Extreme sensitivity of myelinating optic nerve axons in a rodent model of perinatal ischemic injury

Christian Zammit, M.D.,[1] Mario Valentino, Ph.D.,[2] James J. P. Alix, MRCP., Ph.D.,[3] Art Riddle, Ph.D.,[4] Charles K. Meshul, Ph.D.,[6] Stephen A. Back, M.D., Ph.D.,[4,5] Robert Fern,Ph.D.[3]

[1]Department of Anatomy, [2]Physiology and Biochemistry, University of Malta, Malta. [3]Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, UK.
[4]Department of Pediatrics, [5]Neurology, Oregon Health & Sciences University, Portland, Oregon, USA. [6]Veteran Affairs Medical Center, Portland, Oregon, USA.

Objectives and Principal findings

- Developing central white matter is subject to ischemic-type injury during the period that precedes myelination. At this stage in maturation, axons initiate a programme of ion channel re-distribution.
- Here we test the hypothesis that during radial expansion axons display heightened ischemic sensitivity, when clusters of Ca^{2+} channels decorate future node of Ranvier sites.
- Our results show that this axon population is more sensitive to ischemia than neighbouring myelinated axons and to smaller axons yet to initiate radial expansion. Injury is mediated by Ca^{2+} influx through Ca^{2+} channels expressed in axolemma clusters.
- A pharmacological strategy designed to protect both small and large diameter pre-myelinated axons proved 100% protective against acute ischemia studied under modelled ischemia *in vitro.*

Introduction

- Cerebral white matter injury (WMI) is increasingly recognized as a common form of perinatal brain injury that predisposes to cerebral palsy as well as cognitive and learning disabilities.
- Despite the growing impact of WMI, it is unclear whether common cellular and molecular mechanisms define WMI pathogenesis in preterm and term neonates.
- Although numerous studies have defined a role for glial vulnerability in WMI, there has been limited study of the susceptibility of immature axons.

Methods and Results

Maturation-dependent axonal vulnerability in the optic nerve

- We compared the magnitude of axonal damage at post-natal day 10 (P10), 20 (P20) and adult Thy-1/GFP-M mice optic nerves, using confocal microscopy. Pathological changes including local swelling, axonal beading and fragmentation were observed (Fig 1 C-E).
- Consistent with earlier studies[1], there is heightened sensitivity to ischemic conditions in axons of the P20 optic nerve.
- Electron microscopy revealed generalized areas of axonal swelling with dissolution of axoplasmic inclusions. Less damaged axons showed localized regions of sub-myelin swelling where the sheath occasionally buckled from the axolemma and formed vesicular debris (Fig 1 A)

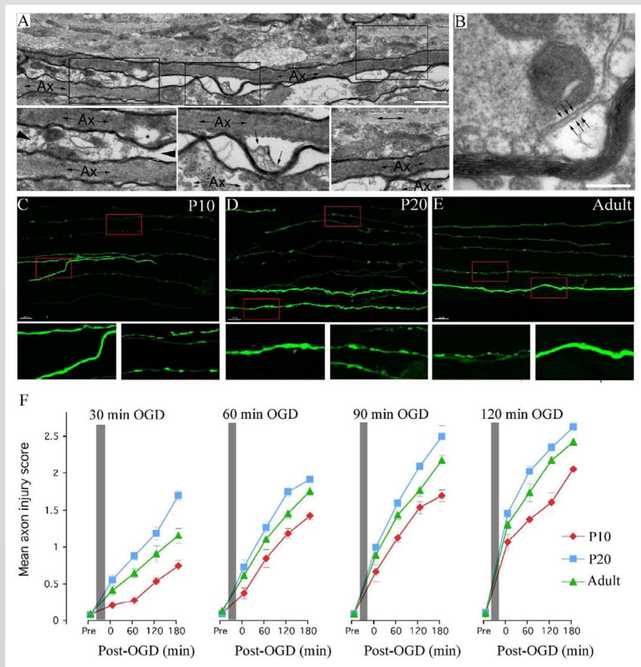

Figure 1: OGD-induced injury in rodent axons

***A**: Ultra-micrograph from post-OGD RON, showing regional myelin detachment from the axoplasm, shown in cross section in "B". "Ax" = myelinated axon. Bar = 100 nm.*

***C-E** Pathological changes in GFP-M axons in mice at P10 , P20 and adult exposed to 60 min OGD + 60 min recovery. Boxed regions are expanded below. Bar = 10 µm.*

***F**: The mean axon injury score of GFP-M axons collected after 30, 60, 90 or 120 min of OGD . Data collected from P10 (red), P20 (blue) and adult (green) MONs is included. Axon injury in P20 MONs is higher under all conditions than at P10 or adult.*

Axons preparing for myelination have a heightened sensitivity to OGD

- Ultra-micrograph morphometric analysis of wild-type P20 MON revealed populations of premyelinated (0.10-0.56 µm), ensheathed (0.35-0.73 µm) and myelinated (0.54-2.20 µm) axons (Fig 2 A-D).
- A range of GFP-M(+) axon diameters were observed in Thy-1/GFP-M mice of the same age over the range 0.43-1.90 µm (Fig 2 E, F). Therefore our model allows imaging of large pre-myelinating, ensheathed and myelinated axons but not small pre-myelinating axons.
- Following a standard 60 min OGD + 60 min recovery protocol applied to the transgenic P20 MON, injury scoring revealed an inverse relationship between axon pathology and diameter, with the large pre-myelinated axons damaged most and injury tailing off as axons progressed through ensheathment and myelination (Fig 2G).

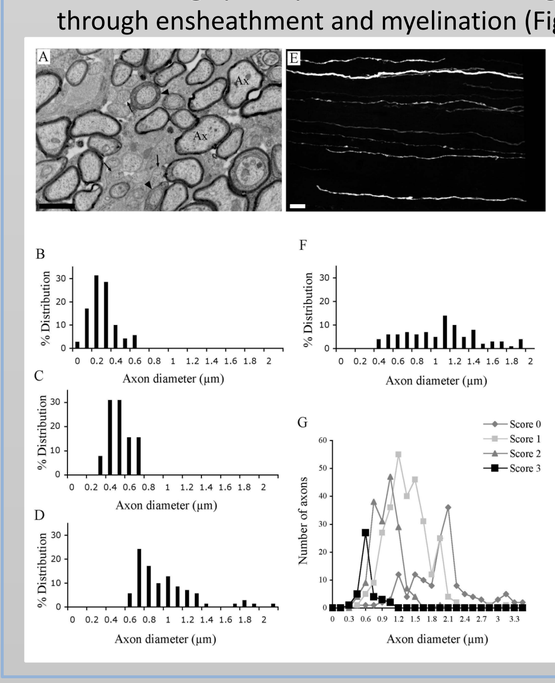

Figure 2: Central axons preparing for myelination have a heightened sensitivity to OGD.

***A**: Electron micrograph of wild-type P20 MON showing pre-myelinated axons (arrows), ensheathed axons (arrow heads) and early myelinated axons ("myl") in cross section. Bar = 1 µm*

***B-D**: Diameter spectra for pre-myelinated axon (B), ensheathed axons (C) and myelinated axons (D).*

***E**: GFP-M fluorescent axons in transgenic P20 MO. Bar = 5 µm.*

***F**: Diameter spectrum of fluorescent axons.*

***G**: Injury scores for fluorescent axons following 60 min OGD + 60 min recovery, plotted against axon diameter. Note that the smallest fluorescent axons corresponding to large pre-myelinated and enstheathed axons, are the most damaged.*

Block of intracellular Ca^{2+} release does not protect P10 RON axons

- Developing RON axons contain numerous inclusions that are potential intracellular Ca^{2+} stores (Fig 3 A).
- The addition of neither thapsigargin (a sarco/endoplasmic reticulum Ca^{2+}-ATPase pump inhibitor) nor diltiazem (an L-type VGCC blocker) increased the protection against OGD-induced injury from that seen in zero Ca^{2+} alone (Fig 3 B – E).

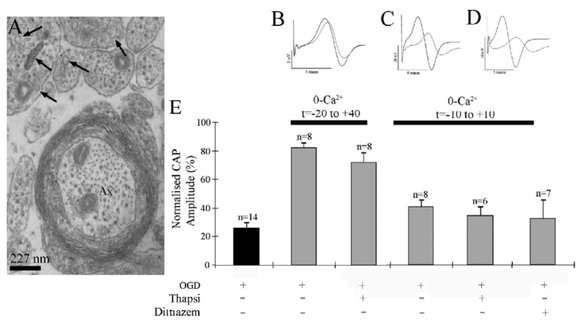

Figure 3: Central axons preparing for myelination have a heightened sensitivity to OGD.

***A**: High-power electron micrograph showing the presence of intracellular inclusions with the features of endoplasmic reticulum in premyelinated and actively myelinating axons (e.g., arrows).*

***B- D**: CAPs recorded from a RON that was exposed to zero Ca2+/50 µM EGTA, plus thapsigargin (B-C), and diltiazem (D)*

***E**: Data summary showing that neither thapsigargin protocol nor diltiazem significantly increased the degree of recovery compared to the relevant control.*

Combined block of glutamate receptors and voltage gated calcium channels is highly protective against degeneration of premyelinated axons

- Electrophysiology recordings of compound action potential (CAP) across optic nerves showed that the combined action of VGCC blockers and GluR blockers resulted in complete recovery from 60 min of OGD (Fig 4 A).
- Immuno-staining of axons for either neurofilament light (NF-L) or heavy (NF-H) corroborated this finding (Fig 4 B).
- Ultrastructural analysis further proved that addition of VGCC block to GluR block augmented protection to include the larger pre-myelinated axons (Fig 4 C-G).

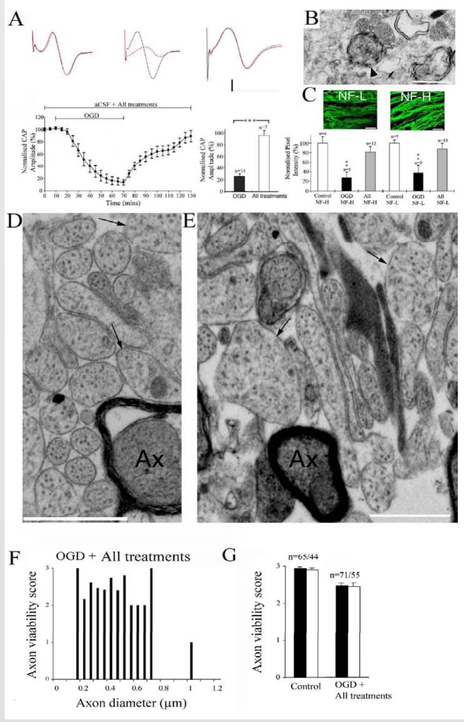

Figure 4: Combined GluR and VGCC block is highly protective of all premyelinated axons

***A**: In the presence of ω-agatoxin VIA+diltiazem+MK-801+NBQX ("All treatments") CAP area recovered fully from a 60 min period of OGD. Bars = 2 mV/ 1 msec.*

***B**: Electron-micrograph showing typical ultrastructural changes following the OGD protocol in the absence of drugs. Larger diameter ensheathed axons (arrow head) has lost all microtubules. Bar = 0.5 µm.*

***C**: NF-L and NF-H staining was also protected during OGD by the "all treatments" protocol.*

***D, E**: The ultrastructural features of axons in nerves exposed to 60 min OGD and recovery in the presence of 'all treatment'. Even large pre-myelinated axons retain membrane integrity and contain microtubules (arrows). Myelinated axons ("Ax") retain membrane integrity but have few microtubules and exhibit myelin pathology. Bar = 1 µm.*

***F**: Axon health was assessed using (axon viability score), where a score of 3represents a normal appearance and 0 represents total axon breakdown*

***G**: Mean viability score in treated RONs for small (<0.4µm diameter, black bars) and large (>0.4µm diameter, white bars) pre-myelinated axon does not differ significantly from control.*

Combined block of glutamate receptors and voltage gated calcium channels protected larger pre-myelinated axons.

- Examination of microtubule numbers in cross section electron micrographs showed that in small diameter (< 0.4 µm) axons microtubule disassembly was prevented following OGD by GluR block and by "all treatments" (Fig 5 A).
- On the other hand microtubules in larger diameter (> 0.4 µm) pre-myelinated axons were not protected from OGD by GluR block but were by "all treatments" (Fig 5 A), consistent with a role for VGCC in damage to this axon group.
- OGD resulted in a significant reduction in the number of tubules in axons of all diameters. GluR block prevented tubule loss following OGD only in axons <0.4 µm, whilst the addition of VGCC provided protection also to the larger pre-myelinating axons. Fig 4 B)

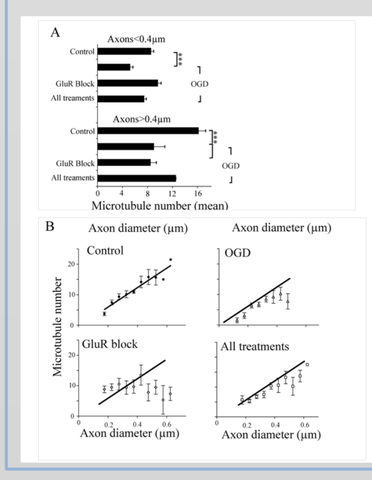

Figure 5: Addition of VGCC block to GluR block protected larger pre-myelinated axons

***A**: Microtubule number in small axons (<0.4 µm) falls significantly following OGD, an effect that is countered by GluR block; this effect is not augmented by combined GluR + VGCC block ("All treatments"). Microtubule number in larger axons (>0.4 µm) falls significantly following OGD, an effect that is not reduced by GluR block but is prevented by combined GluR and VGCC block.*

***B**: Mean microtubule number plotted against axon diameter (0.05 µm bins) for pre-myelinated axons. Note that OGD produces a significant reduction in the number of microtubules at any given axon diameter while GluR block protects the smaller axons and "all treatments" prevents the loss of microtubules across the whole diameter spectrum ("*" = P<0.05 vs., control).*

Conclusion

- By imaging GFP-M expression that was restricted to pre-myelinated, ensheathed and early myelinated axons, we found highly selective injury of the smallest fluorescent axons. These corresponded to the larger pre-myelinated axons.
- We found no evidence for a contribution from intracellular Ca^{2+} release to ischemic axon injury at this developmental stage.
- Prior to the onset of myelination, axons initiate radial expansion and undergo ion channel re-organization in preparation for the formation of nodes of Ranvier and myelination. The appearance of P/Q- and L-type voltage-gated VGCC clusters at putative node sites is an early event in this process[2], and our results indicate that these VGCCs act as a significant route of toxic Ca^{2+} influx during hypoxia-ischemia, making axons more vulnerable to ischemic injury than at any other point in their maturation

Clinical Relevance

- Early maturing axons having initiated diameter expansion and expressing clusters of functional VGCC, are exquisitely sensitive to ischemic injury. This is a property that this axon population shares with preOLs, previously regarded as the most ischemia-sensitive element with immature white matter. By contrast, axons yet to enter this developmental window, or which have passed through it to initiate myelination, have a much higher ischemic tolerance.
- Recent clinical data highlight the importance of axon pathology in developing white matter injury. The elevated susceptibility of early maturing axons to ischemic injury described here may significantly contribute to selective white matter pathology and places these axons alongside pre-oligodendrocytes as a potential primary target of both injury and therapeutics
- Since human white matter begins active myelinating in the third trimester[3], the VGCC-mediated axonal injury identified here may contribute to WMI from late mid-term into the post-natal period and following milder insults not characterized by necrosis.
- The high degree of protection afforded to larger pre-myelinated axons by the multiple agent pharmacological strategy tested here suggests a strategy to reduce preterm WMI during the transition to active myelination, although detailed testing with in vivo models is first required.

References

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