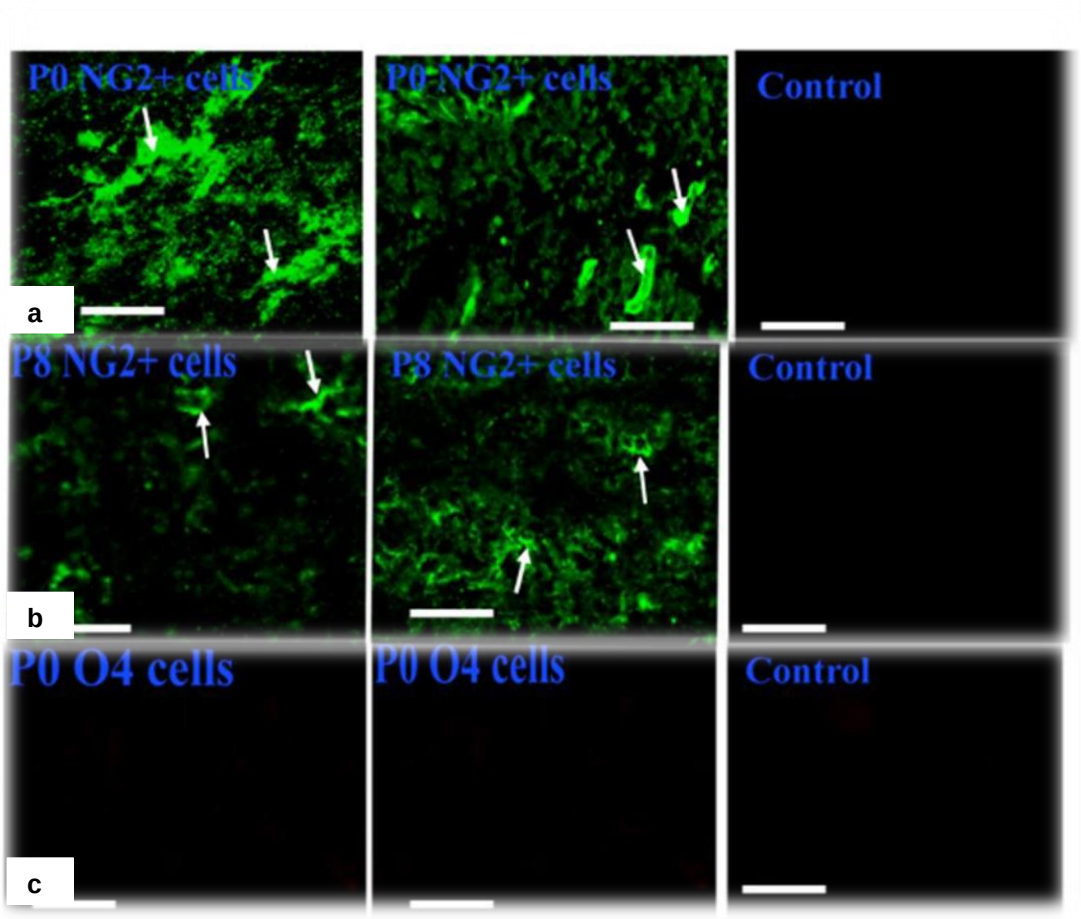

Figure 63- Expression of NG2^{+} cells on P0 and P8 RONs, and absence of O4 cells.

a, and b, NG2+ cells in P0 RON and P8 RON with mix mouse monoclonal NG2+ antibody showed in the left and middle panels with green staining. **c,** O4 marker showed absence of O4 cells in P0 RON in the left and middle panels. Controls: Primary antibody omission controls were performed for P0 and P8 NG2+ cells in which no NG2+ cells staining observed in right panels.

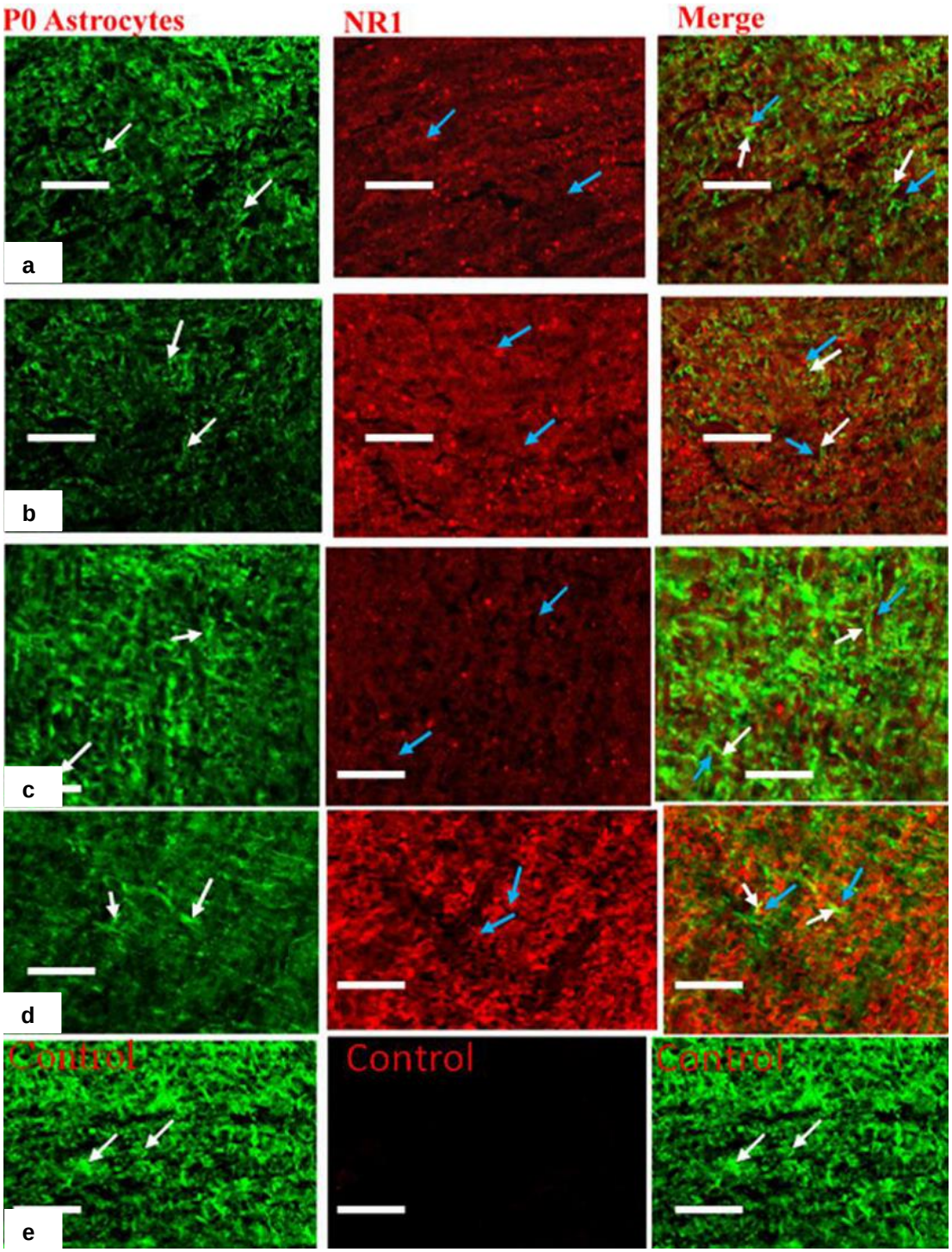

Figure 64- NRI expression in P0 RON astrocytes.

A, and d, left: co-staining for GFAP+ astrocytes (green), middle: NR1 (red) protein in P0 RON, and right: co-localization with GFAP+ astrocytes at P0. **e,** control: Primary antibody omission controls showed no NR1 protein staining in middle red panel. (GFAP+ astrocytes (green) represented by (white arrows) and NR1 (red) expression by (blue arrows).

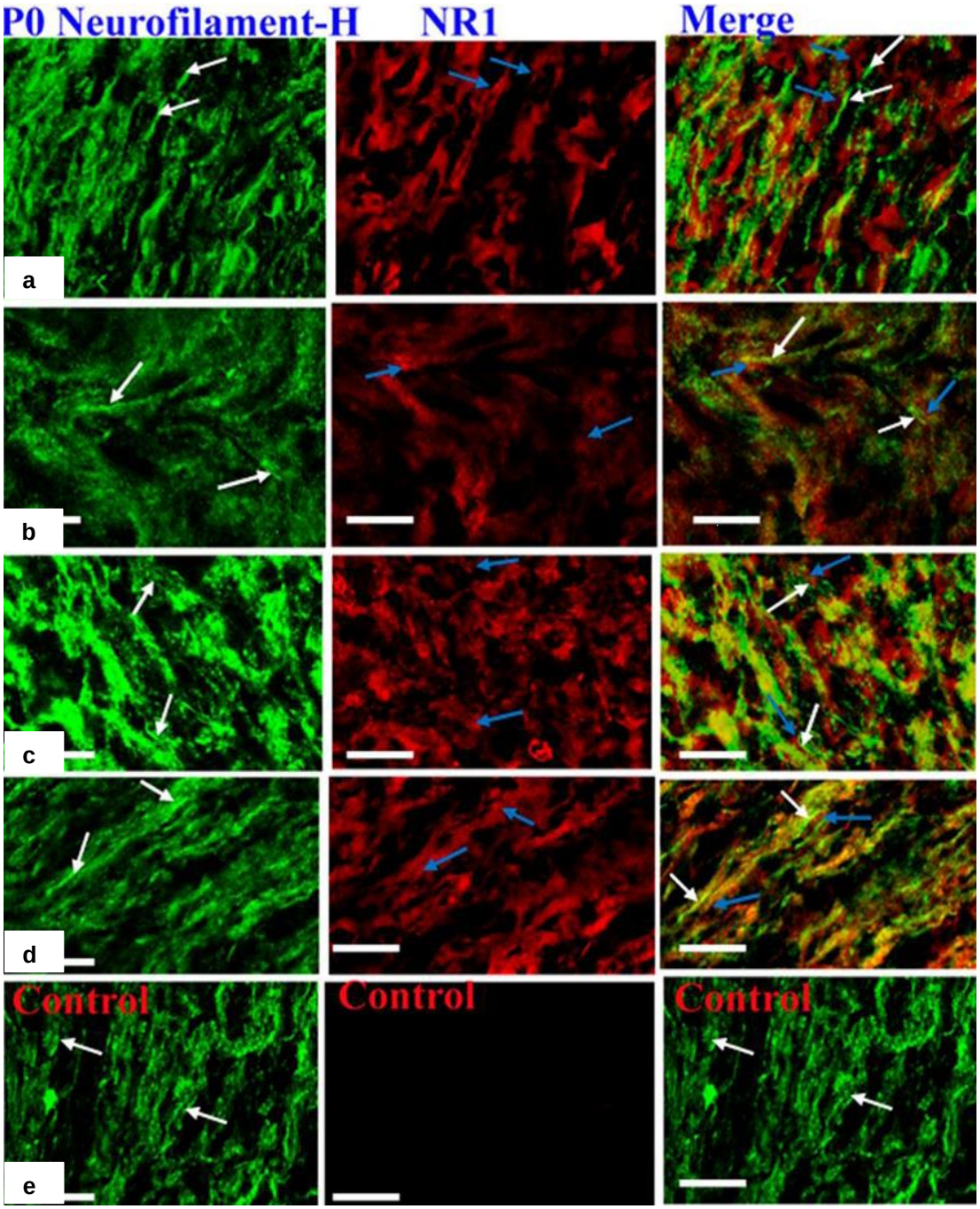

Figure 65- NRI expression in P0 RON axons.

a, b, c, and d, left: co-staining for NH-F (+) (green), middle: NR1 (red) protein in P0 RON, and right: co-localization with NF-H (+) axons at P0. **e,** control: Primary antibody omission controls showed no NR1 protein staining in middle red panel. (NF-H (+) axons (green) represented by (white arrows) and NR1 (red) expression by (blue arrows).

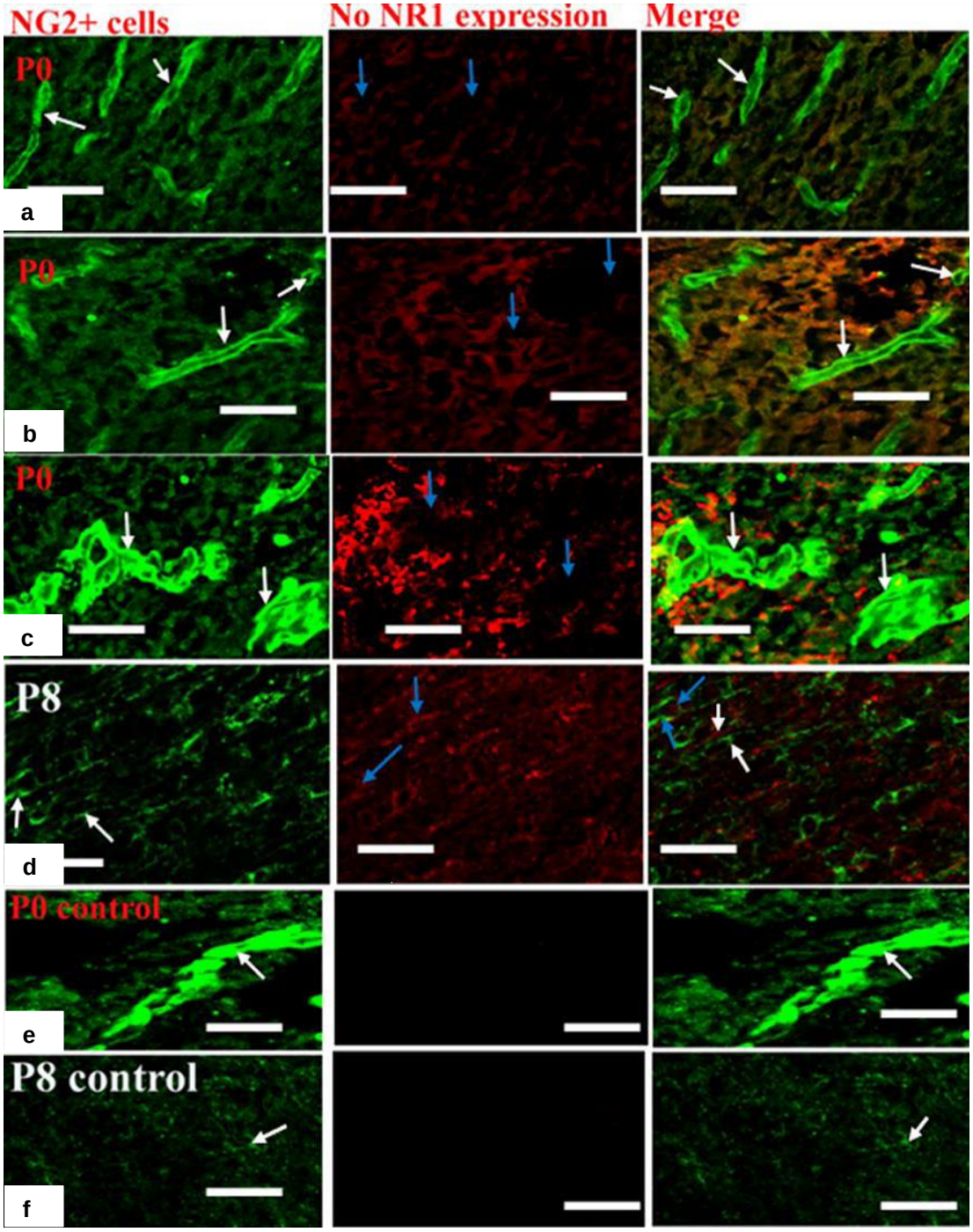

Figure 66- NR1 expression on NG-2(+) ON p0 and P8 RONs.

a, b, and c, left (green): NG-2(+) cells co-stained with mouse monoclonal NG-2(+) marker, middle (red): NR1 protein, right: The images showed no co-localization of NR1 mRNA on NG-2(+) cells in the PO RONs. **d,** left (green): NG-2(+) cells in P8, middle (red): NR1 protein, right: co-localization of NR1 with NG-2(+) at P8 RONs. **e, f,:** control.

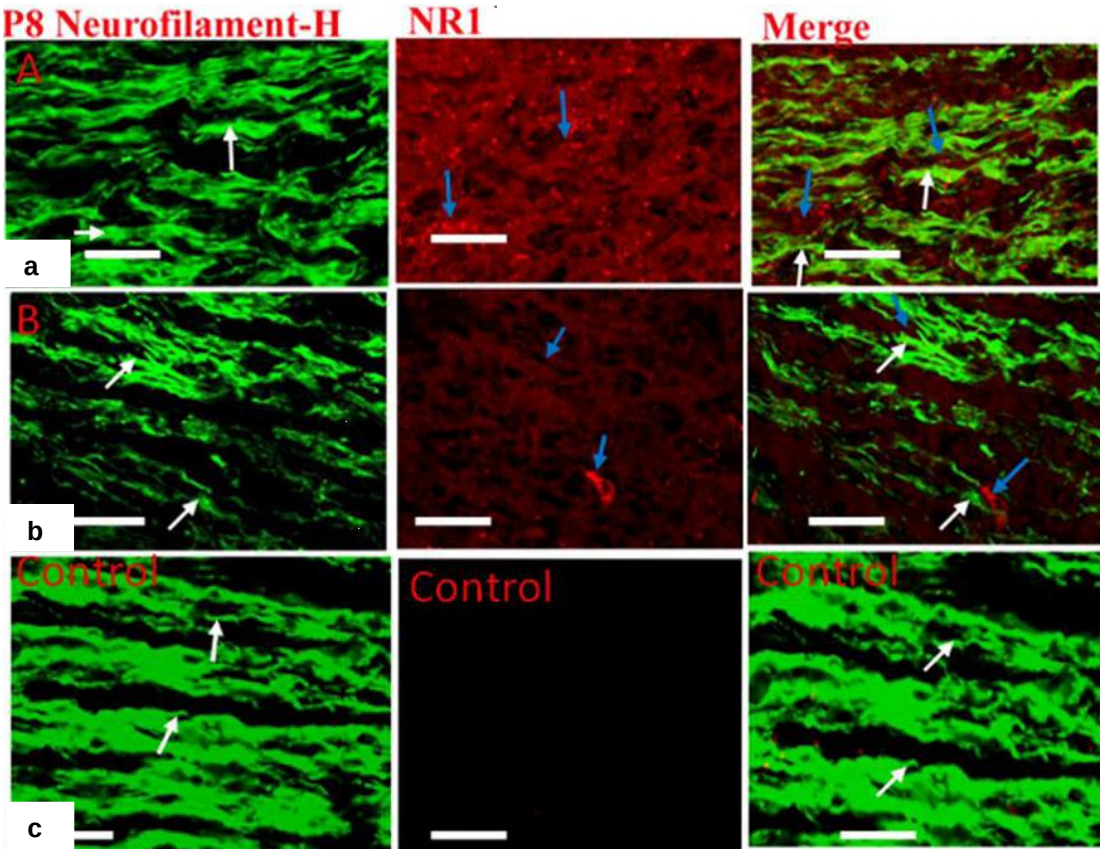

Figure 67- NRI expression in P8 RON axons.

a, and b, left: co-staining for NH-F (+) (green), middle: NR1 (red) protein in P8 RON, and right: co-localization with NF-H (+) axons at P8. **c,** control: Primary antibody omission controls showed no NR1 protein staining in middle red panel. (NF-H (+) axons (green) represented by (white arrows) and NR1 (red) expression by (blue arrows).

Discussion

The pathogenesis of immature CNS damage by ischaemia has become a challenge to scientists and researchers. A contribution from NMDA-Rs and other forms of neurotransmitter receptors-mediated excitotoxicity has not previously been found in P0 rat optic nerves. In addition to the electrophysiological study in developing white matter during 90 min OGD that has been shown in chapter IV, ultra-micrographs were taken blindly from P0 RONs cross-sections to study the appearance and shape of both axons and astrocytes during normal and ischaemic conditions. Furthermore, immunohistochemistry was used to reveal the expression of the NR1 obligatory subunit of NMDA-Rs in axons and glia during and after the initiation of myelination.

OGD-induced injury in pre-myelinated white matter axons

All the pre-myelinated axons in P0 RON are ≤ 0.4 μm in diameter and contain axolemma, microtubules and cylindrical structures as seen by the electron-microscope. An ultra-micrograph analysis of the extent of axonal and glial injury following 90 min OGD in P0 RONs showed that axonal injury is highly variable, with some axons showing axoplasmic swelling, mitochondrial swelling and loss of microtubules with other axons spared. The number of identifiable axons and the score of axonal viability were low following OGD, and the extent of astrocytic injury was high, indicating that both the axons and glia of immature central white matter are susceptible to OGD-induced injury. This is consistent with the findings of previous studies that hypoxia-ischaemia causes injury to axons as well as glia *in vivo* and *in*

vitro (Yoshioka *et al.,* 1995; Fern and Moller, 2000; Follett *et al.,* 2000; Deng *et al.,* 2003).

Alix *et al.* (2012) studied ischaemia in immature central axons (P10). They found that pre-myelinated axons of diameter ≤ 0.4 μm are protected by GluR blockade, whereas in pre-myelinated axons of diameter > 0.4 μm a combined GluR and VGCC blockade is protective; this indicates the role of VGCC and GluRs in OGD-inducing damage to these axons (Alix *et al.,* 2012). These axons were assessed at P8-P12 RONs and the mechanisms of injury appear to be fundamentally different from those shown in this study in pre-myelinating axons in P0 RONs, which are mediated by NMDA-Rs. Several reports indicate that axonal injury of immature white matter is an early event in the development of focal and diffuse components of PVL (Haynes *et al.,* 2008; Hirayama *et al.,* 2001; Deguchi *et al.,* 1999), and might be central to the formation of the injury (Dammann *et al.,* 2001). The expression of NMDA-Rs in both the growth cone and the neighbouring axon cylinder during axon elongation and synaptogenesis has been documented in a variety of neuron populations *in vitro* (Ehlers *et al.,* 1998; Herkert *et al.,* 1998; Wang *et al.,* 2011; Hosie *et al.,* 2012). Expression of the NR1 subunits is found in immature white matter by using immuno-staining techniques in this study, which is an indication of the presence of NMDA-Rs that result in the acute injury of pre-myelinated optic nerve axons during 90 min OGD. The data of this study showed that the results of 90 min OGD-induced axonal and glial injury in P0 RONs using ultra-micrograph cross-sections (chapter V) correlate with the results of electrophysiology during 90 min OGD-induced injury in P0-P4 RON axons (chapter IV). A study using ultra-micrographic

sections in P1 RONs during OGD performed in the presence of MK-801 showed the lower number of identifiable axons and the higher number of injured axons and astrocytes normally produced by OGD were significantly prevented by the presence of MK-801 (Huria *et al.,* 2013). MK-801 protected P0-P4 RON axons from OGD-induced injury assessed electrophysiologically by significant and complete recovery in the CAPs as seen in chapter IV. It is therefore clear that the degree of MK-801 protection was similar in pre-myelinated RONs during OGD evaluated by using different techniques. This indicates that OGD-induced injury in pre-myelinated RONs is mediated by the over-activation of the axonal NMDA-Rs which enhances the vulnerability of developing white matter structures to the excitotoxicity. This may be of a special significance in the understanding of the pathophysiology of neonatal brain.

OGD-induced astrocyte injury in pre-myelinated white matter

There is a correlation between synaptogenesis and astrocyte development which strongly suggests that signals from astrocytes might regulate the initiation of synapse formation (Ullian *et al.,* 2004). *In vitro* studies with purified retinal ganglion cells showed that astrocytes secrete extracellular signals that induce synapse formation (Eroglu, 2009); and the presence of this regulatory step in the timing of synaptogenesis might be necessary to ensure that all axons reach their targets (Eroglu, 2009). Astrocytes are essentially important to the maintenance and proper functioning of the CNS (Salter and Fern, 2008). During the period of development when myelination

is occurring, white matter astrocytes are particularly sensitive to ischaemic injury and their failure to regulate glutamate during ischaemia may be an important aspect in excitotoxic injury (Salter and Fern, 2008). Astrocytes in developing white matter are significantly more sensitive to ischaemic injury than grey matter astrocytes (Shannon *et al,* 2007). This may be relevant to developmental disorders such as cerebral palsy that typically involve selective injury to white matter regions (Back and Rivkees, 2004; Volpe, 1995). However, Rose *et al.* (1998) found that developed white matter astrocytes are temporary protected during ischaemia because they have their own glycogen to use as energy and are anaerobic in nature.

In this study, 90 min OGD of immature white matter astrocytes induced cellular swelling and disruption of cell membranes with severe damage to the organelles. The susceptibility of astrocyte to OGD-induced injury may be secondary to axonal damage and excitotoxicity mediated by over-activation of NMDA-Rs due to accumulation of extracellular glutamate. Salter and Fern (2008) studied the ischaemic mechanism in immature white matter astrocytes using transgenic mice, they found that OGD-induced injury of immature astrocytes follows a cytotoxic ion influx mediated in part by Na^+–K^+–Cl^- co-transport and in part by Na^+ and K^+ dependent HCO_3 transport. Other studies found that astrocytes are injured and swell during energy deprivation due to release of glutamate extracellular. There are two pathways are required for astrocyte swelling-mediated by glutamate, an early phase release of glutamate mediated by EAATs (Rutledge *et al.,* 1996; Kimelberg and Mongin, 1998), and a delayed phase by Na^+–K^+–Cl^- co-transport required for high K^+ and induced astrocyte swelling (Su *et al.,* 2002). Astrocytes in developing

white matter are subject to loss of cell processes (known as clasmatodendrosis) during energy deprivation, which will release intracellular glutamate into the extracellular space (Fern, 2011).

The role of $GABA_A$, Gly, nACh and AR antagonists in OGD-induced injury in developing white matter

Axonal and glial injury during energy deprivation in developing white matter is not solely mediated by NMDA-Rs; it is clear therefore that neurotransmitters receptors other than glutamate participate in white matter injury at this critical period, such as GABA and glycine receptors. The role of these receptors during ischaemia in pre-myelinated white matter was investigated electrophysiologically in P0-P4 RONs (chapter IV). Correspondingly; their role has been confirmed in this result chapter by using ultra-micrographic cross-sections in P0 RONs. The ability of blockade of these receptors to protect the pre-myelinated RONs from OGD-induced injury at this age group has not been shown before. It was found that the number of identifiable axons following exposure to 90 min OGD was significantly restored by $GABA_A$, Gly and nACh receptor antagonists (picrotoxin, strychnine, and mecamylamine respectively). In addition, a glycine or nACh receptor antagonist significantly protected the axonal viability scores, while a $GABA_A$-R antagonist produced a minimal effect on the axonal viability score. Combined-AR antagonists were protective only in restoring the number of identifiable axons; it was not protective in restoring the axonal viability scores. $GABA_A$, Gly or nACh receptor antagonist produced significant preservation of the normal structures of the astrocytes of P0 RONs following

exposure to OGD-induced cellular damage. However, $GABA_A$-R and Gly receptor activation evoked depolarisation in neonatal astrocytes and cells of the oligodendroglial line due to the opening of a chloride conductance (Constantinou and Fern, 2009; Sakatani *et al.,* 1991; Sakatani *et al.,* 1992; Sakatani *et al.,* 1994). No study has yet revealed that GlyRs or $GABA_A$-Rs elevate intracellular Ca^{2+} in glia (Constantinou and Fern, 2009). Some studies showed that nACh-Rs increase intracellular Ca^{2+} in both astrocytes and oligodendrocytes in neonatal RONs, whereas α-adrenoceptors have been reported to elevate intracellular Ca^{2+} in astrocytes (Khorchid *et al.,* 2002; Constantinou and Fern, 2009). Results presented here regarding the protective effect of $GABA_A$-R and GlyR antagonists against OGD-induced axonal and glial injury in P0-RONs using ultra-micrograph study is consistent with electrophysiological data presented here showing protective effects against OGD-induced axonal injury in P0-P4 RONs. Both produced a mild decline in the CAPs in a reversible fashion post-OGD. The results of both electrophysiology and microscopy are consistent with a potential role of $GABA_A$ and GlyR-mediated injury in pre-myelinated central white matter.

Although a nACh-R antagonist produced a significant protection against OGD-induced injury in neonatal axons and glia using ultra-micrograph study, the effect is different electrophysiologically during OGD, where it exhibited only limited protection in the CAPs in pre-myelinated optic nerves. The absence of the protective effects of combined-adrenoceptors blockade shown by ultra-micrograph section during OGD in P0-RONs is correlated to their effects electrophysiologically – They failed to protect from an irreversible decline in the CAPs in P0-P4 RONs post-OGD. These observations indicate

that adrenoceptors are not involved during ischaemia in developing brain at this stage of development. Constantinou and Fern (2009) found that nACh-Rs or ARs induced partial conduction failure in P7-P15 RONs that was not associated with any obvious axon pathology but associated with evident widespread glial cell damage significantly more astrocyte than oligodendroglial cell damage. The absence of morphological changes in P7-P15 optic nerve axons suggests that it is the glial injury that underlies the non-reversible conduction failure evoked by 80 min activation of nACh-Rs or ARs (Constantinou and Fern, 2009). The data collected in this chapter shows that in addition to NMDA-Rs, GABA and GlyRs play a major role in sequencing the ischaemic injury in developing white matter; consequently, antagonising of GABA, Gly or nACh receptors is potentially protective.

Correlation between electrophysiological and electron microscopy results regarding the role of neurotransmitters in OGD-induced injury.

CAP during control conditions in P0-P4 RONs was stable for several hours and reached to 107 ± 5.5 %. OGD for 90 min produced profound injury to the axons of P0-P4 RONs and the CAP declined to 34.5 ± 3.9 %. The following table showed the electrophysiological effects of several neurotransmitters than glutamate on the CAP recovery during 90 min OGD-induced axonal injury in P0-P4 RONs. $GABA_A$-R and GlyR antagonists provide high protection against OGD-induced injury in P0-P4 RONs which is represented by a large recovery in the CAPs post OGD.

Table 2

CAP recovery (%)	Experiment	Receptor type
107 ± 5.5	Control	
34.5 ± 3.9	OGD	
72.1 ± 3.6	OGD +picrotoxin	$GABA_A$-R
70.7 ± 6.6	OGD +strychnine	GlyR
26.9 ± 8.8	OGD +prop. & phent.	Combined-ARs
45.0 ± 10.8	OGD +mecamylamine	nACh-Rs

The following table showed the effects of the above mentioned neurotransmitters on 90 min OGD-induced axonal and glial injury in P0

RONs using electro-microscopy techniques. Blinded counting of axonal density showed a high number of identifiable axons in control nerves (mean= 11 ± 0.737axons/μ²), viability scoring showed that the majority of axons have a score of "3", which correlates to no pathology (mean= 2.9 ± 0.09). Viability scoring of glia in P0 RONs showed that the majority of glia also had a score of "3" (mean= 2.9 ± 0.05). Blinded counting of axonal density showed a significantly lower number of identifiable axons following OGD (mean= 2.7 ± 0.74 axons/μ²; P<0.0001 vs control perfused RONs). Blinded axonal viability scores were significantly lower in identifiable axons following OGD; most scored 1.5 (mean= 1.7 ± 0.31; P<0.0001 vs control perfused RONs). Blinded assessment of astrocyte injury following OGD resulted in an extremely low score of "1" in many cells (mean= 1.0 ± .018; P<0.001 vs control perfused RONs). $GABA_A$-R and GlyR antagonists restore the lower number of identifiable axons in P0 RONs post OGD.

Table3

Axonal density (axons/μ^2)	Axonal injury Score "3"	Glial injury Score "3"	Experiment	Receptor type antagonist
11 ± 0.737	2.9 ± 0.09	2.9 ± 0.05	Control	
2.7 ± 0.74	1.7 ± 0.31	1.0 ± .018	OGD	
6.0 ± 1.19	1.9 ± 0.10	1.7 ± 0.20	OGD+ picrotoxin	$GABA_A$-R
7.75 ± 0.67	2.6 ± 0.02	3 ± 0.01	OGD +strychnine	GlyR
4.6 ± 0.146	1.6 ± 0.07	1.3 ± 1.2	OGD+ prop. & phent.	Combined-ARs
7.0 ± 2.6	2.5 ± 0.7	3 ± 0.0	OGD+mecamylamine	nACh-R

Table4

CAP recovery (%)	Axonal density restoring %	Experiment
34.5	24.5	OGD
72.1	54.5	OGD+ $GABA_A$-R antagonist
70.7	70.4	OGD+ GlyR antagonist
26.9	41.8	OGD+ Combined-ARs antagonist
45.0	63.6	OGD+ nACh-Rs antagonist

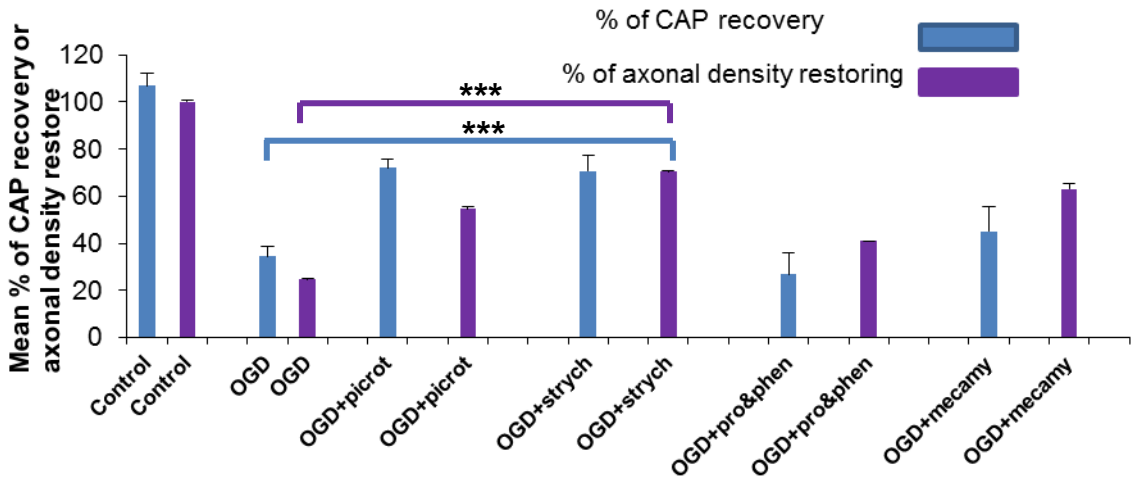

Figure-68: Histogram illustrating the correlation between the results of CAP recovery recorded electrophysiologically and axonal density restoring collected by electron-microscopy.

It shows the protective effect of $GABA_A$-R and GlyR antagonists against OGD-induced axonal injury in P0-RONs using ultra-micrograph study is consistent with electrophysiological data in P0-P4 RONs. ***P< 0.0001. Error bars are SEM.

Results presented here regarding the protective effect of $GABA_A$-R and GlyR antagonists against OGD-induced axonal and glial injury in P0-RONs using ultra-micrograph study is consistent with electrophysiological data presented here showing protective effects against OGD-induced axonal injury in P0-P4 RONs. Both produced a large protection against 90 min OGD-induced injury which is exhibited by significant recovery in the CAP. They also reversed the lower number of identifiable axons during 90 min OGD. The results of both electrophysiology and microscopy are consistent with a potential role of $GABA_A$ and GlyR-mediated injury in pre-myelinated central white matter.

Although a nACh-R antagonist produced a significant protection against OGD-induced injury in neonatal axons and glia using ultra-micrograph study, the effect is different electrophysiologically during OGD, where it exhibited only limited protection in the CAPs in pre-myelinated optic nerves. The absence of the protective effects of combined-adrenoceptors blockade shown by ultra-micrograph section during OGD in P0-RONs is correlated to their effects electrophysiologically – They failed to protect from an irreversible decline in the CAPs in P0-P4 RONs post-OGD. These observations indicate that adrenoceptors are not involved during ischaemia in developing brain at this stage of development. The data collected in this study shows that in addition to NMDA-Rs, GABA and GlyRs play a major role in sequencing the ischaemic injury in developing white matter; consequently, antagonising of GABA, Gly or nACh receptors is potentially protective. To avoid false +Ve results, axonal density data collected by the electron-microscopy is correlated with the CAP recovery data recorded electrophysiologically. The

data of viability axonal injury score does not correlated with the result of the electrophysiology, because some axons can be visible but not viable and not functioning probably.

These results raised numerous questions that needed to be answered?

Constantinou and Fern (2009) stimulated the neurotransmitter receptors initially and then exposed these receptors to the neurotransmitter blockade in late point of development. The current study was investigating the role of these neurotransmitter receptors in OGD-induced injury in the axons of neonatal nerves at very early point of development. Interestingly, the P0-P4 RON axons was not stimulated by exogenous administration of the receptor agonists, but blockade of these receptors particularly protect the neonatal axons from OGD-induced injury. Is it an indication that the axons at early point of development can release these neurotransmitters during OGD even if there are no synapses? If so, where is the location of these neurotransmitters in the axons? In addition, because these axons are generally found in clusters embedded within few astrocyte processes, are these glial processes considered the source of releasing the neurotransmitters during injury and lead to stimulation of their receptors located on the axons?

Immuno-staining revealed the expression of the NR1 NMDA-Rs subunit in both P1 and P8 RONs

A central theme to this work is that NMDA-Rs are involved in the development and injury of both axons and glia in pre-myelinated central white matter. Immuno-staining was used to describe the expression of NR1, the obligatory subunit of NMDA-Rs, at early developmental time points. Some studies were focused on later development point when axon myelination has started, they found that NMDA-Rs are predominantly localised to the processes of developing oligodendrocytes (Karadottir *et al.,* 2005; Salter and Fern, 2005; Micu *et al.,* 2006). The NR1 subunit is also localised on some populations of rat astrocytes, microglia and sub-plate neurons (Gottlieb and Matute, 1997; Schipke *et al.,* 2001). Functional glutamatergic synapses on NG-2(+) glial cells have been identified in various brain regions in immature and mature animals (Paukert and Bergles, 2006), while vesicular glutamate signalling between axons and neighbouring NG-2(+) cells has been documented in the corpus callosum and immature rat optic nerve (Kukley *et al.,* 2007; Ziskin *et al.,* 2007). Some studies showed that glutamate receptors can be expressed on both astrocytes (Gallo and Ghiani, 2000) and oligodendroglia (Fern and Moller, 2000). The final piece of data offered in this thesis is the expression of NMDA-Rs in P0 RONs. High levels of NR1 expression are found within axons (NF-H (+)) of P0 RONs, while low levels of NR1 expression are found within astrocytes (GFAP (+)); there is no evidence of NR1 expression within developing oligodendrocytes (NG-2+).

A similar series of images showed high expression of NR1 in NG-2(+) cells of P8 RON. Very low or absent NR1 co-localisation was found within NF-H (+) axons at P8 RON. These findings imply that NMDA-Rs play an important role in regulating central white matter development and injury. These results suggest that both perinatal and post-natal white matter injury are NMDA-R-dependent processes. In light of these data, NMDA-Rs expressed on the axons of P0 RONs play an important role in mediating white matter injury during this early period of development, whereas NMDA-Rs expressed on the developing oligodendrocytes of P8 RONs are the key in mediating white matter injury in the post-natal period. The presence of the NR1 subunit on the axons of P0 optic nerves suggests that axonal damage and excitotoxicity occurs directly because of the over-activation of NMDA-Rs, whereas the low levels of NMDA-R expression within some astrocytes populations might be non-functional and the susceptibility of glial cells to OGD-induced injury is a subsequent of the axonal injury and excitotoxicity. In P8 optic nerves, the excitotoxicity of the oligodendroglia occurs directly because of the over-activation of the NMDA-Rs.

In conclusion, axons are the vulnerable Part to ischaemic insult in perinatal period that matches the time of PVL. Axonal injury and excitotoxicity during this time of development is mediated in part by NMDA-Rs and by GABA and GlyRs in another part. In addition a specific antagonist of each receptor is highly protective. Therefore, a deeper knowledge of the mechanisms leading to developing white matter injury and excitotoxicity mediated by NMDA-Rs and other neurotransmitter receptors will facilitate

new pharmacological strategies in the treatment of neonatal brain disorders such as PVL.

Chapter VI

General discussion

This thesis investigated the effect of energy deprivation in the optic nerve axons of developed and developing white matter. Energy deprivation in the form of ischaemia is a leading cause of serious diseases in the human brain, for example stroke in adults and the lesion PVL (the major cause of cerebral palsy) in neonates. I have investigated the axonal injury and dysfunction in both adults and neonates illustrated by electrophysiological recording of CAPs, and ultra-micrographic cross sections. The significance of NMDA and other neurotransmitter receptors expressed in developing white matter have been examined in this thesis, the functions of which are not known. NMDA, $GABA_A$, Gly, nACh and ARs can all significantly modulate the conduction of action potentials in white matter and contribute large effects to the regulation of glial membrane potential; effects that are developmental and fade with maturation. The significance of these receptors for injury to this tissue at this point in development has previously not been studied.

Why is the brain particularly sensitive to ischaemia?

The most plausible answer is that there are endogenous excitatory amino acids in the brain such as glutamate which once released into the extracellular space induces central neurotoxicity under hypoxic/ischaemic conditions (Choi and Rothman, 1990). The relationship between glutamate and brain ischaemia was anticipated over 50 years ago by Van Harreveld (1959), who studied cortical spreading depression in the rabbit. The neurotoxic effect of extracellular glutamate in the retina during ischaemia was recognised slightly earlier by Lucas and Newhouse (1957), and 12 years after this in the brain (Olney and Sharpe, 1969). Interestingly, the term ‘neurotoxicity’ was changed to ‘excitotoxicity’ by Olney and De Gubareff in 1978, and became a common expression for excitatory neurotransmitters on central neurons (Olney and De Gubareff, 1978). Glutamate activates NMDA and non-NMDA-Rs; these receptors mediate the ability of glutamate to induce excitotoxicity in the brain (Choi and Rothman, 1990). Unfortunately, glutamate excitotoxicity is not the only explanation for the brain damage that occurs during hypoxia/ischaemia, however, as neurotransmitters other than glutamate can also mediate excitotoxicity in the brain. Dopamine under certain conditions in the brain can form reactive oxygen species that lead to mitochondrial dysfunction which is involved in neurodegenerative diseases such as Parkinson`s disease and methamphetamine-induced neurotoxicity (Berman and Hastings, 1999).

But are there other neurotransmitters than glutamate and dopamine in the brain can mediate excitotoxicity?

The collected data from this study is the first data to indicate that glutamate is not the sole cause of ischaemic damage in pre-myelinating white matter, there are neurotransmitters other than glutamate in the brain such as GABA and glycine can mediate excitotoxicity during energy deprivation, while others such as nACh and adrenoceptors are not involved. The significance of these neurotransmitters and their receptors for inducing injury to neonatal white matter structures at ages coincident with the time peak of PVL has previously not been studied. Neurotransmitters excitotoxicity is not the only reason for the sensitivity of the brain to ischaemia, the high metabolic rate and low energy reserve of the brain can contribute to ischaemic susceptibility as does the particular vasculature.

Ischaemia for 60 min resulted in severe injury in optic nerve axons of adult rodents which assessed by loss of the evoked CAP. MON showed no permanent loss of function during ischaemic insults that exhibited by a recovery in the CAP in compare to RON. However, activation of NMDA-Rs by exogenous administration of the receptor agonists resulted in irreversible injury in MON assessed by irreversible ablation in the CAP at the same degree of RON CAP. MON is less sensitive to ischaemic insults possibly due to its smaller diameter and low level of NMDA-Rs will be activated during ischaemia. Ischaemia of 60 min did not produce any permanent damage to the neonatal optic nerve axons; while longer exposure did result in significant damage that assessed by a large ablation in the evoked CAP with mild

recovery. The rate of CAP recovery was irreversibly lost during activation of NMDA-Rs by exogenous administration of the receptor agonists. This prominent role of glutamate excitotoxicity mediated by NMDA-Rs depends on extracellular Ca^{2+}, since only the NMDA-Rs opens as membrane channel that is highly permeable to Ca^{2+} (MacDermott *et al.,* 1986). Although, one should keep in mind that additional Ca^{2+} entry probably occurs through voltage-activated Ca^{2+}-channels, the Na^{+}/Ca^{2+} exchanger, and non-specific membrane leakage (Choi, 1988). However, the ischaemic damage to the optic nerve axons at this age group of pre-myelination that matches the time of PVL was completely protected by the administration of NMDA-R antagonist MK-801. Remarkably, $GABA_A$ and GlyRs mediate novel excitotoxicity in developing white matter during the risky time of PVL. Activation of these receptors by the endogenous agonists during energy deprivation resulted in a profound injury and disruption to the neonatal optic nerve axons and glia as assessed by electrophysiological recording of CAP and blind collection of microscopic images of both axons and glia; the effects were significantly prevented by administration of $GABA_A$ and GlyR antagonists (picrotoxin and strychnine, respectively). On the other hand, nACh and ARs do not participate in ischaemic damage in pre-myelinated white matter, the effect assessed electrophysiologically and microscopy. A multiplicity of questions regarding the pathophysiology and therapy of white matter ischaemia need to be answered before any wide-ranging clinical management can be considered.

But why is white matter in the neonate at risk for ischaemic insult?

The answer is that it is not only a story of neurotransmitters excitotoxicity or ionic based mechanism; the vasculature of the periventricular area of the preterm infant predisposes the foetal brain to ischaemic insult (Alix, 2006). The blood supply to the periventricular area is small, consisting of branches from the middle cerebral artery which are not fully developed until the end of gestation (Rorke, 1992). These branches are quite some distance away from the periventricular area, which adds to the susceptibility of this region to ischaemic insult (Rorke, 1992). The immature white matter receives a very small amount of blood – about 1.6-3 ml / 100 g / min – which is 25% of that of the grey matter and less than half that of the adult white matter (Altman *et al.,* 1988; Greisen and Borch, 2001). The blood flow in the human preterm infant is therefore particularly low, which increases the susceptibility of the foetal brain to ischaemic insult. The data collected in the current study suggests that PVL is ischaemic in origin, which is consistent with the conclusion of Paneth *et al.* (1994) – that the arterial blood supply to the white matter is an important component in the pathogenesis of such injury (Paneth *et al.,* 1994).

Based on the results in this study and others, it is proposed that, both myelinated and pre-myelinated white matter is susceptible to excitotoxic injury mediated by over-activation of NMDA-Rs. Energy deprivation in pre-myelinated white matter damages axons and glia through disrupting ionic homeostasis following a reduction in ATP, with subsequent release of axonal

glutamate into the extracellular space leads to over-activation of axonal NMDA-Rs and consequent excitotoxicity that appears to enhance glial injury. At the same time elevated levels of glutamate are accompanied by an increase in the concentration of GABA and glycine in the extracellular space, which during ischaemia lead to over-activation of $GABA_A$ and GlyRs that result in further excitotoxic damage to the axons and glia. These findings indicate that brain ischaemia and excitotoxic injury are associated with an increase in both excitatory and inhibitory neurotransmitters. For that reason, combined therapy may be of value in reducing the damaging effects of ischaemic insults in neonate. However, the hypothesis is that NMDA, $GABA_A$ and GlyRs are the key to excitotoxicity in PVL; while nACh and ARs are not involved.

List of abbreviation

-CNS: Central Nervous System

-PLP: proteolipid protein

-MBP: Myelin Basic Protein

-MAG: Myelin-associated glycoprotein

-GalC: Galactocerebroside

-GalDG: Galactosulfaide glycolipids

-GM1: Ganglioside

-ATP: Adenosine triphosphate

-LIF: Leukaemia Inhibitory Factor

-GFAP: Glial fibrillary acidic protein

-E21: Embryonic day 21

-E17: Embryonic day 17

-PNS: Peripheral Nervous System

-$E_{K:}$ Equilibrium potential of K^+ ion

-$E_{Cl:}$ Equilibrium potential of Cl^- ion

-AP: Action potential

-AIS: Axon Initial Segment

-cAMP: Cyclic Adenine Mono Phosphate

-cGMP: Cyclic Guanine Mono Phosphate

-DAG: Di Acyl Glycerol

-IP3: Inositol tri-phosphate

-TTX: Tetrodotoxin

-$_{I}Na_{T:}$ Inactivating transient Na+ current

-$_{I}Na_{P:}$ Persistent Na+ current

- I_{Na_R}: Resurgent Na+ Current

-VGCC: Voltage gated calcium channel

-P2XRs: Purinergic receptors of subunit named 2X

-P2YRs: Purinergic receptors of subunit named 2Y

-ADP: Adenosine diphosphate

-sGLU: Synaptic glutamate

-eGLU: Extra- synaptic glutamate

-GLTs: Glutamate transporters

-EAAT: Excitatory amino acid transporter

-EAAC1: Excitatory amino acid carrier

-GLAST: Glutamate aspartate transporter

-VGLUTs: Vesicular glutamate transporters

-GluRs: Glutamate receptors

-LTP: Long term potentiation

-LTD: Long term depression

-MS: Multiple sclerosis

-PVL: Periventricular leukomalacia

-Glu: Glutamate

-Gln: Glutamine

-iGluRs: Ionotropic glutamate receptors

-mGluRs: Metabotropic glutamate receptors

-NMDA: N-methyl-D-aspartate

-NMDA-Rs: N-methyl-D-aspartate receptors

-Non-NMDA-Rs: Non- N-methyl-D-aspartate receptors

-AMPA: α-amino-3-hydroxy-5-methyl1-4-isoazolepropionic acid

-AMPA-Rs: α-amino-3-hydroxy-5-methyl1-4-isoazolepropionic acid-receptors

-TMD: Trans-membrane domain

-D-AP5: 2-amino-5-phosphonopentanoate

-SDZ EAB515: (S)-alpha-amino-5-phosphono-methyl [1, 1´-biphenyl]-3-propanoic acid

-hERG: Ether-à-go-go-Related Gene

- Con G: Conantokin G

-ALQTS: Acquired long QT syndrome

-KA-Rs: Kainate receptors

-PEPA: 4[2-(phenyl sulphonyl amino) ethylthio]-2, 6-difluorophenoxy acetamide

-ATPA: 5-tert-butyl-4-isoxazolepropionic acid

-CNQX: 6-cyano-7nitroquinoxaline-2, 3-dione

-NBQX: 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

-ACET: (S)-1-(2-amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methylpyrimidine-2, 4-dion

-VFD: Venus flytrap domain

-ESC: Extra cellular space

-E/R: Endoplasmic reticulum

-Cyt-c: Cytochrome complex

-GABA: ɣ-amino-butyric acid

-GAD: Glutamic acid decarboxylase

-GABA-T: GABA-transaminase

-GABA-Rs: ɣ-amino-butyric acid-receptors

-TBPs: t-butylbicyclophosphorothionate

-IPSPs: Inhibitory postsynaptic potentials

-EPSPs: Excitatory postsynaptic potentials

-ARs: Adrenergic receptors

-GPCRs: G protein coupled receptors

-PLC: phospholipase-C

-IP3: Inositol triphosphate

-IP2: Inositol diphosphate

-PKC: Protein kinase-C

-AC: adenylyl cyclase

-CO: Cardiac output

-BP: Blood pressure

-NTS: Nucleus tractus solitarius

-BPH: Benign prostatic hyperplasia

-α-AR: Alpha-adrenoceptors

-β-AR: Beta-adrenoceptors

-BARK: Beta-adrenergic receptor kinas

-HSP72: Heat shock protein 72

-GlyRs: Glycine receptors

-nACh: Nicotine acetylcholine

-Glra: Glycine receptor alpha (gene encoding protein)

-Glrb: Glycine receptor beta (gene encoding protein)

-Ach-Rs: Acetylcholine receptors

-nACh-Rs: ionotropic nicotinic receptors

-mACh-Rs: metabotropic muscarinic receptors

-VTA: ventral tegmental area

-d-TC: d-tubocurarin

-DHBE: dihydro-β-erythroidine

-aCSF: Artificial cerebrospinal fluid

-OGD: Oxygen and glucose deprivation

-P0-P4: Post-natal day from 0-4

-RON: Rat optic nerve

-MON: Mouse optic nerve

-NMDG: N-methyl-d-glutamine

-EGTA: Ethylenebis (oxyethylenenitrilo) tetraacetic acid

-MK-801: (+)-5-methyl-10,11-dihydro-5H- dibenzo [a,d] cyclohepten5,10-imine maleat

-CAP: Compound action potential

-IHC: Immunohistochemistry

-EM: Electron-microscopy

-NF-H: Neurofilament-Heavy

-NF-L: Neurofilament-Light

-GFAP: glial fibrillic acid protein

-FOV: Fields of view

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