Cerebellar Organotypic Slice Culture System: A Model of Developing Brain Ischaemia

Mohamed A. Al-Griw[1], Ian C. Wood[2] and Michael G. Salter[3]

[1]Develomental Biology Division, Zoology Department, Faculty of Science, University of Tripoli, Tripoli-Libya
[2]School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds-UK.
[3]Present address: Invista (UK) Ltd, Redcar, Cleveland, UK.
m.algriw@zo.uot.edu.ly

Abstract: Ischaemic injury during brain development correlates with long-term neurological problems resulting in part from oligodendrocytes (OL) damage and a loss of appropriate myelination. The molecular and cellular mechanisms responsible remain partially understood and there is no effective clinical treatment. Here we develop and characterise an *ex-vivo* slice culture ischaemia model to elucidate the cellular mechanisms to aid the search for therapeutic interventions. Cerebellar slices from 7 day-old rats were cultured for 10 days and their developmental profile in culture and their response to oxygen-glucose deprivation (OGD) was assessed. During the culture period development of white matter progressed as *in-vivo*, the numbers of oligodendrocyte precursor cells (OPC) decreased and the numbers of mature OLs increased and there was extensive myelination of axons as judged by colocalisation of myelin basic protein and neurofilament. Cultured slices were exposed to a short period of OGD at 7 days *in-vitro* and reperfused to mimic *in-vivo* conditions. Twenty minutes of OGD was found to result in significant injury as judged by a 58.6% reduction in cell viability 3 days post-injury. Treatment of cultures with OGD resulted in a loss of OLs and a loss of myelination of axons. In summary we have developed a paradigm for studying the damage to OLs and loss of myelination associated with ischaemic periods during development which should facilitate the search for understanding the mechanisms responsible and identifying potential therapeutic interventions.
[Mohamed A. Al-Griw, Ian C. Wood and Michael G. Salter. **Cerebellar Organotypic Slice Culture System: A Model of Developing Brain Ischaemia.** *Life Sci J* 2017;14(4):89-98]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 11. doi:10.7537/marslsj140417.11.

Keywords: Myelin; oligodendrocyte; white matter; organotypic; slice culture; ischaemia

1. Introduction

During the development of the central nervous system a key process is the formation of the myelin sheath that envelops axons to greatly enhance the transmission of signals, particularly over longer distances [1-2]. As myelination progresses there is a period of heightened sensitivity to ischaemic insult where developing OLs are particularly susceptible to injury as pre-OLs develop into immature OLs. Injury at this time has a catastrophic effect on the prospects of an individual with those affected suffering from conditions ranging from minor problems with motor function to severe motor and cognitive disorders usually known collectively under the banner of cerebral palsy [3-6]. There has been a considerable research effort applied to understanding the causes of this injury with a number of groups seeking to identify the mechanisms by which oligodendrocytes become more vulnerable during this vital period (reviewed by [7]. Of particular importance in the understanding of any biological system is the model that is used to study that system. Within study of the central nervous system (CNS) there are a number of models currently employed that utilize whole animals for *in-vivo* experiments, isolated tissue models for *ex-vivo* experiments and isolated cell culture where specific cell types are isolated and maintained for investigation. Each of these systems have their own merits, however within the study of neonatal ischaemia there has been a tendency to use either *ex-vivo* tissue models or whole animal models [8-10]. This reflects the need to study the mechanism of injury in the context of the complex cellular matrix that exists in CNS white matter where the injury to one cell type has an effect on other cell types [11-14]. Glutamate release from axons and astrocytes has a direct affect on oligodendrocytes, activating NMDA and AMPA glutamate receptors causing a fatal influx of Ca^{2+} [8, 15]. In addition, the two-dimensional nature of cell culture systems means that changes in the concentration of particularly K^+ and Na^+ ions as well as pH are not reflected as accurately in cell culture systems as they are in a complex tissue matrix exemplified in *ex-vivo* tissue models [16]. Perhaps most importantly in the normal tissue environment the close proximity of the different cells may well result in a more significant increase in local glutamate concentrations than would be seen within a perfused single cell type culture system. Finally cultured developing OLs express a different isoform of the Na^+-dependent glutamate transporter that is significant in the release of glutamate into the extracellular space [8, 11, 17].

Traditionally the *ex-vivo* tissue model has been used to study the early effects of OGD with both rodent optic nerve and spinal cord white matter tracts proving particularly useful in identification of mechanisms of necrotic cell death [9, 18-19]. Where longer term effects have been studied carotid artery occlusion has been used in whole animal models and in particular this has demonstrated that downstream of the initial injury secondary damage occurs due to the accumulation of reactive oxygen species [20]. For the study of how these downstream effects interact and are as a result of those mechanisms identified as being important in early injury the carotid artery model system a high level of animal usage would be required. Also, within the carotid artery occlusion model it is difficult maintain the precise levels of control required to ensure a fully repetitive experimental system where the surgical conditions may alter and post operatively the response of particular individuals may differ. Here we sought to create a model of ischaemia that would enable us to investigate how early influx of Ca^{2+} impacts on the downstream effects of an ischaemic insult whilst maintaining precise control of experimental conditions throughout the course of a three day experiment. A particular priority was to develop a protocol that enabled us to deliver a precise and measured deprivation of oxygen and glucose so that in all experiments we could be sure that the nature and degree of insult was constant. To achieve this we sought to use an *ex-vivo* tissue model whereby a cerebellar slice culture could be generated from animals where the white matter is developing and maintain it whilst exposing it to a short ischaemic insult at some defined point in the culture cycle. Here we describe the methodology used and the protocols developed for the Scientific Procedures (1986) act study of the extended effects of a short term neonatal insult.

2. Material and Methods

2.1 Animals

Cerebellar organotypic slice cultures (OSCs) were generated from 7 day old postnatal (P7) Wistar rats. All experimental procedures involving the use of animals were carried out in accordance with United Kingdom Home Office guidance and regulations and were in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.2 Cerebellar organotypic slice cultures

Male and female Wistar rats were used for cerebellar organotypic slice cultures (OSC) which were prepared as described previously [21] and later modified by others [22-23]. Briefly, animals were sacrificed by cervical dislocation in the brain was removed under aseptic conditions and immediately placed into a Petri dish containing ice-cold DMEM (minimum essential medium) supplemented with 2.3 mM (v/v) penicillin/streptomycin (Invitrogen). The cerebellum was dissected away from the brain, embedded in a mold containing cool, molten 4 % agar (37 ºC, Melford) and 300-µm sagittal slices of the cerebellum were generated using a vibratome (Leica). Intact slices were then cultivated on cell culture inserts (Millipore, 1.0 µm, Falcon) placed in 6-well culture plates (Falcon) containing 1 ml culture medium. The nutrient culture medium containing 50 % DMEM with earls salts (Sigma), 25 % HBSS (Hanks balanced salt solution 1X) (GIBCO, Invitrogen), 20 % normal horse serum (Invitrogen), 4.6 mM L-glutamine (Sigma), 21 mM D-glucose (Fisher Scientific), penicillin/streptomycin solution stabilized (Invitrogen), 4.2 µM (v/v) L-ascorbic acid (Aldrich-Sigma) and 11 mM NaHCO3 at pH 7.2-7.4. The culture medium was filter-sterilized and pre-warmed in a humidified aerobic incubator (5% CO_2) at 37 °C for 15 minutes before use. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was changed to serum free and supplemented with 0.3 % B27 growth supplement (Invitrogen) and thereafter the media was refreshed every 72 hours with cultures maintained for up to 40 days.

2.3 Generation of ischaemia *in-vitro*

Ischaemia was generated by combined OGD as descried previously [24]. P7-derived rat cerebellar slices were cultured for 7 days before being used for OGD. The cerebella tissues were transferred into filter-sterilized, deoxygenated glucose-free culture medium and placed in an airtight chamber (Figure 1) containing 95% N_2 and 5% CO_2 constant positive pressure gas flow. The temperature was maintained at 37 ± 0.5 °C by circulation of water through a re-circulating, thermostatically controlled water bath (Grant) and temperature within the chamber was monitored using a thermocouple immersed in media. At the end of a predetermined period of OGD treatment, the slices were washed at least three times with fresh oxygenated culture medium containing 5 mg/ml D-glucose and supplemented with 2% B27 and returned to normoxic conditions (5% CO_2) at 37 °C. Control sections which had no exposure to OGD were washed with normal culture medium in the same manner. Subsequent to the insult, cultures were incubated under reperfusion conditions for a defined period prior to analysis.

2.4 Adenoviral preparation and infection

Recombinant Adenovirus (AVV) expressing GFP under the control of the CMV promoter has been described previously [25-26]. High titre virus was prepared by four rounds of infection of HEK cells and subsequent CsCl gradient density centrifugation [27].

For Adenoviral delivery, slices were cultured with culture media containing approximately 10^9 pfu/ml of Adenoviral particles.

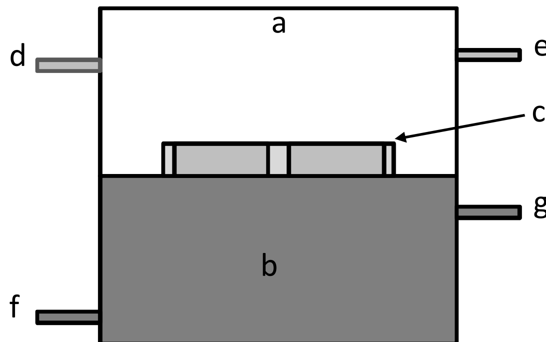

Figure 1. Chamber designed to enable ischaemic insult to cerebellar slice cultures. Representation of the ischaemia chamber which was constructed from clear perspex sheeting bonded together with silicone cement. A, airtight chamber, b water tank for temperature control, c, culture dish containing CSC, d, gas ingress, e, gas egress, f heated water ingress, g, heated water return.

2.5 Measurement of cerebellar slice viability

Cell survival was measured using the Live/dead Viability/Cytotoxicity kit (Invitrogen) in accordance with manufacturer's instructions. Briefly, cultured cerebellar slices and were incubated in the presence of a solution containing 4 µM ethidium homodimer-AM (EthD-1) and 2 µM calcein-AM at 37°C for 30-45 minutes, and were then fixed in 4% paraformaldehyde (PFA, Sigma) in phosphate-buffered saline (PBS) for 30 minutes at room temperature. Using confocal microscopy, viable cells showed the green fluorescence of calcein-AM whilst nuclei of dead cells were indicated by the red fluorescence of EthD-1. The extent of cellular survival was counted in 3-5 visual microscopic fields per slice with at least three slices per group assignment. Quantitation was performed by using a manual count, independently by an observer blinded to the experimental conditions, within four predetermined grid sections outlined by hand using imageJ (NIH analysis software).

2.6 Evaluation of apoptosis

Cells undergoing apoptosis were identified by labeling of DNA strand breaks using a fluorescence based Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection kit in accordance with manufacturer's instructions (ApopTag Fluorescein in Situ Apoptosis Detection kit, Chemicon). Briefly, cerebellar slices prepared and cultured as described above, and were fixed with 4% PFA in PBS for 30 minutes at room temperature. The fixed slices were incubated with terminal deoxynucleotidyl transferase (TDT) enzyme for 1 hour at 37 °C in the dark, and the reaction was stopped by stop/wash buffer, and finally 4,6-Diamidino-2-Phenylindole- (DAPI) was applied. Non-specific staining was examined by omission of the TDT in the labeling procedure. The total number of $TUNEL^+$ cells was calculated across 3-5 random non-overlapping microscopic fields for each slide. The number of $TUNEL^+$ and $DAPI^+$ nuclei were counted and data expressed as percentage $TUNEL^+$ cells.

2.7 Evaluation of cell proliferation

Cell proliferation was assessed by determining the incorporation of 5-bromo-2`-deoxyuridine (BrdU) using a standard incorporation assay. Cultured slices were incubated in 20 µM 5-bromo-2`-deoxyuridine (BrdU, Aldrich-Sigma) during the 24 hours prior to fixation, and then fixed in 4% PFA for 30 minutes at room temperature. The fixed slices were incubated with 1N HCL for 10 minutes on ice followed by 10 minutes incubation with 2N HCL at room temperature before placing them in an incubator at 37 °C for 20 minutes. To neutralize acid, slices were incubated with borate buffer (0.1 M, pH=8.5) for 12 minutes at room temperature, followed by three 5 minute washes in PBS (1 M, pH7.4) with 1% Triton 100-X. Permeabilisation was achieved with a solution of PBS (1 M, pH7.4) with 1% Triton 100-X, glycine (1 M) and 5% normal goat serum for 1 hour prior to incubation overnight with anti-BrdU mono-antibody (eBioscience) at a dilution 1: 50 in PBS followed by DAPI labelling of nuclei.

Quantification of $BrdU^+$ cells was performed in 2-4 fluorescence confocal images for at least three slices per experiment with respect to the total number of DAPI-stained nuclei. Confocal analysis was restricted to the top of the cerebella slice where BrdU antibody was reliable. To examine the identity of the phenotype of newly generated cells and their fate under tested conditions, double immunostaining with fluorescent-conjugated antibodies against BrdU, Chondroitin Sulphate Proteoglycan (NG2), myelin basic protein (MBP), glial fibrillary acidic protein (GFAP) or CD11b (OX-42) was performed. Cell counting of BrdU incorporating cells was performed by a blinded observer using ImageJ software. Quantification was carried out in 2-3 slices per animal: a total of 6-8 fields per section at each time point of experimental groups.

2.8 Immunocytochemistry

Cerebellar OSCs were fixed in 4% PFA in PBS for 30 minutes at room temperature. Following three washes with PBS, the slices were mounted on glass and were then blocked for 1 hour at room temperature with 10% normal goat serum (MP Biomedical), 0.25% Triton 100-X (Sigma) in PBS. After washing for three times 5 min in PBS, the slices were exposed

to primary antibodies (1:300 dilution in PBS) and incubated for 2 hours at 4°C in the dark. The slices were washed three times 5 min in PBS and secondary antibody (1:1000 in PBS) was added over night at 4°C. Finally, the slices were washed three times 5 minutes in PBS, nuclei were counterstained with DAPI, before mounting. The following primary antibodies were used for immunostaining: anti-NG2 (rabbit monoclonal clone, Millipore), anti-MBP (rabbit monoclonal clone, MBL Sigma) and anti-neurofilament (NF) H 200 (mouse, rabbit monoclonal clone, Sigma). All secondary antibodies were conjugated with Alexafluor-488 or Alexafluor-633 (All from Molecular Probes, Invitrogen). The secondary antibodies were diluted at 1:1000 in PBS and incubated for 2 hours at room temperature in the dark. To examine the specificity of immunolabelling with the antibodies, control tissues were processed without primary antibodies, which resulted in no immunostaining. The survival of specific cell populations was counted in a series of confocal fluorescence images.

2.9 Evaluation of myelination

A Series of confocal immunofluorescence images was used to evaluate the status of immunostaining of MBP, and its alignment with neurofilament (NF) protein. Each image was scored by an observer blinded to the experimental conditions using a procedure similar to [28]. Images were given a score of 0-6 based on the following: 6 - extensive colocalization, fibers show complete myelination and processes are long and intact with no fragmentation, 5 fibres are mostly myelinated and processes are long, there are some short fragments, 4 many of the fibers and processes are short intact with some long fragments, 3 – half of the fibers and processes are short and half are long, 2 - most of the fibers and processes are short with short fragments, 1- most of the fibers and processes are short and mostly non-myelinatied,0 –most of the fibers and processes are fragmented and show very little or no myelination as judged by lack of colocalisation between NF and MBP. Average scores across the images for each treatment were calculated Percent of myelination of axons was measured across a minimum of four grid sections. The length of each axon was measured based on NF staining, as was the length of the axon that showed colocalisation with MBP. The proportion of the axon showing colocalisation with MBP was then calculated.

2.10 Statistics

Statistical analysis was performed using SPSS software (version 20). All data represent the mean ± SEM of at least five independent experiments performed in triplicate. Statistical differences were assessed by student t test or by ANOVA test with Tukey post hoc analysis, as appropriate. Results were considered to be statistically significant at $P < 0.05$.

3. Results

3.1 Modelling brain ischaemic injury *ex-vivo*

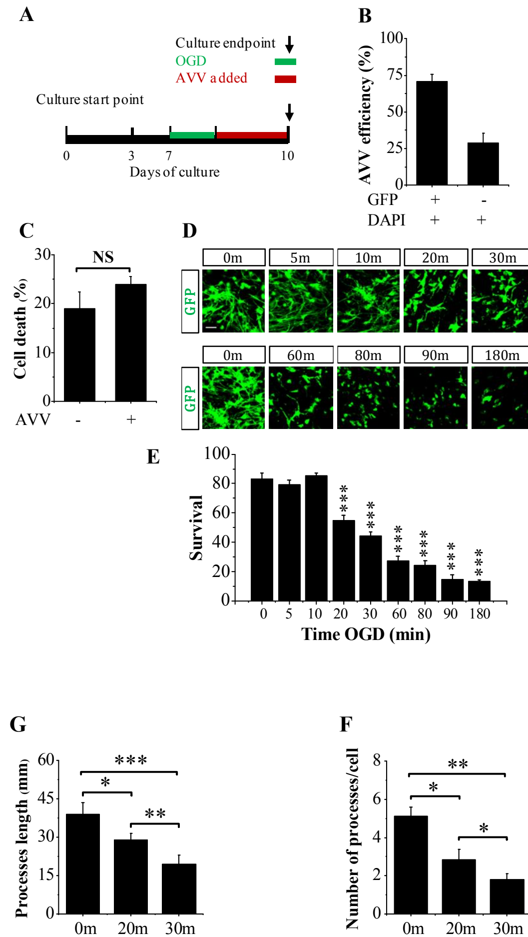

Figure 2. Time course analysis of neural cell survival in cerebellar OSCs after ischaemia. (A) Schematic representation of the time course of the experiment. Cultured slices at 7 DIV were non- or infected with AVV and the proportion of cells expressing GFP was assessed at 10 DIV. (B) Quantification of the AVV efficiency to deliver GFP gene into cultured cerebellar slices. (C) Quantification of neural cell death after AVV infection. (D) Confocal micrographs of cultured slices subjected to defined periods of OGD followed by reperfusion. Viable cells appear in green. Scale bar: 40 µm. (E) Quantitative analysis of the course of neural cell survival after ischaemia. (F-G) Quantification of the GFP $^{+}$ cell process number (F), and length (G). Bars represent means ± SEM. Each experiment was performed in triplicate from three separate rats. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$.

Student's *t* test (B, C) or One-way ANOVA followed by Post-Tukey test (E-G).

In the first instance we sought to develop an experimental protocol that allowed for a short ischaemic insult that was sufficiently severe to measure but not catastrophic that it resulted in complete death. Such a level would allow us to study the potential intervention without destruction of all cells within the slice. To determine the optimum period for cell survival after ischaemic injury, we tested the effects of varying durations of OGD on organotypic slices cultured for 7 days *in-vitro* (DIV) and monitored neural cell survival using an AVV expressing GFP (Figure 2A). The effects of ischaemic injury on the somata and process integrity of cells were then measured. Cerebellar slices were infected with AVV, cultured for a further 3 DIV and were incubated with ethidium homodimer-1 to asses cell viability. Control slices showed 18.99 ± 3.46 % dead cells which were not different from those treated with AVV, 24.02 ± 1.53 %, illustrating that the virus had no detrimental effect on cell survival in the infected slices (Figure 2B, C). The time course of cell survival in slice cultures revealed that ischaemia caused a significant reduction in neural cell survival in a manner dependent on the duration of OGD (Figure 2D, E). There was no difference ($P > 0.05$) in cell survival between control (0 minutes) and those slices subjected to 5 or 10 minutes OGD (Figure 2E). Comparably, slice cultures subjected to 20 and 30 minutes OGD resulted in ~ 58.6 % and 52.3 % reduction in cell viability, respectively (Figure 2E). Although there were no differences ($P > 0.05$) in the number of viable cells between slices subjected to either 20 or 30 minutes OGD, loss of process integrity (number and length of the processes per cell) reached significance ($P < 0.01$) after 30 minutes compared with that seen after 20 minutes OGD (Figure 2F-G). Prolonged OGD, 60, 80, 90 or 180 minutes triggered a significant ($P < 0.001$) cell loss (Figure 2D, E), illustrating that cell viability within the slice is dependent on the duration and severity of the ischaemic insult in this model. In all subsequent experiments, slices were exposed to 20 minutes ischaemia to induce mild cell death as a culture model of infant ischaemia that caused measurable damage over a 3 day period without causing catastrophic injury to the tissue slice.

3.2 Astrocytes and microglia *ex-vivo* model

To examine the responses of astrocytes and microglia in the slice culture model, we analyzed expression of the astrocyte marker GFAP and the microglia marker OX-42 proteins immunocytochemically at 0, 3, 7, 8, and 10 DIV (Figure 3A-B). At 7 DIV the GFAP$^+$ and OX-42$^+$ cells represented 12% and 10 % of total cells respectively (Figure 3C, D). At 8 DIV GFAP$^+$ and OX-42$^+$ cells represented 16 % and 7 % of total cells respectively (Figure 3C, D). At 10 DIV, percent GFAP$^+$ and OX-42$^+$ cells were 14% and 9 % of total cells, respectively (Figure 3C, D). Over time, there was no significant ($P > 0.05$) difference in the proportion of cells expressing OX-42 (Figure 3D). Overall, these data suggest that there is no progressive reactive gliosis over an extended period of time in these cultures. Note that the microglial marker OX-42 was never expressed by cells expressing either NG2 or GFAP (Figure 3E, F).

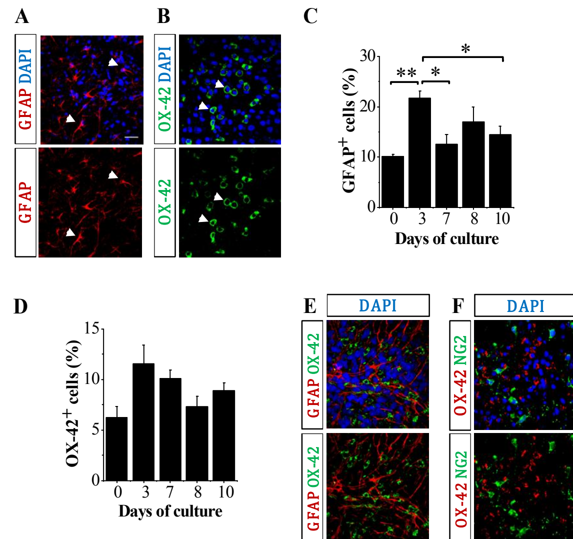

Figure 3. Astrocytes and microglia in cerebellar OSCs. (A, B) Representative immunostained sections for GFAP (A, red) and OX-42 (B, green). Blue color shows DAPI-stained nuclei. Arrowheads point to astrocytes (A) and microglia (B). Scale bar: 30 µm. (C, D) Quantification of GFAP$^+$ cells (C) and OX-42$^+$ cells (D). (E, F) Double immunofluorescence labelling of (E) OX-42 (red) and GFAP (green) or (F) OX-42 (red) and NG2 (green). Arrowheads indicate exemplar microglia. Bars represent means ± SEM. $P < 0.05$. One-way ANOVA followed by Post-Tukey test.

3.3 Maturation of oligodendrocytes *ex-vivo* model

To examine the OPCs responses in white matter of this slice culture model, the proportions of NG2$^+$ OPCs (NG2, an early oligodendroglial marker) (Figure 4B) and the colocalization with the proliferation marker BrdU (Figure 4C) in 7-10 day old cultures were examined. During development OPCs differentiate to mature OLs which no longer express the OPC marker NG2 but instead express MBP. Over time, the proportions of NG2$^+$ OPCs and NG2$^+$/BrdU$^+$ double-labelled cells were reduced. The percentage of NG2$^+$ cells was significantly ($P = 0.015$) reduced by up to1.75-fold (17%) in 10 day old cultures compared

to their percentage (28%) in 7 day old cultures (Figure 4E). There was no difference in the percentage of NG2$^+$ cells in 9 day old cultures from those examined at 7, or 10 DIV (Figure 4E). The analysis of cell proliferation using BrdU labeling revealed that the percentage of NG2$^+$/BrdU$^+$ double-labelled cells was reduced over time. For example, in 10 day old cultures, the percentage of BrdU$^+$ NG2$^+$ cells was decreased by up to 1.6-fold (24.71 ± 1.73%) and 1.4-fold (34.44 ± 3.08%) compared to 7 and 9 day old cultures, respectively, (Figure 2, 4E). There was no significant difference in the percentage of BrdU$^+$ NG2$^+$ cells in 7 and 9 day old cultures (Figure 4E). Mature oligodendrocytes (OLs) were monitored immunocytochemically using antibodies to the mature OL marker MBP (Figure 4A). Quantitative analysis showed that the proportion of mature MBP$^+$-OLs within the cultured slices increased by up to 1.4-fold (15.98 ± 2.02% to 22.83 ± 4.08%) in 9 day old cultures compared to 7 day old cultures (Figure 4E). Continued analysis revealed that the MBP$^+$ cells percentage was further increased (P = 0.002) up to 1.9-fold (28.67 ± 2.74%) in 10 day old cultures compared to 7 day old cultures (Figure 4E). Taken together these data suggest that enhanced mature OL numbers and the reduction in OPCs over the culture period follow similar developmental changes in slice culture as in intact CNS where in most vertebrates, OPC proliferation and maturation takes place mainly during postnatal life [29-30].

3.4 Development of myelination *ex-vivo*

Having established that OLs were developing and maturing in our culture system we next investigated the extent to which myelination around axons was taking place. We used immunohistochemistry to observe overlap between MBP and NF, a marker of neural axon (Figure 5A). After three days of culture, MBP-expressing OLs were observed but there was little alignment of MBP$^+$ OLs processes with axons as judged by the level of colocalisation of MBP and NF (Figure 5A, B). Alignment levels between MBP and NF staining increased over time and the levels of MBP/NF colocalisation were significantly higher at 7 and 10 DIV compared to 3 DIV (Figure 5A, B). At 7 DIV (P7+7 DIV), the number of maturing MBP$^+$ oligodendrocytes was increased as was the expression of MBP as judged by the intensity of MBP staining (Figure 5C) and their processes were oriented largely parallel to axons (Figure 5B). By 10 DIV, the extent of alignment between axons and mature MBP$^+$ OL processes was more evident in slice culture (Figure 5A). Levels of myelination were also assessed using ranking analysis of myelinated cerebellar slice cultures (where the highest rank represents most myelination, see methods) of myelinated cerebellar slices (Figure 5A. B). At birth the human brain has little myelin. During the early postnatal life, OPCs migrate into axon tracts and they initiate the myelination necessary for skilled acquisition motor behavior throughout development [4]. In each brain region, myelination takes place at different times. Generally, formation of myelin peaks in the first 3 weeks of the rodents and in the first year of human life [31-33]. It has been demonstrated that the timing of myelination onset peaks in the first postnatal month [33]. This may explain why at 10 DIV (P7+10 DIV) some axons are not ensheathed by adherent MBP$^+$ cells processes as well as some MBP$^+$ cells processes do not show any alignment with the axonal marker although there was a high alignment degree between myelin and axonal markers over time.

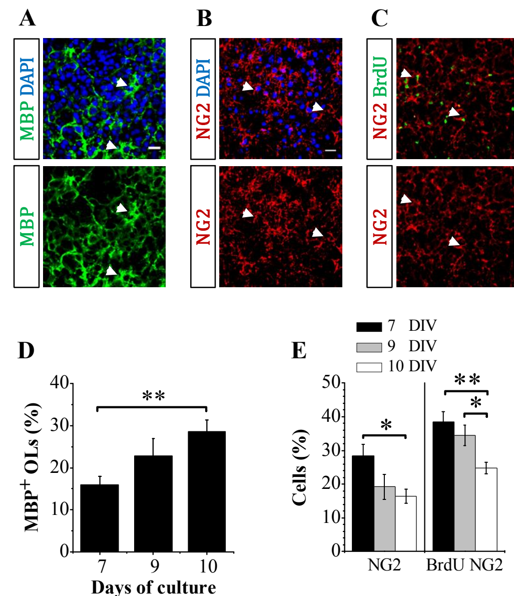

Figure 4. OL survival and responses of OPCs in cerebellar OSCs. (A-C) Representative slice sections stained for MBP (A, green). NG2$^+$ (B, red) and NG2$^+$/BrdU$^+$ (C, red/green). Blue color shows DAPI-stained nuclei. Scale bar: 50 µm (A) and 20 µm (B, C). (D) Quantification of MBP$^+$-OLs. (E) Quantification of NG2$^+$ and BrdU$^+$ NG2$^+$ cells. Bars represent means ± SEM., *P < 0.05, **P < 0.01. One-way ANOVA followed by Post-Tukey.

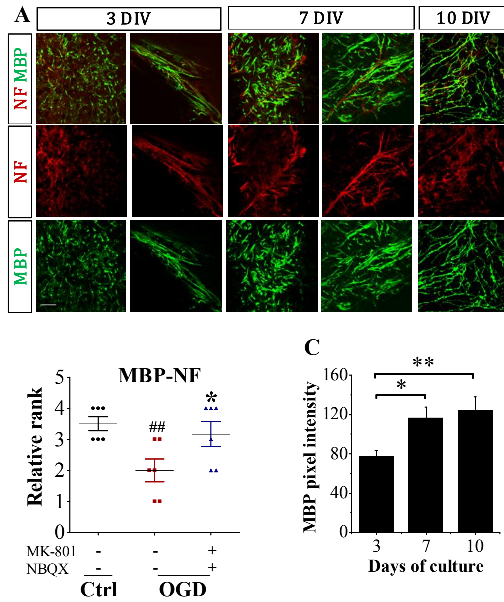

Figure 5. Development of myelination in cerebellar OSCs. (A) Cultures fixed at 3, 7 or 10 DIV and immunolabeled for and myelin marker MBP (green) and axonal marker NF (red). Scale bar: 40 µm. (B) Rank analysis. Highest rank represents most myelination (NF/MBP staining) (see methods). (C) Graph showing pixel intensity of MBP staining. Data are represented as mean ± SEM for at least 5 independent biological replicates. *$P < 0.05$, **$P < 0.01$. Kruskal-Wallis H test followed by Mann-Whitney U test (B) or One-way ANOVA followed by Post-Tukey test (C).

3.5 Ischaemic injury mediated delayed cell death

We investigated the early and long-term effects of ischaemia on neural cell survival in the slice cultures. Ischaemic injury (20 minutes OGD) followed by a reperfusion triggered a significant ($P < 0.05$) reduction in cell survival (59.48 ± 2.86 in OGD vs. 72.74 ± 6.5 in Ctrl), and the damage reaching a peak 72 hours after injury (41.1 ± 3.27 in OGD vs. 78.46 ± 2.31 in Ctrl), (Figure 6A-B). TUNEL staining was used to examine whether the neural cell death occurred by apoptosis. There was no difference in the proportion of apoptotic cell death at 20 minutes after OGD treatment compared to control (11.51 ± 2.32 in OGD vs. 8.61 ± 1.62 in Ctrl), whereas 72 hours after OGD treatment there was a significant ($P < 0.001$) elevation in the proportion of TUNEL$^+$ cells (39.25 ± 2.82 in OGD vs. 11.25 ± 1.22 in Ctrl) (Figure 6C, D), indicative of the delayed cell death that was apoptosis in its nature.

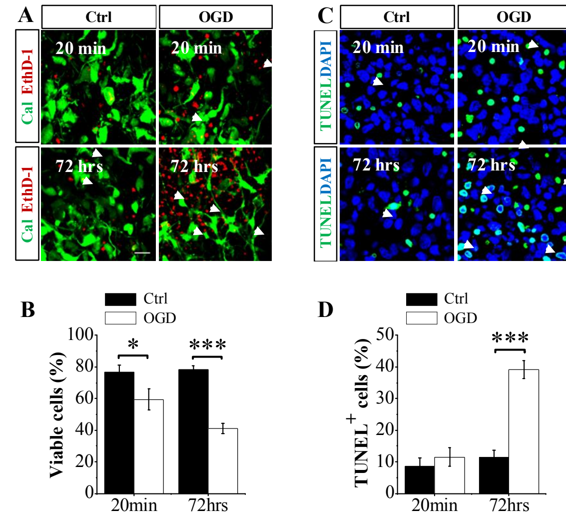

Figure 6. Ischaemia causes delayed cell death in cerebellar OSCs. Confocal micrographs of cultured cerebellar slices exposed to 20 min OGD followed by reperfusion examined 20 min or 72 hr post-OGD (A) stained with ethidium homodimer (red) and Calcein (green) or (C) labeled using TUNEL assays (green). Scale bar: 20 µm. (B) Quantification of neural viable cells as judged by ethidium homodimer and calcein staining (D) Percent of TUNEL$^+$ cells in slice cultures after 20 min and 72 hr post OGD. Bars represent means ± SEM. Each experiment was performed in triplicate from three separate rats. # $P < 0.05$, ### $P < 0.001$. Student's t test.

3.6 Ischaemia causes myelination disturbance

Next we investigated the long-term effect of OGD-induced cellular damage on myelin-axon alignment (myelination), co-staining of OSCs for myelin marker MBP and axonal marker NF was carried out. Under control conditions, extensive colocalization between MBP and NF was observed after 10 DIV (Figure 7A). The morphology of OLs conformed to prior descriptions, characterized by primary process running parallel to axons [9, 34]. Cultured slices treated to 20 min of OGD at 7 DIV and then returned to normal culture conditions for a further 3 days showed extensive loss of MBP and NF colocalisation and myelination of axons (Figure 7A, B). Together, these results demonstrate that this culture system can be a useful tool to study the damage to developing white matter in response to ischaemia induced cell damage.

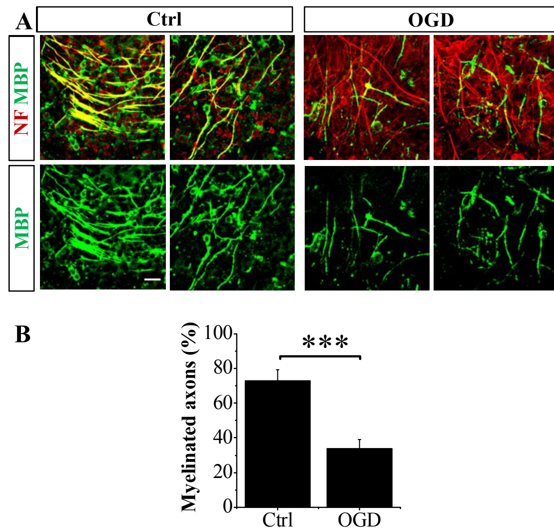

Figure 7. Myelin disruption in cerebellar OSCs after simulated ischaemia. (A) Representative co-staining of cerebellar OSCs for NF (red) and MBP (green). Scale bar: 30 µm. (B) Quantification of percentage of myelination of axons (%). ###*P* < 0.001. Data is represented as mean ± SEM for at least 4 independent biological replicates. Student's t test.

4. Discussions

Here we present a system that enables the modeling of the long term effects of a short ischaemic insult within tightly controlled conditions. The system utilizes established methods of culturing cerebellar slices as described by [21] and modified by [22-23, 35] however within our system we have introduced treatment regimens that include ischaemic insult to the cultures at 7 days *in-vitro*. Previous systems have exposed *ex-vivo* tissue preparations to continuous ischaemic insult until the tissue is effectively completely dead [9, 36]. In this preparation we have identified a regimen that enables survival of the tissue whilst achieving some level of cell death/ injury giving us the opportunity to study the tissues capability to recover from the insult and more closely mimicking a clinically relevant event (Figure 2E).

We have previously used GFP transgenic animals for such imaging with both OLs [9] and astrocytes [36] and these systems give excellent opportunities to follow the progress of injury during a persistent ischaemic insult. Although these methods may well work within our protocol we decided in the first instance to use transient labeling of all cells with adenovirus transformation of all cells using a vector containing GFP under the control of the CMV promoter, [37-38]. This approach enables us to label the majority of cells within the slice and in our preparation this method was effective, (Figure 2D), whilst having no affect on cell viability, (Figure 2C). Using AVV transformation made it possible to assess global injury to the slices during ischaemia and given the correct protocols AVV transformation of slices with GFP would certainly be useful for assessment of differing treatment conditions to alleviate injury (Figure 2D, E). Within the conditions of our culture we found that the staining of cells remained active for in excess of the 3 days required for our protocol (Figure 2D, E).

In many instances it will be particularly desirable to observe and quantitate the effect of the ischaemic insult on specific cell types. In this way the progress of the different cells types and the benefits or otherwise of a particular intervention can be demonstrated for these cell types. In this work we used antibodies to neurofilament 200, neurons; MBP, OLs and myelin formation [39]; chondroitin sulfate proteoglycan, NG2 glia; GFAP [40], astrocytes [41]; OX-42 [CD11b], microglia [42]. Here we demonstrate that this strategy can be applied successfully to cerebellar slices held in culture and that staining is sufficient to enable analysis of injury levels within a tissue sample when subject to ischaemic insult (Figures 3-5). We also demonstrate that we can effectively follow the progress of myelination in this preparation with dual labeling of OLs and neurons.

The final analytical method we utilized involved analysis of delayed cell death and myelination disturbance. Previous studies using *ex-vivo* tissue preparations have investigated short term effects of an ischaemic insult however it is well documented that delayed cell death occurs where the affected individual survives following an ischaemic insult [14, 43]. Using this preparation we are able mimic this paradigm *in-vitro* and using a TUNEL assay and immunocytochemistry were able to assess the delayed cell death and demyelination following ischaemic insult 72 hours post-insult (Figure 6-7).

CNS myelination formed by mature OLs is essential for enhancing the velocity of transmission signals and is required for the normal functions of the brain [1-2]. Failure of remyelination following injury/disorder impairs saltatory conduction of transmission of signals, particularly over longer distances, leading to axonal degeneration, and is correlated with acquired and inherited disorders, including multiple sclerosis, cerebral palsy, and leukodystrophies [44-46]. Clarification of re myelination process has implications in the management of the CNS in health and disease. Cerebellar OSCs enable the *in-vitro* study of myelination in the developing brain and provide a mechanism for the study of the effects of an ischaemic insult on the progress of myelination through this vital period.

In summary we have developed and characterized an *ex-vivo* culture system with which we can model ischaemic insult and study the effects of such an insult on white matter. By allowing a precise and consistent control over the conditions this model system will be useful in studying the molecular mechanisms involved in white matter injury and identifying potential therapeutic avenues with which the resulting damage can be minimised.

Acknowledgements:

Work was funded by a Higher Education Libyan scholarship from University of Tripoli to M. Al-Griw

Corresponding Author:
Dr. Mohamed A. Al-Griw
Division of Developmental Biology
Zoology Department
University of Tripoli
Tripoli-Libya
E-mail: m.algriw@zo.uot.edu.ly

References

1. Volpe, J.J., *Neurology of the Newborn, 4th Ed., W. B. Saunders Co., Philadelphia, PA.* 2004.
2. Ishii, A., et al., *ERK1/ERK2 MAPK Signaling is Required to Increase Myelin Thickness Independent of Oligodendrocyte Differentiation and Initiation of Myelination.* Journal of Neuroscience, 2012. 32(26): p. 8855-64.
3. Back, S.A., et al., *Selective vulnerability of late oligodendrocyte progenitors to hypoxia–ischemia.* Journal of Neuroscience, 2002. 22: p. 455-463.
4. Dewar, D., S.M. Underhill, and M.P. Goldberg, *Oligodendrocytes and Ischemic Brain Injury.* Journal of Cerebral Blood Flow & Metabolism, 2003. 23: p. 263-274.
5. Grossman, S.D., L.J. Rosenberg, and J.R. Wrathall, *Temporal–spatial pattern of acute neuronal and glial loss after spinal cord contusion.* Experimental Neurology, 2001. 168: p. 273-282.
6. Deng, W., *Neurobiology of injury to the developing brain.* Nature | Reviews | Neuroscience, 2010. 6: p. 328-336.
7. Matute, C., *Glutamate and ATP signalling in white matter pathology.* Journal of Anatomy, 2011. 219: p. 53-64.
8. Fern, R. and T. Moller, *Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop.* Journal of Neuroscience, 2000. 20: p. 34-42.
9. Salter, M.G. and R. Fern, *NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury.* Nature, 2005. 438(7071): p. 1167-71.
10. Lorenzini, P., et al., *Metabotropic glutamate receptors and brain ischemia: differential effects on phospoinositide turnover in young and aged rats.* Ann Ist Super Sanita, 2000. 36(4): p. 453-7.
11. Tekko¨k, S.B., Z. Ye, and B.R. Ransom, *Excitotoxic mechanisms of ischemic injury in myelinated white matter.* Journal Cerebral Blood Flow & Metabolism, 2007. 27: p. 1540-1552.
12. Fern, R., *Intracellular calcium and cell death during ischemia in neonatal rat white matter astrocytes in situ.* Journal of Neuroscience, 1998. 18: p. 7232-7243.
13. Matute, C., *Calcium dyshomeostasis in white matter pathology.* Cell Calcium, 2010. 47: p. 150-157.
14. Stys, P.K., *White matter injury mechanisms* Current Molecular Medicin, 2004. 18: p. 113-130.
15. Back, S.A., et al., *Hypoxia-ischemia preferentially triggers glutamate depletion from oligodendroglia and axons in perinatal cerebral white matter.* Journal of Cerebral Blood Flow Metabolism, 2007. 27: p. 334-347.
16. Fern, R., *Ischemia: Astrocytes show their sensitive side.* Progress in Brain Research, 2001. 132: p. 405-411.
17. Domercq, M., et al., *Expression of glutamate transporters in rat optic nerve oligodendrocytes.* European Journal of Neuroscience, 1999. 11: p. 2226-2236.
18. Thomas, R., et al., *Acute ischemic injury of astrocytes is mediated by Na-K-Cl cotransport and not Ca^{2+} influx at a key point in white matter development.* Journal of Neuropathology & Experimental Neurology, 2004. 63: p. 856-871.
19. Peasley, M.A. and R. Shi, *Resistance of isolated mammalian spinal cord white matter to oxygen-glucose deprivation.* Am J Phys Cell Phys, 2002. 283: p. C980-989.
20. Volpe, J.J., *Neurobiology of periventricular leukomalacia in the premature infant.* Pediatric Research, 2001. 50: p. 553-562.
21. Stoppini, L., P.-A. Buchs, and D. Muller, *A simple method for organotypic cultures of nervous tissue.* Journal of Neuroscience Methods, 1991. 37: p. 173-182.
22. Kluge, A., et al., *Tracing of the entorhinal-hippocampal pathway in vitro.* Hippocampus, 1998. 8: p. 57-68.
23. Ghoumari, A.M., E.E. Baulieu, and M. Schumacher, *Progesterone increases oligodendroglial cell proliferation in rat cerebellar slice cultures.* Neuroscience, 2005. 135: p. 47–58.

24. Wang, Y., et al., *The Effects of 17â-estradiol on Neuronal PC12 Cells Injuried by OGD-R and NO/iNOS System Mechanism.* Life Science Journal, 2010. 7: p. 34-40.
25. Bingham, A.J., et al., *The repressor element 1-silencing transcription factor regulates heart-specific gene expression using multiple chromatin-modifying complexes.* Mol Cell Biol, 2007. 27(11): p. 4082-92.
26. Wood, I.C., et al., *Interaction of the repressor element 1-silencing transcription factor (REST) with target genes.* J Mol Biol, 2003. 334(5): p. 863-74.
27. He, T.C., et al., *A simplified system for generating recombinant adenoviruses.* Proc Natl Acad Sci U S A, 1998. 95(5): p. 2509-14.
28. Xiang, Z. and S.A. Reeves, *Simvastatin induces cell death in a mouse cerebellar slice culture (CSC) model of developmental myelination.* Experimental Neurology, 2009. 215: p. 41-47.
29. Miller, R.H., *Regulation of oligodendrocyte development in the vertebrate CNS.* Progress in Neurobiology, 2002. 67: p. 451-467.
30. Baumann, N. and D. Pham-Dinh, *Biology of oligodendrocyte and myelin in the mammalian central nervous system.* Physiological reviews, 2001. 81: p. 871-926.
31. Rivkin, M.J., et al., *Oligodendroglial development in human fetal cerebrum.* Annual Neurology, 1995 38: p. 92-101.
32. Back, S.A., et al., *Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury.* Journal of Neuroscience, 2001. 21: p. 1302–1312.
33. Benjamins, J.A. and P. Morell, *Proteins of myelin and their metabolism.* Neurochemistry Research, 1978. 3: p. 137-174.
34. Butt, A.M. and B.R. Ransom, *Morphology of astrocytes and oligodendrocytes during development in the intact rat optic nerve.* Journal of Comparative Neurology, 1993. 338: p. 141-158.
35. Harrer, M.D., et al., *Live imaging of remyelination after antibody-mediated demyelination in an ex-vivo model for immune mediated CNS damage.* Experimental Neurology, 2009. 216: p. 431-438.
36. Salter, M.G. and R. Fern, *The mechanisms of acute ischemic injury in the cell processes of developing white matter astrocytes.* Journal of Cerebral Blood Flow & Metabolism, 2007. 28: p. 1-14.
37. Forsayeth, J.R. and P.D. Garcia, *Adenovirus-mediated transfection of cultured cells. University of California at San Francisco.* BioTechniques, 1994. 354: p. 354-6, 357-8.
38. Foecking, M.K. and H. Hofstetter, *Powerful and versatile enhancer-promoter unit for mammalian expression vectors.* Gene, 1986. 45: p. 101-105.
39. Groome, N.P., et al., *Region-specific immunoassays for human myelin basic protein.* Journal of Neuroimmunology, 1986. 12: p. 253-264.
40. Butt, A.M., et al., *Cells expressing the NG2 antigen contact nodes of Ranvier in adult CNS white matter.* Glia, 1999. 26: p. 84-91.
41. Rodríguez-Pérez, L.M., et al., *Immunocytochemical characterisation of the wall of the bovine lateral ventricle.* Cell Culture Research, 2003. 314: p. 325-235.
42. Milligan, C.E., T.J. Cunningham, and P. Levitt, *Differential immunochemical markers reveal the normal distribution of brain macrophages and microglia in the developing rat brain.* Journal of Comparative Neurology, 1991. 314: p. 125-135.
43. Han, B.H., et al., *BDNF blocks caspase-3 activation in neonatal hypoxia–ischemia.* Neurobiology of Disease, 2000. 7: p. 38-53.
44. Franklin, R.J.M., *Why dose remyelination fail in multiple sclerosis?* Nanture Review | Neuroscience, 2002. 3: p. 705–714.
45. Trapp, B.D., et al. *Cell Biology of Myelin Disorder*. 2004: California: Elsevier Academic Press.
46. Mar, S. and M. Noetzel, *Axonal damage in leukodystrophies.* Pediatric Neurology, 2010. 42: p. 239-242.

4/21/2017