Glutamate Receptor-Mediated Ischemic Injury of Premyelinated Central Axons

James J.P. Alix and Robert Fern, PhD

Objective: Ischemic injury of axons is a feature of periventricular leukomalacia, a pathological correlate of cerebral palsy. Recent evidence suggests that axons are damaged before they receive the first layer of compact myelin. Here we examine the cellular mechanisms underlying ischemic-type injury of premyelinated central axons.

Methods: Two-thirds of axons in the postnatal day 10 (P10) rat optic nerve are small premyelinated axons (<0.4 μm in diameter), and one-third have undergone radial expansion in preparation for glial contact and the onset of myelination. Compound action potential recording and quantitative electron microscopy were used to examine the effect of modeled ischemia (oxygen-glucose deprivation) upon these two axon populations. Glutamate receptor (GluR) expression was investigated using polymerase chain reaction (PCR) and immunostaining approaches at the confocal light and ultrastructural levels.

Results: Oxygen-glucose deprivation produced action potential failure and focal breakdown of the axolemma of small premyelinated axons at sites of contact with oligodendrocyte processes, which were also disrupted. The resulting axon loss was Ca²⁺-dependent, Na⁺- and Cl⁻-independent, and required activation of N-methyl-D-aspartic acid (NMDA) and non-NMDA GluRs. NMDA receptor expression was localized to oligodendrocyte processes at sites of contact with premyelinated axons, in addition to expression within compact myelin. No periaxonal NMDA receptor expression was observed on oligodendrocyte processes ensheathing large premyelinated axons and no protective effect of GluR block was observed in these axons.

Interpretation: NMDA receptor-mediated injury to oligodendrocyte processes navigating along small premyelinated axons precedes damage to the underlying axon, a phenomena that is lost following radial expansion and subsequent oligodendrocyte ensheathment.

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Selective injury of developing white matter is the most frequent cause of cerebral palsy, the most common human birth disorder. The underlying pathology (periventricular leukomalacia [PVL]), may feature focal necrotic damage of periventricular white matter and diffuse white matter injury. White matter injury may arise following hypoxia-ischemia at birth, but the largest patient cohort suffers injury during mid-gestation.¹,² The motor, sensory, and cognitive deficits that characterize the lesion are associated with injury to immature cells of the oligodendroglial lineage, which are susceptible to a form of non-N-methyl-D-aspartic acid (NMDA)-type glutamate receptor (GluR)-mediated excitotoxic injury.³–⁸ In addition to non-NMDA receptors, these cells express NMDA GluRs selectively on their processes,⁹–¹¹ although the role of NMDA receptors in ischemic injury of myelinated white matter is controversial.¹²–¹⁵

Oligodendroglial injury has been the focus of research into the cellular mechanisms of developing white matter injury for the past decade. However, axon damage is consistently reported in histological examinations of PVL.²,¹⁶,¹⁷ Axonal changes include truncation of the axon cylinder¹⁸ and the formation of “retraction balls and clubs.”¹⁶ Recent reports indicate that axonal injury is an early event in the formation of both focal and diffuse components of PVL,¹⁹–²¹ and may be central to the evolution of the injury.¹⁷ Despite these considerations, almost nothing is known regarding the injury mechanisms operating upon premyelinated central axons. In mid-gestation telencephalic white matter that is subject to PVL, axons have an immature expression profile for developmental markers and oligodendroglia are changing from the precursor to the immature form.²²,²³ The first evidence of early myelination is apparent, which includes myelin basic protein expression around some axons of the optic radiation.²³ There is evidence that injury to these axons during PVL can result in optic nerve hypoplasia.²⁴ Central axon development has been studied in detail in the rat optic nerve (RON), where radial expansion corre-
sponding to an approximately three-fold increase in diameter within an ~4-day period precedes first contact by oligodendrocyte processes and the subsequent onset of myelination. The first postmitotic oligodendrocytes appear at postnatal day 6 (P6) and start to extend processes parallel to axons. The first ensheathed axons appear at this age and correspond to the largest premyelinated axons with diameters of ~0.4 μm. By P10 the first wraps of compact myelin appear, corresponding to ~1% of axon profiles with ~4% of axons ensheathed but not myelinated, at which point the axons become sensitive to acute ischemic injury. Here we examine the mechanisms of acute ischemic-type injury at this crucial developmental point and test the significance of GluR expression in the injury process.

Materials and Methods
All animal procedures conformed to United Kingdom (UK) home office regulations. RONs were dissected from Lister-hooded rats between P8 and P12. Nerves were perfused with artificial cerebrospinal fluid (aCSF), composition (in mM): NaCl, 126; KCl, 3; NaH2PO4, 2; MgSO4, 2; CaCl2, 2; NaHCO3, 26; and glucose, 10; pH 7.45, bubbled with 5% CO2/95% O2 and maintained at 37°C. Zero-Na aCSF was comprised of (in mM): NMDG/Cl, 124; KCl, 3; CaCl2, 2; MgSO4, 2; choline-HCO3, 26; KH2PO4, 2; and glucose 10. Zero-Cl aCSF was comprised of (in mM): Na-cyclamate, 124; K-gluconate, 3; Ca gluconate, 2; MgSO4, 2; NaHCO3, 26; NaH2PO4, 2; and glucose 10. In zero-Ca2 aCSF, CaCl2 was omitted and 50 μM ethylene glycol tetraacetic acid (EGTA) was added. Glucose was omitted from solutions when oxygen-glucose deprivation (OGD) was required and the solution was prebubbled with 5% CO2/95% N2 for at least 60 minutes. The chamber atmosphere was switched to 5% CO2/95% N2 during perfusion with OGD. The osmolarity of all solutions was measured and adjusted using NaCl or sucrose, as required. Data are mean ± standard error of the mean (SEM), significance determined by t test or analysis of variance (ANOVA) as appropriate. GluR antagonists were from Tocris (Bristol, UK), all other reagents were from Sigma (Gillingham, UK) unless stated otherwise.

Electrophysiological recordings used the same equipment described recently. In brief, extracellular compound action potentials (CAPs) were evoked and recorded with suction electrodes. Peak-to-peak amplitude was used to assess....
changes in the number of unitary action potentials since CAP area can not be applied reliably to recordings from neonatal nerves due to the long stimulus duration required for supramaximal stimulation.\textsuperscript{29} CAPs were evoked via square-wave constant current pulses of 150 to 600\textmu sec (Iso Stim A320; World Precision Instruments, Stevenage, UK). The signal was amplified (Cyber Amp 320; Axon Instruments, Union City, CA), subtracted from a parallel differential electrode, filtered (low-pass: 800Hz), digitized (25,000Hz, 1401 mini; Cambridge Electronic Design) and displayed on a PC running Signal software (Cambridge Electronic Design, Cambridge, UK). CAP loss is taken to indicate irreversible failure of axon function,\textsuperscript{29} and is well correlated with pathological changes in axons such as dissolution of microtubules/neurofilaments, disruption of mitochondria, loss of axoplasmic integrity, and loss of neurofilament staining.

For immunohistochemistry, P10 RONs were dissected into 0.1M phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 minutes. Nerves were subsequently incubated in 0.1M PBS plus 20% sucrose weight/volume for 5 minutes prior to freeze-sectioning (20\mu m sections) and subsequent blocking for 60 minutes in 0.1M PBS, 10% fetal goat serum plus 0.5% Triton-X 100. Section were then incubated in this solution plus primary antibody at 4\degree C overnight. Antibodies raised against NR1 (1:200; Upstate-Millipore, Billerica, MA), GluR4 (1:200, Upstate), neurofilament-200 (NF-H), neurofilament-70 (NF-L) (1:200 and 1:100, respectively; Chemicon-Upstate), neurofilament-200 (NF-H), neurofilament-70 (NF-L) (1:200 and 1:100, respectively; Chemicon-Millipore, Billerica, MA), and CNPase (an oligodendroglial marker, 1:100; Sigma) were detected using appropriate Alexa-conjugated secondary antibodies (1:1000; Cambridge Bioscience, Cambridge, UK; see Ref. 10 for further details), and imaged as single planes via laser scanning confocal microscopy. Primary antibody omission controls were performed and are included in the figures at appropriate points (Fig 6). Intensity levels of neurofilament staining were assessed using mean pixel intensity levels within standard regions of interest, performed using Metamorph (Molecular Devices, Sunnyvale, CA).

For polymerase chain reaction (PCR), P10 RONs were digested in MELT (Ambion, Austin, TX) and subjected to a triazol extraction. RNA was then column-purified and the resulting RNA was treated to remove DNA contamination. The RNA was purified by extraction with phenol, pH 4.2 and triazol extraction. RNA was then column-purified and the resulting RNA was treated to remove DNA contamination. The RNA was purified by extraction with phenol, pH 4.2 (Sigma) and resuspended to a concentration of 1\mu g/\mu l. PCR was then performed using Omniscript (Qiagen, Crawley, UK) according to the manufacturer’s instructions. All primers were tested on whole brain as a positive control (see Ref. 10 for further details).

For electron microscopy, P10 RONs were collected at the end of electrophysiological experiments, washed in Sorensen’s buffer, and postfixed in 3% glutaraldehyde/Sorensen’s. Nerves were then fixed with 2% osmium tetroxide and dehydrated prior to infiltration in epoxy. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined with a Jeol 100CX (Jeol, Tokyo, Japan) electron microscope. Glial cell processes could sometimes be identified on ultrastructural grounds.\textsuperscript{6} For example, the astrocyte process in Fig 6E contains characteristic wide-bore endoplasmic reticulum and glycogen particles, and the oligodendrocyte process in Fig 4E has myelinated a nearby axon. Since all axons in the adult RON are myelinated, nonmyelinated axons at P10 are in either an early or late stage of preparation for myelination. For postembedding immunolabeling of NR1 (1:100), GluR2/3 (1:50; Upstate), and KA2 (1:50; Upstate), primary antibody was applied to the sections overnight and appropriate 20nm gold-particle secondary antibodies were applied following washing. Primary antibody omission controls were blank.

For morphometric analysis and viability scoring, axons within a minimum of three grid sections were outlined by hand using Image-J software (National Institutes of Health [NIH]) and axon area and perimeter were measured. Grids were selected at random and all identifiable axons within the area were included to avoid bias. Axon profiles were often far from round and the closest corresponding circular diameter was calculated from the perimeter values. 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: (1) the presence of a relatively complete axolemma; (2) the presence of normal microtubules; and (3) the presence of a clear and debris-free axoplasm. Axons that showed all three of these features were therefore given a viability score of “3,” and axons with none were given a score of “0,” with intermediate scores between this range. Examples of axon scores are shown in Supplementary Fig 1, where a healthy axon (score of “3”), and degrees of injury (“2” to “0”) are shown.

**Results**

Action potential conduction in the isolated P10 RON was stable for several hours under control conditions,
Figure 2
while 60 minutes of OGD resulted in conduction failure that was only partially reversible after 60 minutes of recovery in aCSF (25.6 ± 4.2% CAP recovery; n = 14 nerves; \( p < 0.001 \) vs. 130 minutes of control perfusion; Fig 1A–C). Injury was largely prevented by removing extracellular Ca\(^{2+}\) (84.2 ± 4.2% CAP recovery; n = 8 nerves; \( p < 0.001 \) vs. injury in normal Ca\(^{2+}\); Fig 1A–C), an effect confirmed at the ultrastructural level (Fig 2D). Staining for the axonal markers NF-L and NF-H, which label small-diameter and larger-diameter axons, respectively, was also disrupted in axons following 60 minutes of OGD + 60 minutes of recovery (Fig 1E, F). Confocal imaging of post-OGD staining revealed areas denuded of neurofilament

Fig 3. The ionic-dependence of injury is novel. (A) (left) Representative CAP recorded before and after perfusion with zero-Na\(^+\) solution; note the full recovery following washout. (right) Representative example of the absence of protection against OGD by zero-Na\(^+\) solution; note the limited recovery following washout. (B) Mean CAP amplitude, showing the low level of recovery following OGD in zero-Na\(^+\); note that after 20 minutes of zero-Na\(^+\) perfusion the action potential is blocked. (C) Data summary for zero-Na\(^+\) experiments. (D) (left) Recordings before and after perfusion with zero-Cl\(^-\) solution; note the full recovery following washout. (right) Representative example of the absence of protection against OGD by zero-Cl\(^-\) solution; note the limited recovery following washout. (E) Mean CAP amplitude, showing the low level of recovery following OGD in zero-Cl. (F) Data summary from zero-Cl\(^-\) experiments.

Fig 4. GluRs mediate premyelinated axon injury. (A) NMDA receptor block (MK-801) increases mean CAP recovery following OGD. (B, C) Block of non-NMDA GluR (NBQX) has a similar effect (B), as does combined application (C). (D) The extent of recovery following these procedures. (E, F) Small diameter premyelinated axons (“S”) and oligodendrocyte processes (“op”) are protected from OGD in the presence of MK-801 + NBQX, while larger axons (“L”) are not. (G) A large diameter premyelinated axon has no axoplasmic structures intact while a glial process wrapped around the axon is undamaged (arrowheads). \( * p < 0.05; ** p < 0.001. \) Scale bars: (E) = 1\( \mu m; \) (F) = 400\( nm; \) (G) = 100\( nm. \)
Figure 4

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reactivity and ball-like structures that may correspond to swollen axon regions. Staining levels were quantified to reveal a similar decline in reactivity to NF-L and NH-H following the insult (Fig 1F).

Control perfused nerves had the ultrastructural features typical of this age. Small (<0.4 μm in

Fig 5. Morphometric analysis of OGD induced axon injury. (A) The density of premyelinated axons (the number of axons in an area) of different diameter (the diameter that corresponds to the axon perimeter) present within the P10 RON. (left) Control nerve, showing a peak in diameter between 0.25 and 0.3 μm and a range between 0.15 and 0.70 μm. (right) The corresponding analysis for nerves exposed to OGD for 60 minutes followed by 60 minutes of recovery (black bars). Note the much lower density of axons indicating loss of identifiable axons and expansion of the extracellular space. The corresponding analysis from nerves exposed to OGD in the presence of NBQX and MK-801 (“GluR block”) shows that many identifiable axons are present (white bars). (B) The mean axon viability score is shown for premyelinated axons <0.4 μm in diameter (black bars) and those of ≥0.4 μm (white bars). Note that following OGD, both small and large premyelinated axons are significantly injured, with the small premyelinated axons effected more than the large premyelinated axons. Following OGD in the presence of GluR blockers only the small premyelinated axons are significantly damaged. (C–E) Individual size spectra of the viability scores, showing greatly improved viability in axons <0.4 μm in the presence of GluR blockers. ***p < 0.001 vs. control.

Fig 6. GluRs in developing white matter. (A) NR1 (green, left) and CNPase (red, middle) appear to be colocalized (right) using fluorescent confocal imaging. Note that the NR1 clusters appear orange in the overlay. (B) NR1 (green, left) and NF-H (red, middle) appear poorly colocalized (right). Note that the NR1 clusters appear green in the overlay. (C) Absence of staining when NR1 antibody was omitted. (D) mRNA for NMDA receptor subunits in P10 RON. (E–G) Localization of GluR NR1 subunit at the ultrastructural level by immunogold labeling. (E) A small-diameter premyelinated axon (“s”) is aligned between an astrocyte process (“ap”) and two myelinated axons (“Ax”). The boxed region is shown at higher gain to the right. Three immunogold beads (short arrows) are present on the junction between an oligodendroglial process (“op”) and the small premyelinated axon. Note the absence of NR1 reactivity on the astrocyte process. Also note the tubulovesicular complex apparently fusing with the axolemma at this point (“**”), and the NR1 reactivity within the compact myelin of a neighboring axon (short arrow). (F) An oligodendrocyte somata (“Oli”) is extending a process showing NR1 reactivity (short arrows) along a small premyelinated axon. Note that no reactivity is seen in the somata itself. The boxed area is shown at higher gain to the right and reveals NR1 reactivity on the outer edge of an actively myelinating oligodendrocyte process. (G) Two oligodendrocyte processes (boxes) are navigating along small premyelinated axons (shown in great detail to the right). In both cases, NR1 reactivity is present on the axon side of the process. “gp” indicates that this glial process can not be positively identified as an oligodendrocyte process. Scale bars: (A–C) = 10 μm; (E–F) = 500 nm.
Non-NMDA GluRs are expressed in oligodendrocyte processes and the axolemma. (A, B) The effect of GluR activation upon CAP amplitude in P10 rat optic nerve. (A) Exposure to 200μM AMPA + 200μM kainic acid in the presence of 30μM cyclothiazide evokes a small reversible reduction in CAP amplitude. Sample CAPs are shown at the top, taken from the times indicated. Times “1” and “2” were used to calculate mean data for the histogram, revealing a significant reduction in amplitude in experiments performed in either the presence or the absence of cyclothiazide (*p < 0.05; **p < 0.01 vs. control; “n” numbers as indicated). (B) Exposure to 200μM AMPA + 200μM kainic acid + 1mM NMDA + 50μM glycine in the absence of Mg2+ also produced a small reversible fall in CAP amplitude (***p < 0.01 vs. control; “n” numbers as indicated; times “1” and “3” were used for the histogram data). Cyclothiazide has no effect applied alone. (C–I) Localization of GluR 2/3 (C–E) and KA2 (F–I) subunit at the ultrastructural level by immunogold labeling. (C) Strong staining (short arrows) is apparent for GluR 2/3 in a myelinating axon (“Ax”), with gold particles present within and under the myelin (“my”). The axon contains a mitochondria (“mit”). Staining is also present on the axoplasmic side of the axolemma (the axolemma is indicated by arrowheads) of a neighboring small premyelinated axon (“s”). (D) Gold particles largely located on the axoplasmic side of the myelin of a myelinating axon. (E) Three gold particles are located on the interface between a glial process (“gp”) and a small premyelinated axon (axolemma indicated by arrowheads). (F) Immunolabeling for KA-2 reveals focal expression associated with the myelin of an actively myelinating axon. (G) Focal axolemma staining of a premyelinated axon facing an ensheathing oligodendrocyte process (“op”). (H) Focal staining on the axolemma underneath an actively myelinating oligodendrocyte process. Note the nearby astrocyte processes (“ap”). (I) KA-2 reactivity in the axoplasm and axolemma of small premyelinating axons contacted by glial processes. Scale = 500nm.
diameter) and large (>=0.4μm) premyelinated axons where separated by a narrow extracellular space and by glial processes (“S” and “L” premyelinated axons, respectively; Fig 2A), with the occasional myelinated axon apparent (“Ax”; Fig 2A). Some of the large premyelinated axons were ensheathed by glial processes (“**”; Fig 2A), shown in long-section in Fig 2C (arrow). Mitochondria in all cell compartments had a typical internal structure and showed no evidence of swelling. Following OGD, the extracellular space was expanded, and nondescript cellular debris was common (Fig 2B). Many premyelinated axons showed a loss of microtubules and regions of axolemma breakdown (Fig 2B), and extensive disruption of glial processes was widespread (intact glial processes were only occasionally seen). In long-section, regions of intense focal premyelinated axon disruption were seen (Fig 2E; “Axon” arrowheads), generally aligned parallel to damaged regions of neighboring glial processes (Fig 2E; “gp” arrowheads). Identification of axons in post-OGD micrographs was based upon the presence of identifiable microtubules, which were sometimes rather sparse (eg, Fig 2E; “Axon” arrow).

Unlike mature optic nerve axons,33,34 replacing extracellular Na⁺ (Fig 3A–C; Supplemental Fig 2) or Cl⁻ (Fig 3D–F) was not protective against injury. This was not due to any toxicity of ion substitution at this age since control ion substitutions had no significant nonreversible effect (Fig 3A, C, D, F). This result suggests a fundamentally different ionic basis to injury in premyelinated and myelinated axons, and rules out a significant role for Na–Ca exchange or Cl⁻ channels in the injury process.33,34 In particular, Na⁺ replacement leads to rapid depletion of axoplasmic Na⁺ and block of reverse-mode Na–Ca exchange, a major source of Ca²⁺ influx in mature myelinated axons during anoxia.33 The finding was confirmed at the ultrastructural level where wide-scale disruption of axons following OGD in zero-Na⁺ conditions was confirmed (Supplemental Fig 2). No changes were observed in the ultrastructure of nerves perfused with zero-Na⁺ aCSF for 140 minutes in the absence of OGD (data not shown).

Oligodendrogial GluRs are implicated in the Ca²⁺-dependent injury of their processes and somata,3,4,9–11 while axonal expression of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and kainate receptors is important for ischemic-type injury of myelinated spinal axons.35,36 Block of NMDA-receptors (10μM MK-801) or non-NMDA receptors (20μM NBQX) was protective against OGD-induced injury of premyelinated axons, as was combined block (67.9 ± 7.0% CAP recovery during combined block; n = 8 nerves; p < 0.001 vs. OGD alone; Fig 4A–D). There was complete preservation of the small premyelinated axons during combined GluR block (Fig 4E, F; “s”), while large premyelinated axons showed lower levels of protection (Fig 4E–G; “L”). Note in Fig 4G that a large diameter premyelinated axon has lost all axoplasmic differentiation but a glial processes wrapped around the axon is intact (arrowheads).

In control nerves, the diameter spectra of premyelinated axons ranged between 0.15 and 0.70μm, with a peak between 0.25 and 0.30μm (n = 100 axons; Fig 5A, left). This corresponds closely to earlier studies.25 Following OGD, the density of recognizable premyelinated axons fell from 11.50 axons/μm² to 1.26 axons/μm² (p < 0.001; n = 91 axons; Fig 5A, right, filled bars). The loss of recognizable premyelinated axons was significantly reduced by GluR block (to 6.59 axons/μm²), in particular in small premyelinated axons (Fig 5A, right, open bars). The axon diameter spectra following OGD may be affected by expansion of the extracellular space and swelling of glial processes. To better assess the degree of injury within small and large premyelinated axons, we employed a numerical viability scoring system. The mean viability score for small premyelinated axons (<0.4μm in diameter) and large premyelinated axons (>=0.4μm in diameter) are shown in Fig 5B. Under control conditions, the mean viability score of both groups of axons was close to “3,” indicating no significant pathology. Following OGD, the viability scores of both groups declined significantly, with the smaller axons showing a significantly greater decline than the larger axons (p < 0.001). Combined GluR block prevented the appearance of pathology in identifiable small premyelinated axons but had no comparable effect upon the larger premyelinated axons. The viability scores for all premyelinated axons examined is shown in Fig 5C–E, confirming the selective protection of small premyelinated axons by GluR block.

The pattern of NMDA receptor protein (Fig 6A–C) and mRNA (Fig 6D) expression in P10 RON was similar to that reported in the P10 mouse optic nerve.10 Confocal imaging indicated that NR1 subunit protein is colocalized with CNPase (+) oligodendroglial processes but not with N-FH (+) axon profiles (Fig 6A, B). GluR4 protein (non-NMDA subunit) appeared primarily in CNPase (+) oligodendroglial somata (Supplemental Fig 3). At the ultrastructural level, intense NR1 protein reactivity was found in regions where oligodendrocyte process membrane ran parallel to the axolemma of small premyelinated axons, in addition to expression within compact myelin as previously reported9,11 (Fig 6E, G, arrows; Supplemental Figs 4 and 5). NR1 reactivity appeared to translocate from the axolemmal face of oligodendrocyte processes aligned along small diameter premyelinated axons into the myelin as development progressed. On no occasion was NR1 reactivity seen in the cell membrane of axons or astrocytes and it was unusual to see it in cell membranes of oligodendroglial somata.

Perfusion with AMPA+kainate produced a small
but significant fall in the CAP (Fig 7A), which achieved higher significance in the presence of the AMPA desensitization inhibitor cyclothiazide. Addition of NMDA to the test condition did not produce any significant augmentation of this effect (Fig 7B; “all agonists”). Immunolocalization using an antibody against the AMPA receptor Glur2/3 subunit revealed high expression in and under myelin and in the axolemma of premyelinated axons (Fig 7C–E). The kainate receptor KA2 subunit showed more focal expression at sites within and under myelin, in addition to the axolemma of premyelinated axons (Fig 7F–I).

Discussion
Recent findings suggest that the intricate morphological arrangement that forms between axons and oligodendrocyte processes during central myelination is orchestrated in part by vesicular-type glutamate release from axons and subsequent detection by GluR on processes. We have shown here NMDA receptor expression on oligodendrocyte processes, as required by this hypothesis. We have also shown that the presence of these NMDA receptors results in acute injury of small premyelinated axons during OGD. Possible mediators that might link NMDA receptor-mediated oligodendrocyte process injury with axon injury include local release of intracellular potassium resulting in axon depolarization and cytotoxic swelling; physical disruption of the axolemma during oligodendrocyte process swelling and dissolution, which will also disrupt the extracellular matrix; release of free radicals; and release of intracellular components, such as calpains and lipases, that are likely to attack the axon directly.

In addition to expression on oligodendrocyte processes, NMDA receptors were found in early compact myelin. Similar myelin expression in mature RON can mediate cytotoxic Ca$^{2+}$ influx. As a result, NMDA receptors contribute to OGD-induced injury in this preparation, although apparently not in mouse optic nerve. The current findings indicate that oligodendroglial processes express NMDA receptors where they navigate along premyelinated axons, while expression shifts to the myelin as it is deposited. NMDA receptor block failed to protect larger premyelinated axons, which is consistent with loss of receptors from the axonal face of oligodendrocyte processes once axon ensheathment has been achieved. At this point in central axon development, injury may involve the voltage-gate calcium channels transiently expressed in these large premyelinated axons.

NMDA or AMPA/kainate receptor block was equally protective against OGD-induced injury. Combined block had no additional protective effect and resulted in sparing of small but not large premyelinated axons. Since oligodendrocyte NMDA receptors require coactivation of AMPA/kainate receptors to overcome Mg$^{2+}$-block even during modeled ischemia, this is consistent with the current observation that both classes of GluR are present on oligodendrocyte processes. Immunolabeling at the light level appears to show low AMPA/kainate receptor expression on oligodendrocyte processes, but the current ultrastructural analysis reveals high levels of membrane expression in these structures in addition to clear expression in the axolemma. AMPA/kainate receptor activation produced a ~10% decline in the CAP, consistent with expression of functional receptors in the axolemma. Although a small contribution from AMPA/kainate receptors to axonal injury cannot be ruled out by the current findings, the absence of an additive effect of coreceptor block indicates that axonal non-NMDA GluRs are not directly involved in injury. This contrasts with the situation in mature central axons, and may indicate that the axonal nanocomplexes incorporating AMPA/kainate receptors and NO synthase that are involved in acute OGD-injury have yet to form in premyelinated white matter.

The results may have significant clinical ramifications, since magnesium sulfate is a recognized antenatal intervention associated with a decreased risk of gross motor deficit in low-birth-weight babies. While the significance of this effect requires further investigation, elevated extracellular Mg$^{2+}$ will inhibit NMDA receptors in developing white matter. The current findings raise the possibility that this prophylactic strategy may prove effective at protecting small premyelinated central axons in the fetus at risk of cerebral palsy, while interventions designed around NMDA receptor block may protect both oligodendroglial cells and their partner axons.

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