White Matter Axon Vulnerability to AMPA/Kainate Receptor-Mediated Ischemic Injury Is Developmentally Regulated

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Periventricular white matter injury (PWMI) is the leading cause of neurodevelopmental morbidity in survivors of premature birth. Cerebral ischemia is considered a major etiologic factor in the generation of PWMI. In adult white matter (WM), ischemic axonal damage is mediated by AMPA/kainate receptors. Mechanisms of ischemic axonal injury during development are not well defined. We used a murine brain slice model to characterize mechanisms of ischemic axonal injury in developing WM. Acute coronal brain slices were prepared from *thyl*-yellow fluorescent protein (YFP) mice at postnatal day 3 (P3), P7, P10, and P21. Ischemia was simulated by oxygen-glucose deprivation (OGD). YFP-positive axon morphology in the corpus callosum was preserved for at least 15 h under normoxic conditions. OGD resulted in delayed degeneration of YFP-positive axons, characterized by axonal beading, fragmentation, and loss of YFP. AMPA and cyclothiazide damaged WM axons at P7, P10, and P21 but not at P3. The AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione (NBQX) decreased OGD-induced axonal degeneration and oligodendrocyte loss at P10 and P21. At P3 and P7, NBQX protected oligodendrocytes but did not prevent axonal degeneration after OGD. The NMDA receptor antagonist MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate] provided no protection at any age. These results indicate that developing WM axons are susceptible to ischemic injury. However, mechanisms of axonal degeneration are developmentally regulated. At P3 and P7, corresponding developmentally to the window of peak vulnerability to PWMI in humans, ischemic axonal injury is not mediated by AMPA/kainate receptors. Strategies to protect WM during this period may be substantially different from those used at later developmental stages.

Key words: axon; excitotoxicity; ischemia; oligodendrocyte; periventricular leukomalacia; white matter

Introduction

Periventricular white matter injury (PWMI) is the leading cause of neurodevelopmental disability in survivors of prematurity (Back and Rivkees, 2004). Cerebral ischemia contributes to the generation of PWMI (Greisen and Borch, 2001; Volpe 2001). Recent research has focused on mechanisms of injury to developing oligodendrocytes (OLs), which are a major target of injury in PWMI. Axons are damaged in PWMI, and axonal injury is a key predictor of outcome in CNS disease (Deguchi et al., 1999; Hirayama et al., 2001; Medana and Esiri, 2003; Bell et al., 2005). However, mechanisms of axon injury remain uncharacterized. Understanding mechanisms of axon injury may translate into improved outcomes in PWMI.

Energy depletion during ischemia causes failure of energy-dependent homeostatic mechanisms, resulting in axonal Ca²⁺

overload, conduction failure, and structural injury (Stys, 2005). Excessive glutamate receptor activation, or excitotoxicity, contributes to ischemic white matter (WM) axon injury. Intracerebral injection of AMPA damages axons (Fowler et al., 2003; Cuthill et al., 2006). AMPA/kainate receptors mediate ischemic injury to myelinated WM axons *in situ* (Tekköt and Goldberg, 2001; Tekköt et al., 2005) and *in vivo* (Kanellopoulos et al., 2000; McCracken et al., 2002). However, isolated axons are not injured by direct exposure to ionotropic glutamate receptor agonists, nor are they protected from oxygen-glucose deprivation (OGD) by glutamate receptor antagonists (Underhill and Goldberg, 2007).

Excitotoxic injury of axons in myelinated white matter may occur indirectly through interactions with neighboring cellular elements that express glutamate receptors. One hypothesis is that toxic interactions are mediated by activation of OL glutamate receptors (Tekköt and Goldberg, 2001). OLs express functional glutamate receptors (Gallo et al., 1994) and are injured *in vitro* via AMPA/kainate receptor activation (Yoshioka et al., 1995; McDonald et al., 1998; Fern and Møller, 2000; Li and Stys, 2000). AMPA/kainate receptor antagonists have been shown to reduce OL loss in ischemic WM (Tekköt and Goldberg, 2001). OL processes express NMDA receptors, which may also contribute to ischemic injury (Karadottir et al., 2005; Salter and Fern, 2005;

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Micu et al., 2006). Specific developing OL populations in the developing brain are particularly vulnerable to ischemic and excitotoxic injury (Follett et al., 2000; Back et al., 2002; Deng et al., 2003; Rosenberg et al., 2003). The period of peak vulnerability to PWMI in humans correlates to the presence of susceptible OL populations (Back et al., 2001; Riddle et al., 2006), corresponding to the first postnatal week in mice, before the onset of myelination (Craig et al., 2003).

The vulnerability of WM axons to ischemic injury varies during development. In isolated rodent optic nerve, premyelinated WM is highly resistant to ischemic injury, whereas early and late myelinating WM are increasingly vulnerable (Fern et al., 1998). Mechanisms of ischemic axon injury may also vary during development. We characterized the role of ionotropic glutamate receptor activation in ischemic axonal degeneration in premyelinated WM in murine brain slices and compared this with early and late myelinating WM. Our results suggest that AMPA/kainate receptor mediation of ischemic axonal degeneration in WM is developmentally regulated.

Materials and Methods

Acute brain slice preparation. Methods were modified from those reported previously for acute brain slice preparation in adult mice (Tekkok and Goldberg, 2001). Brains from *thyl*-yellow fluorescent protein (YFP)-16 (C57BL/6) mice (Feng et al., 2000) at postnatal day 3 (P3), P7, P10, and P21 were dissected out into ice-cold 95%-oxygenated modified artificial CSF (aCSF) slicing solution composed of the following (in mM): 87 NaCl, 2.5 KCl, 3 MgCl₂, 0.5 CaCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 25 glucose, and 75 sucrose, pH 7.4 (Valentino et al., 2004). Coronal sections, 400 μ m, were cut using a vibratome. Only slices containing corpus callosum were included in perfusion experiments (two per animal). Some slices were fixed immediately after slicing for use in immunohistochemical analysis. Eight to 12 slices from four to six littermates were transferred to two Haas-type interface chambers (Harvard Apparatus, Natick, MA), and superfused with 95%-oxygenated aCSF composed of the following (in mM): 126 NaCl, 3.5 KCl, 1.3 MgCl₂, 2 CaCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, and 10 glucose pH 7.4. Each chamber consisted of two compartments with parallel perfusion circuits, allowing simultaneous comparison of two perfusion conditions (i.e., drug vs control). Slices were allowed to recover at 25°C for 2–3 h after slicing. Perfusion temperature was then increased to 37°C over 2 h before the onset of experimental conditions and maintained at that temperature throughout the remainder of the experiment.

Oxygen-glucose deprivation. In OGD experiments, one chamber was maintained under normoxic control perfusion, whereas the second chamber underwent OGD. OGD was initiated by switching from normal aCSF to superfusion with glucose-free aCSF (supplemented with 10 mM sucrose to maintain osmolarity) saturated with 95% N₂/5% CO₂. On the completion of OGD, slices were reperfused with normal glucose-containing aCSF saturated with 95% O₂/5% CO₂ at 37°C until the completion of the experiment. Total perfusion time after slice preparation was at most 15 h (3 h recovery, 2 h to increase temperature, 1 h experimental condition, and 9 h reperfusion). In experiments involving inhibition of ionotropic glutamate receptor activity, the following drugs were added to superfusate 1 h before onset of OGD and continued throughout the period of OGD and reperfusion: the AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX) (30 μ M; Sigma, St. Louis, MO) and/or the NMDA receptor antagonist MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohept-5,10-imine maleate] (10 μ M; Sigma).

Excitotoxicity. Slices were superfused for 1 h at 37°C with aCSF containing the ionotropic glutamate receptor agonists glutamate (100 μ M; Sigma), AMPA (100 μ M; Sigma), or NMDA (100 μ M, Sigma) and then returned to drug-free aCSF for 9 h. AMPA was used in combination with the AMPA/kainate receptor desensitization inhibitor cyclothiazide (100 μ M, Sigma). In some experiments, the AMPA/kainate receptor antagonist NBQX (30 μ M; Sigma) and/or MK-801 (10 μ M; Sigma) were added

to the superfusate during the period of glutamate receptor agonist exposure.

Immunohistochemistry. At the conclusion of each experiment, slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h at room temperature. Each slice was then cryoprotected for at least 48 h in 30% sucrose in PBS. Sections, 16 μ m, were cut from each 400 μ m brain slice using a cryotome and collected onto glass slides (Fisher Scientific, Pittsburgh, PA). Sections from the outer 80 μ m of each slice were excluded to avoid tissue that may have been damaged during slice preparation. Because YFP fluorescence in the corpus callosum of *thyl*-YFP-16 mice was dim during the first postnatal week, all sections were stained with anti-green fluorescent protein (GFP) antibodies to improve visualization of YFP-positive (YFP⁺) axons. Sections were rehydrated in PBS and then blocked and permeabilized in a solution of 5% normal goat serum (Sigma) and 0.1% Triton X-100 (Sigma) in PBS for 30 min at room temperature. For visualization of YFP⁺ axons, sections were incubated with Alexa Fluor-488-conjugated rabbit anti-GFP antibodies (1:200; Invitrogen, Carlsbad, CA) for 2 h at room temperature in blocking/permeabilization solution. Cells of the OL lineage were labeled with stage-specific antibodies. For visualization of late OL progenitor cells/pre-OLs (O4⁺/O1[−]) and immature OLs (O4⁺/O1⁺), sections were incubated with mouse anti-O4 or anti-O1 antibodies (1:100; Chemicon, Temecula, CA) overnight at 4°C. Mature OLs were labeled with anti-adenomatous polyposis coli (APC) (CC-1) antibodies (1:100; Calbiochem, La Jolla, CA). Triton X-100 was omitted when using the O4 and O1 antibodies. Primary antibodies were visualized with appropriate Alexa Fluor-546 anti-mouse secondary antibodies (1:200; Invitrogen) for 2 h at room temperature. In some experiments, nuclei were labeled with Hoechst 33258 (5 μ g/ml; Invitrogen) for 1 min at room temperature. After thorough washing in PBS, sections were coverslipped using the Prolong Gold antifade kit (Invitrogen). Slides were kept in the dark at 4°C overnight to ensure antifade treatment before imaging.

Assessment of axon damage by confocal microscopy. Axons in the corpus callosum of each slice were imaged using a Zeiss (Oberkochen, Germany) LSM 5 Pascal inverted laser scanning confocal microscope. Sections were scanned with a 488 nm argon laser for YFP/Alexa Fluor-488 fluorescence. Every third section per slice was imaged for a total of three sections per slice. A total of 10 (1 μ m) optical sections were collected in the *z*-axis from a single microscopic field in the midline of the corpus callosum of each section using a 40 $\times$ (water immersion; numerical aperture, 1.2) objective lens under fixed gain and pinhole settings. Optimal settings were obtained from tissue fixed immediately after slicing. Projected images were acquired with Zeiss LSM imaging software. Z-stacks were projected into a single-plane image before analysis and assessment of axonal damage. Axon damage was quantified by visual scoring. Images were divided into a 5 $\times$ 5 grid, and each grid box was scored by a blinded observer for the presence of axon damage using the following system: 0, no damage; 1, axon swelling and/or beading; 2, axon fragmentation and/or loss of fluorescence. The total score for a single section (0–50) was divided by the number of grid boxes to give a mean damage score (0–2). Damage scores from three sections were averaged and recorded for each slice.

Assessment of oligodendrocyte injury. The central corpus callosum was imaged with a confocal microscope, as above, using 405 and 543 nm laser lines for visualization of Hoechst dye and Alexa Fluor-546, respectively. Both the total number of OLs and the number of pyknotic OLs were counted in three sections per slice by a blinded observer. OLs were considered to be nuclei surrounded completely by O4, O1, or APC labeling. Pyknotic OLs were identified by specific morphologic characteristics: pyknotic nuclei, condensed cytoplasm, and/or fragmented processes (Back et al., 2002). The number of viable OL lineage cells per field was recorded as total cells minus pyknotic cells.

Statistical analysis. All data are expressed as means $\pm$ SEM. Statistical significance was determined by Student's unpaired *t* test or one-way ANOVA, followed by Tukey's *post hoc* test for multiple comparisons between groups. *P* values are reported in the figure legends. Data for each experimental condition was obtained from 8–12 individual slices (two slices per animal) in two to four separate perfusion experiments.

Results

Visualization of axons in developing white matter of *thy1*-YFP-16 mice

thy1-YFP mice express YFP in subsets of neuronal populations throughout the nervous system. Each variant line of *thy1*-YFP mice expresses cytoplasmic YFP in a specific pattern (Feng et al., 2000). We selected line 16 for our experiments because it expresses YFP in a subset of cortical neurons that project axons across the corpus callosum, beginning in the embryonic period. In the corpus callosum, this labeling is axon specific, because glia and other CNS cellular components do not express YFP. Because only a subset of axons in the corpus callosum are labeled with YFP, it is possible to visualize the morphologic details of individual axons. YFP expression was dim in corpus callosum axons during the first postnatal week. To improve YFP visualization, we used immunohistochemical labeling of YFP with fluorophore-conjugated anti-GFP antibodies. Images were acquired via laser scanning confocal microscopy, which allowed for collection of precise optical sections in the z-axis, to decrease optical overlap of labeled axons. We were able to follow the courses of individual axons within the corpus callosum over long distances with high resolution and to evaluate WM axon morphology in detail (Fig. 1).

Detection of axon damage in *thy1*-YFP-16 mice

The morphologic characteristics of axon degeneration in axons from *thy1*-YFP mice have been characterized using *in vitro* models of ischemia and aglycemia (Valentino et al., 2004; Underhill and Goldberg, 2007) and *in vivo* models of peripheral nerve and spinal cord injury (Beirowski et al., 2004; Bareyre et al., 2005; Kerschensteiner et al., 2005). Beading and fragmentation of YFP⁺ axons are sensitive markers of injury, comparable with traditional methodologies such as electron microscopy, toluidine blue staining, and neurofilament immunohistochemistry (Beirowski et al., 2004) (M. Valentino and M. P. Goldberg, unpublished observations). In our brain slice preparation, YFP⁺ WM axons perfused under control conditions maintained a linear appearance without beading or fragmentation for at least 15 h (Fig. 2*A,D*). YFP⁺ axons subjected to OGD became diffusely swollen, beaded, and fragmented by the end of the reperfusion period. There was also a decrease in the density of labeled fibers (Fig. 2*B,E*). In comparison, signs of axon damage were less appreciable in WM labeled with the pan-axonal neurofilament marker antibody SMI-312. Some axon beading was

Figure 1. Visualization of white matter axons in *thy1*-YFP-16 mice. Sections of *thy1*-YFP-16 brains, 20 μ m, were labeled with anti-GFP antibodies to aid in visualization of YFP⁺ axons. Confocal images were collected as 10 μ m Z-stacks and then projected in a single plane. **A**, Low-magnification view demonstrating YFP⁺ cortical neurons and axonal projections across the corpus callosum. Scale bar, 100 μ m. View of midline corpus callosum (**B**) (scale bar, 25 μ m) and highly magnified view (**C**) (100 $\times$; scale bar, 10 μ m) demonstrating detailed axon morphology.

Figure 2. Detection of axon damage after OGD by morphologic changes in axoplasmic YFP. **A, D**, YFP⁺ axons in the corpus callosum at the onset of perfusion (**A**) and after 10 h perfusion at 37°C (**D**). **B, E**, YFP⁺ axons at the onset of perfusion (**B**) and after 1 h OGD and 9 h normoxic reperfusion (**E**). **C, F**, Axons labeled with the pan-axonal neurofilament marker SMI-312 at the onset of perfusion (**C**) and after 1 h OGD and 9 h normoxic reperfusion (**F**). The morphology of many individual axons is visible in WM of *thy1*-YFP-16 mice but not in SMI-312-labeled WM. Axon beading and fragmentation, characteristic of injury, are clearly visible in YFP- and SMI-312-labeled axons (arrowheads). However, areas of fiber loss visible in YFP⁺ WM are not seen in SMI-312-labeled tissue. Scale bar, 25 μ m.

present in SMI-312-labeled axons, but axon fragmentation and fiber loss were not visible (Fig. 2*C,F*).

OGD induces delayed axonal degeneration in developing white matter

Myelination in the mouse corpus callosum begins at the end of the first postnatal week and progresses rapidly over the next 2–3

Figure 3. OGD induces axonal degeneration in developing white matter. YFP-labeled axons in the corpus callosum of P3, P7, P10, and P21 mice before onset of OGD (top row), immediately after 1 h OGD, at the onset of reperfusion (RP) (2nd row), after 3 h reperfusion (3rd row), and after 9 h reperfusion (bottom row). Scale bar, 25 μ m.

weeks of development. (Sturrock, 1980). We examined the characteristics of OGD-induced axon degeneration in premyelinated (P3 and P7), early myelinating (P10), and late myelinating (P21) WM. One hour of OGD followed by 9 h of reperfusion in oxygenated glucose-containing aCSF resulted in significant degeneration of YFP⁺ WM axons at P3, P7, P10, and P21 (Fig. 3). Morphologic signs of injury did not appear during or immediately after the period of OGD. During reperfusion, individual axon cylinders became swollen and beaded. By the end of the reperfusion period, axonal beading was prominent in WM at all postnatal ages. However, axon fragmentation and fiber loss were far more notable in WM at P10 and P21 than at P3 and P7. Axonal damage scoring revealed the delayed nature of axonal degeneration resulting from OGD. Significant damage was not detected in P3 WM until 9 h of reperfusion (Fig. 4A). Damage appeared by 6 h of reperfusion in P7 and P10 WM and by 3 h reperfusion in P21 WM (Fig. 4B–D).

Axon vulnerability to OGD-induced degeneration increases during postnatal development

The vulnerability of developing WM axons to OGD-induced functional impairment has been studied in isolated rodent optic nerve. Premyelinated axons are highly tolerant of OGD, whereas myelinating axons are increasingly susceptible to OGD-induced conduction failure (Fern et al., 1998). We evaluated the susceptibility of axons in developing cerebral WM to OGD-induced structural injury. YFP⁺ axons in P3 WM were resistant to OGD-induced structural degeneration compared with axons at later

stages (Fig. 5). In P3 WM, 15 and 30 min OGD produced no axon damage after 9 h reperfusion, whereas 60 min of OGD resulted in significant degeneration. In P7 and P10 WM, 30 min OGD resulted in mild axon damage, whereas in P21 WM, only 15 min of OGD were required to produce axon damage. Similarly, the severity of axon damage after a given length of OGD increased during postnatal development (after 1 h OGD, 0.96 ± 0.05 at P3, 1.23 ± 0.07 at P7, 1.32 ± 0.09 at P10, and 1.76 ± 0.05 at P21) (Fig. 5).

Axon vulnerability to AMPA receptor-mediated excitotoxic degeneration increases during postnatal development

We hypothesized that the increased vulnerability of myelinating axons to injury after OGD is attributable to increased vulnerability to excitotoxic injury. To test this hypothesis, we characterized the effects of glutamate receptor agonist exposure on developing axons. After the recovery period, slices were perfused with aCSF containing the ionotropic glutamate receptor agonists glutamate (100 μ M), AMPA (100 μ M), AMPA/cyclothiazide (100 μ M each), or NMDA (100 μ M) for 1 h and then perfused for 9 h with normal aCSF. In P3 WM, direct exposure to glutamate receptor agonists did not cause structural damage to axons (Fig. 6A). At P7, AMPA/cyclothiazide caused mild but significant axon damage versus control WM (Fig.

6B). At P10, AMPA/cyclothiazide caused significant axon damage (Fig. 6C). At P21, AMPA/cyclothiazide caused severe axon damage (Fig. 6D). In all cases, AMPA/cyclothiazide-induced damage was prevented by simultaneous perfusion with NBQX. In the absence of cyclothiazide, glutamate or AMPA did not damage axons at any developmental stage. Similarly, NMDA did not damage axons at any age studied but did produce significant injury to YFP⁺ neuronal cell bodies in the cerebral cortex (data not shown). The AMPA/kainate receptor antagonist NBQX (30 μ M) prevented AMPA/cyclothiazide-induced axon damage in P10 and P21 WM, demonstrating that AMPA/cyclothiazide damage axons via the expected receptor-mediated mechanism.

AMPA/kainate receptor activation does not contribute to axonal degeneration after OGD in P3 and P7 WM

In coronal brain slices from adult mice, the AMPA/kainate receptor antagonist NBQX prevents axon injury in WM after OGD, possibly indirectly, via preservation of WM OLs (Tekkk and Goldberg, 2001). We defined the contribution of ionotropic glutamate receptor activation to axon damage after OGD in premyelinated WM (Fig. 7). Perfusion of slices from P3 and P7 mice in oxygenated glucose-containing aCSF containing either NBQX (30 μ M) or the NMDA receptor antagonist MK-801 (10 μ M) did not damage axons. Slices exposed to 1 h OGD were perfused with aCSF containing NBQX and/or MK-801 beginning 1 h before the onset of OGD and continuing throughout the OGD and reperfusion periods. Neither drug prevented the development of axon swelling and beading after OGD in P3 or P7 WM. Damage scor-

Figure 4. Delayed axonal degeneration after OGD in developing WM. Slices were exposed to 1 h OGD and then fixed for analysis at multiple time points during the reperfusion period. At all ages examined, OGD-induced axon damage is not present during or immediately after the period of OGD but only appears during reperfusion. **A**, In P3 WM, significant damage is not detected until 9 h reperfusion. In P7 (**B**) and P10 (**C**) WM, damage is present by 6 h reperfusion. **D**, In P21 WM, damage is present by 3 h reperfusion. * $p \leq 0.01$ versus normoxic control at each time point, determined by unpaired Student's *t* test. Error bars represent SEM.

ing demonstrated that neither drug (alone or in combination) decreased axon damage after OGD at P3 or P7 (Fig. 7E,F).

AMPA/kainate receptor activation mediates oligodendrocyte death in developing WM

Next, we characterized the effects of ionotropic glutamate receptor antagonists on OL lineage cell death in developing WM. We

Figure 5. Axonal vulnerability to OGD-induced degeneration increases during postnatal development. Axon damage scored at P21 (black triangles), P10 (white squares), P7 (black squares), and P3 (white diamonds) after various lengths of OGD (0–120 min) followed by 9 h reperfusion. * $p < 0.01$ versus P3 and * $p < 0.01$ versus P7 and P10 for each duration of OGD, determined by one-way ANOVA and Tukey's *post hoc* test. Error bars represent SEM.

used the immunohistochemical markers O4 and O1 to label developing OLs (Sommer and Schachner, 1981; Warrington and Pfeiffer, 1992). We used anti-APC antibodies (CC-1) to label mature OLs (Bhat et al., 1996). Under normoxic conditions, numerous viable O4⁺ cells were identified in the central corpus callosum at P3 (Fig. 8). After OGD reperfusion, many of the remaining cells displayed signs of injury, such as pyknotic nuclei and condensed cytoplasm. NBQX prevented the appearance of morphologic signs of injury in O4⁺ cells. The total number of viable OL lineage cells per field were counted in WM at P3, P7, P10, and P21 after normoxic perfusion, OGD, and OGD in the presence of ionotropic glutamate receptor antagonists (Table 1). Blockade of AMPA/kainate receptors with NBQX prevented viable OL lineage cell loss after OGD in all ages examined and for all types of OL lineage cells. The NMDA receptor antagonist MK-801 did not diminish viable OL loss after OGD at any age studied, nor did it provide any additional protection when used in combination with NBQX.

AMPA/kainate receptor activation contributes to ischemic axonal degeneration in P10 and P21 white matter

Slices from P10 and P21 mice were perfused with aCSF containing NBQX and/or MK-801 under normoxic and OGD conditions, as above. In myelinating WM, NBQX and MK-801 treatments were not toxic to axons under normoxic conditions (Fig. 9). At P10, NBQX decreased axon damage after OGD (OGD plus NBQX, 1.06 ± 0.08 ; OGD alone, 1.51 ± 0.11). The protective effect of NBQX was more pronounced at P21 (OGD plus NBQX, 1.07 ± 0.08 ; OGD alone, 1.81 ± 0.08). MK-801 alone did not decrease axon damage at any age, nor did it provide any additional protection when used in combination with NBQX.

Discussion

We used acute brain slices to assess ischemic axonal degeneration in developing WM. OGD causes delayed axonal degeneration. Vulnerability to ischemia increases during postnatal development. In P10 and P21 WM, AMPA/kainate receptors contribute to axonal injury, in accordance with previous work investigating adult WM (Tekköt and Goldberg, 2001; Tekköt et al., 2005). However, in P3 and P7 WM, ionotropic glutamate receptors do not contribute to OGD-induced axonal degeneration. Axons in

Figure 6. Axonal vulnerability to AMPA/kainate receptor-mediated excitotoxic degeneration increases during postnatal development. Slices were exposed to aCSF containing glutamate (100 μ M), AMPA (100 μ M) and cyclothiazide (100 μ M), or NMDA (100 μ M) for 1 h and then returned to normal aCSF for 9 h. **A**, Axons in P3 WM were unaffected. **B**, Axons in P7 WM were minimally damaged. **C**, **D**, Axons in P10 (**C**) and P21 (**D**) WM were damaged by AMPA/cyclothiazide but not glutamate or NMDA. Supplementation of aCSF with NBQX (30 μ M) during AMPA exposure prevented axon damage. * $p \leq 0.001$ versus drug-free aCSF control, Student's *t* test. Error bars represent SEM.

P3 WM are not injured by glutamate agonists, whereas WM axons at later stages degenerate after AMPA/cyclothiazide exposure. Our results suggest that mechanisms of ischemic axonal degeneration are developmentally regulated.

Brain slices as a model of developing white matter

Previously, we developed an acute slice model of ischemia in adult murine WM (Tekkok and Goldberg, 2001). Here, we adapt this model to developing WM. Acute slice preparations offer many advantages for the study of ischemia. Perfusion allows for precise control over the tissue environment and pharmacologic manipulations and mitigates many confounding factors seen *in vivo*, such as the effects of circulating factors or regional disparities in perfusion. Normal anatomic and functional relationships are maintained. Perhaps more importantly for the purposes of our study, acute slices allow for the study of WM in its appropriate developmental context. Maintenance of *in vivo* developmental relationships is critical to modeling WM pathology. In humans, cerebral WM is immature and unmyelinated during the peak of vulnerability to PWMI, corresponding to the WM of the newborn mouse in the first week life (Kinney and Back, 1998; Craig et al., 2003). Myelination in the mouse corpus callosum begins during the first postnatal week and progresses rapidly over the next 2–3 weeks (Sturrock, 1980). We studied premyelinated (P3 and P7), early myelinating (P10), and late myelinating (P21) WM.

In *thy1*-YFP-16 mice, axons in the corpus callosum are labeled with YFP, allowing for visualization of morphological changes in axoplasm by fluorescence microscopy (Gillingwater et al., 2002; Brendza et al., 2003). Because only some axons are labeled, the morphologic details of individual WM axons can be observed. At P3, YFP fluorescence was too dim to be useful for evaluation of axonal morphology. By P7, the YFP fluorescent signal was bright enough to examine axons in detail. We used anti-GFP immunohistochemistry to improve visualization of YFP⁺ axons. Using this approach, we observed individual axons in developing WM with high resolution by confocal microscopy (Fig. 1).

Axonal degeneration after OGD

We assessed structural axonal degeneration after OGD in developing WM. Analysis of *thy1*-YFP axonal morphology is a reliable method for detecting axonal damage (Bareyre et al., 2005; Kerschensteiner et al., 2005; Underhill and Goldberg, 2007) and compares well with electron microscopy, bright-field microscopy, and neurofilament immunohistochemistry (Beirowski et al., 2004) (Valentino and Goldberg, unpublished observations). Axonal morphology was preserved for at least 15 h. One hour of OGD followed by 9 h reperfusion resulted in swelling, beading, and fragmentation of YFP⁺ axons, characteristic of axonal injury. Damage was more readily visualized in YFP⁺ axons than in axons visualized via neurofilament immunohistochemistry (Fig. 2).

We characterized the time course of ischemic axonal injury. Axons did not demonstrate signs of injury during the period of OGD but underwent delayed degeneration during reperfusion. At P3, axonal damage did not appear until 9 h of reperfusion. In myelinating WM, injury appeared earlier, and most axons were beaded and fragmented by 9 h. The severity of axonal damage after OGD increased during postnatal development (Figs. 3, 4). In addition, P3 axons withstood brief periods of OGD without injury, whereas even brief periods of OGD damaged WM axons at later stages (Fig. 5). Our results demonstrate that axons in P3 WM are susceptible to delayed degeneration after OGD but are more tolerant of energy deprivation than axons at P7, P10, and P21, in accordance with previous work investigating axon conduction in rodent optic nerve (Fern et al., 1998).

Figure 7. Axonal degeneration after OGD is not mediated by ionotropic glutamate receptors at P3 and P7. Slices from P3 and P7 mice were perfused with aCSF containing NBQX (30 μ M) and/or MK-801 (10 μ M) under normoxic conditions, or 1 h before the onset of 1 h OGD, and continuing through the reperfusion period. YFP⁺ axon morphology in P3 WM after normoxic perfusion (**A**), drug-free OGD (**B**), OGD plus NBQX (**C**), and OGD plus MK-801 (**D**). NBQX and MK-801 did not prevent axon beading after OGD. Scale bar, 25 μ m. **E, F**, Quantitative analysis demonstrating no decrease in axon damage after OGD reperfusion in the presence of NBQX, MK-801, or both. Error bars represent SEM.

Mechanisms of axonal degeneration

Ischemia disrupts axonal homeostasis, resulting in accumulation of axoplasmic Na^+ and Ca^{2+} (Ransom et al., 1990; Stys et al., 1990; Stys and Lopachin, 1998; Li et al., 2000; Ouadouz et al., 2003). Excessive intra-axonal Ca^{2+} causes injury (Stys et al., 1990, 1992; Agrawal and Fehlings, 1996; Tekkk and Goldberg, 2001; Ouadouz et al., 2005).

Excessive glutamate receptor activation injures WM. Intracerebral injection of glutamate agonists damages axons and OLs (Marret et al., 1995; Fowler et al., 2003; Cuthill et al., 2006). In adult WM, ischemic injury is mediated by AMPA/kainate receptors (Tekkk and Goldberg, 2001). During ischemia, axons and OLs release glutamate, resulting in activation of WM glutamate receptors and cellular injury (Li et al., 1999; Back et al., 2007). Does glutamate injure axons directly? Glutamate receptor subunits are present in axon cylinders (Li and Stys, 2000). However, functional glutamate receptors have not been demonstrated on the axolemmal surface. Glutamate agonists do not damage isolated axons in culture, and antagonists do not prevent ischemic injury to isolated axons (Underhill and Goldberg, 2007).

Ionotropic glutamate receptor agonists did not damage WM axons at P3 and only minimally damaged axons at P7 (Fig. 6).

Figure 8. AMPA/kainate receptor activation mediates oligodendrocyte death after OGD at P3. Viable cells of the oligodendrocyte lineage in P3 WM labeled with the O4 antibody (arrows) after normoxic perfusion (top row), OGD reperfusion (middle row), and OGD reperfusion in the presence of NBQX (bottom row). Nuclei are visualized with Hoechst. After OGD, most O4⁺ cells display morphologic changes characteristic of cellular degeneration, such as pyknotic nuclei and condensed cytoplasm (arrowheads). NBQX treatment prevented these OGD-induced changes. Scale bar, 25 μ m.

Table 1. Cells of the oligodendroglial lineage are vulnerable to AMPA/kainate receptor-mediated injury after OGD throughout postnatal development

	Total viable cells/field (% viable vs normoxia)				
	Normoxia	OGD	OGD + NBQX	OGD + MK-801	OGD + NBQX/MK-801
P3					
O4 ⁺	24.3 ± 1.6 (100)	11.8 ± 0.9 (49)*	20.1 ± 1.4 (83) [#]	11.6 ± 1.1 (48)*	21.0 ± 0.8 (87) [#]
O1 ⁺					
APC ⁺					
P7					
O4 ⁺	36.3 ± 2.3 (100)	21.2 ± 1.1 (58)*	31.1 ± 1.3 (86) [#]	20.4 ± 1.2 (56)*	31.6 ± 1.6 (87) [#]
O1 ⁺	25.8 ± 1.6 (100)	16.6 ± 1.0 (64)*	23.3 ± 0.7 (90) [#]	14.4 ± 0.7 (56)*	24.7 ± 1.2 (95) [#]
APC ⁺					
P10					
O4 ⁺	21.6 ± 1.3 (100)	11.0 ± 1.7 (51)*	19.3 ± 1.5 (90) [#]	12.9 ± 1.3 (60)*	22.5 ± 1.4 (104) [#]
O1 ⁺	18.4 ± 1.0 (100)	9.3 ± 0.8 (51)*	19.3 ± 0.8 (105) [#]	8.3 ± 1.0 (45)*	16.58 ± 0.8 (90) [#]
APC ⁺	15.7 ± 1.0 (100)	6.8 ± 0.4 (44)*	15.3 ± 0.9 (98) [#]	7.8 ± 0.6 (49)*	16.2 ± 1.0 (103) [#]
P21					
O4 ⁺					
O1 ⁺	32.3 ± 1.2 (100)	17.8 ± 0.8 (54)*	31.8 ± 1.3 (98) [#]	17.6 ± 0.7 (54)*	27.8 ± 1.0 (86) [#]
APC ⁺	37.3 ± 2.7 (100)	14.4 ± 0.8 (39)*	33.5 ± 2.4 (90) [#]	16.2 ± 2.5 (43)*	31.3 ± 2.2 (84) [#]

Slices from P3, P7, P10, and P21 mice were subjected to normoxic perfusion, OGD reperfusion, or OGD reperfusion in the presence of ionotropic glutamate receptor antagonists. Oligodendroglia were labeled with the O4 antibody (pre-OLs and immature OLs), O1 antibody (immature OLs and mature OLs), or anti-APC antibody (mature OLs). The total number of viable cells per field was determined by counting the total number of immunoreactive cells for each individual antibody, minus those cells displaying morphologic signs of injury (pyknotic nuclei, collapsed cytoplasm). OGD reperfusion resulted in a decrease in the number of viable OL lineage cells at each postnatal age studied. NBQX preserved the number of viable cells after OGD, whereas MK-801 did not. * $p \leq 0.01$ versus normoxic control, [#] $p \leq 0.01$ versus drug-free OGD, Student's *t* test.

Figure 9. Axonal degeneration after OGD is mediated by AMPA/kainate receptors at P10 and P21. Slices were exposed to aCSF supplemented with NBQX (30 μ M) and/or MK-801 (10 μ M), under normoxic conditions, or 1 h before onset of 1 h OGD, and continuing through 9 h of reperfusion. At P10 (**A**) and P21 (**B**), NBQX decreased axon damage, whereas MK-801 had no effect. NBQX and MK-801 in combination decreased damage to the same extent as NBQX alone. * $p \leq 0.01$, ** $p \leq 0.001$ versus drug-free OGD, Student's *t* test. Error bars represent SEM.

Glutamate receptor antagonists did not prevent ischemic axonal injury in premyelinated WM (Fig. 7). In myelinating WM, AMPA/cyclothiazide exposure damaged axons, and AMPA/kainate receptor blockade decreased ischemic axonal damage (Figs.

6, 8). Neither glutamate nor NMDA damaged axons at any age studied. The lack of glutamate-induced damage may be attributable to the absence of cyclothiazide during glutamate exposure. Alternatively, rapid uptake of exogenous glutamate by glutamate transporters might limit the exposure of WM glutamate receptors to ligand (Rosenberg et al., 1992). In murine WM at P3 and P7, axons were vulnerable to ischemic injury. However, unlike in myelinating or myelinated WM, AMPA/kainate receptors did not contribute to this injury. Our results suggest a change in the vulnerability of WM axons to excitotoxic injury during early postnatal development. One possibility might be that axons in myelinated WM express functional glutamate receptors on the axolemmal surface, whereas premyelinated axons do not. We hypothesize that axon–OL interactions mediate axonal vulnerability to excitotoxic injury.

Axon–oligodendrocyte interactions

OLs express functional glutamate receptors, and there is significant homology of human and rodent glutamate receptor subunit expression patterns (Talos et al., 2006a,b). Developing OLs are particularly vulnerable to excitotoxicity (Deng et al., 2003; Rosenberg et al., 2003). Glutamate released during ischemia activates OL glutamate receptors, leading to OL excitotoxicity (Fern and Moller, 2000). Both AMPA/kainate and NMDA receptors contribute to ischemic OL injury, with AMPA/kainate antagonists protecting the cell soma and NMDA antagonists protecting processes (Follett et al., 2000; Karadottir et al., 2005; Salter and Fern, 2005). Our results do not support a role for NMDA receptor activation in axonal damage at any developmental age. Similarly, blockade of NMDA receptors alone did not prevent OL loss in our model, highlighting the importance of strategies aimed at protection of both the cell body and processes.

Glutamate may injure WM axons indirectly, via OLs. In myelinated WM, OL excitotoxicity contributes to axonal injury, whereas blockade of OL AMPA/kainate receptors protects axons (Tekkok and Goldberg, 2001). Our results support the hypothesis that OL excitotoxicity damages axons in myelinated WM. This may not be true in premyelinated WM. At P3 and P7, OLs are susceptible to AMPA/kainate receptor-mediated ischemic injury (Table 1). However, prevention of OL excitotoxicity does not decrease axonal damage. Changes in the intrinsic properties of

OLs and axons might account for the change in the relationship between OL injury and axonal damage. Alternatively, although premyelinated axons and pre-OLs interact during WM development, it is possible that mechanisms linking OL injury to axon damage are attenuated in unmyelinated WM but increase during myelination and maturation of the OL–axon unit. The mechanisms linking OL injury to axon damage might include release of toxic substances from injured OLs, loss of trophic support to axons, or loss of glial homeostatic functions. Perturbation of OL–myelin–axon interactions in myelinated WM decreases axonal damage after AMPA injection, suggesting that myelination may increase axonal vulnerability to OL-mediated damage (Fowler et al., 2006). In our model, axons in premyelinated WM are vulnerable to ischemic injury via a non-excitotoxic mechanism. The mechanism of axonal injury likely involves failure of ionic homeostasis and intra-axonal Ca^{2+} overload, as outlined above. The downstream executors of Ca^{2+} -mediated axonal injury remain undetermined. Our results underscore the importance of considering both OLs and axons in investigations of WM injury and suggest that different strategies may be necessary to effectively protect all components of perinatal WM from ischemic damage.

Axonal damage is characteristic of human PWMI. Axons in developing murine WM are vulnerable to ischemia. Our results suggest that the mechanisms of axonal injury vary during development. In P10 and P21 WM, AMPA/kainate receptors contribute to ischemic axonal injury. AMPA/kainate antagonism might provide important WM protection at these developmental stages. In murine WM at P3 and P7, corresponding to the window of peak vulnerability to human PWMI, ionotropic glutamate receptors do not contribute to ischemic axonal injury. Non-excitotoxic neuroprotective strategies may be beneficial during the period of vulnerability to PWMI in humans.

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