Published in final edited form as:

Brain Res. 2010 January 14; 1309: 146–154. doi:10.1016/j.brainres.2009.10.066.

Dysmyelinated axons in shiverer mice are highly vulnerable to α -Amino-3-hydroxy-5-Methylisoxazole-4-Propionic Acid (AMPA) receptor-mediated toxicity.

David Pitt¹, Ernesto Gonzales², Anne H. Cross², Mark P. Goldberg²

¹Department of Neurology, Division of Neuroimmunology, The Ohio State University, Columbus, OH 43210

²Department of Neurology and Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, MO 63110

Abstract

Glutamate excitotoxicity plays a role in white matter injury in many neurological diseases. Oligodendrocytes in particular are highly vulnerable to excitotoxicity, mediated through activation of AMPA/Kainate receptors. Myelin may also be injured independently via NMDA (N-methyl-D-aspartic acid) receptors located on peripheral oligodendroglial processes. Central axons are susceptible to glutamate receptor activation *in vivo*, but it is unclear whether this is mediated directly by activation of receptors expressed on axons, or indirectly through glutamate toxicity of myelin or neighboring glial cells.

We examined axonal vulnerability in mice deficient in myelin basic protein (shiverer), also expressing yellow fluorescent protein (YFP) in a subset of axons. YFP fluorescence, EM, and mouse behavior were assessed 24 hours after microstereotactical injections of S-AMPA or NMDA into lumbar dorsal columns. S-AMPA injection led to impaired rotarod performance and widespread axonal degeneration and was more pronounced in shiverer mice than controls. In contrast, NMDA injection did not cause axonal injury or behavioral changes in either group. These results indicate that spinal cord axons *in vivo* are vulnerable to toxicity mediated by AMPA but not NMDA receptors. The presence of compact myelin is not required for excitotoxic axon damage, and its absence may increase vulnerability. Further understanding of AMPA receptor-mediated axonal toxicity may provide new targets for neuroprotective therapy in WM diseases.

Introduction

Overactivation of ionotropic glutamate receptors plays a central role in the pathophysiology of white matter injury in a number of neurological conditions such as traumatic spinal cord injury, stroke and multiple sclerosis (MS) (Agrawal and Fehlings, 1997; Li et al., 1999; Pitt

Address for Correspondence: David Pitt, Department of Neurology, Division of Neuroimmunology, 790 Biomedical Research Tower, 460 W. 12th Ave, Columbus, OH 43210, Phone: (614) 292 0927 Fax: (614) 292 7544, david.pitt@osumc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

et al., 2000; Rosenberg et al., 1999). AMPA/kainate receptors have been identified as the dominant type of glutamate receptors on oligodendrocytes and astrocytes (Garcia-Barcina and Matute, 1996; Li and Stys, 2000). More recent studies have demonstrated the presence of NMDA (N-Methyl-D-aspartate) receptors on myelinating processes of oligodendrocytes (Karadottir et al., 2005; Micu et al., 2006, {Karadottir, 2005 #129; Salter and Fern, 2005). Data are less clear with regard to glutamate receptor expression in axons. Several studies have concluded that axonal tracts show little if any expression of glutamate receptors (Brand-Schieber and Werner, 2003b; Garcia-Barcina and Matute, 1996; Petralia and Wenthold, 1992). Stys and colleagues reported that the glutamate receptor subtypes GluR3 and 4 are readily detectable on axons of rat dorsal columns (Li and Stys, 2000). However, the question whether these receptors are functional and present on axonal cylinders or are transported within axoplasm e.g. towards the synapse, remains

Overactivation of AMPA/kainate receptors is highly toxic to oligodendrocytes *in vitro* and *in vivo* (Matute et al., 1997; McDonald et al., 1998; Yoshioka et al., 1995). In contrast, astrocytes were found to be resistant to excitotoxicity although astrocyte death can be induced *in vitro* by blocking desensitization of AMPA receptors (David et al., 1996; Yamaya et al., 2002). Axons are susceptible to AMPA toxicity administered *in vivo* (Fowler et al., 2003; Fowler et al., 2006) and are protected by AMPA receptor antagonists in a number of animal models for white matter disease (Pitt et al., 2000; Rosenberg et al., 1999; Tekkok and Goldberg, 2001; Tekkok et al., 2005). Interestingly, isolated axons in culture are resistant to glutamate toxicity (Underhill and Goldberg, 2007) suggesting that excitotoxic injury to unmyelinated axons may require interaction with glia and/or myelin. The latter notion is supported by a recent study by Fowler and colleagues, who observed that lack of the myelin component, proteolipid protein, ameliorates AMPA-mediated axonal loss. In addition, the presence of NMDA receptors on myelinating processes may play a role in mediating excitotoxicity to the ensheathed axon (Bakiri et al., 2008). Here, we investigate whether compact myelin is necessary for induction of excitotoxic injury in axons. Activation of AMPA but not NMDA receptors resulted in damage of unmyelinated and myelinated axons *in vivo* indicating that axonal damage is independent of myelination.

Materials and Methods

Mice -

Shiverer mice (C3HeB/FeJ-MBP^{shi/shi}) (Kirschner and Ganser, 1980) were purchased from Jackson Laboratories and were crossbred at our institution with *thy1-YFP-H* (C57BL/6J) mice (Feng et al., 2000) that express yellow fluorescent protein in a subset of axons. In the dorsal columns, YFP is expressed predominantly in the ventral portion, i.e. in the area of the corticospinal pathways. Female mice bred for at least 3 generations towards the C57BL/6J background were used at an age of 9–12 weeks for spinal cord injection. In addition to wildtype mice, female heterozygous littermates (shi/+) served as controls since these mice have no behavioral abnormalities and produce structurally normal myelin (see results). Animal surgery and care were carried out in accordance with the Institute of Laboratory Animal research (ILAR) Guide for the Care and Use of Laboratory Animals; all procedures were approved by the Animal Studies Committee at Washington University.

Microstereotaxic injection -

Mice were anaesthetized via inhalation of isofluorane. Using sterile technique, the spinal cord was visualized at L2/3 after removing the overlying paraspinal musculature without performing a laminectomy. A micropipette, pulled from a N-51-A borosilicate glass capillary, was attached to a microinjector (Nanoject I, Drummond, Broomall, PA) and lowered into the dorsal columns immediately adjacent to the posterior spinal artery. A volume of 0.3 l S-AMPA, NMDA (Tocris, Evansville, MO; both adjusted for pH and osmolality) or PBS was injected at a depth of 0.5 mm. We injected S-AMPA at concentrations of 10 mM, 20 mM and 30 mM resulting in doses of 3, 6 and 9 nmol per injection. NMDA was injected at a concentration of 50 or 100 mM resulting in doses of 15 or 30 nmol per injection. The injection site was marked in some animals by adding tetramethylrhodamine-coupled low molecular dextran (Microruby, Molecular Probes, Carlsbad, California) to the injectorate. Of note, injection of S-AMPA and less frequently of NMDA induced transient seizure activity upon recovery from isofluorane anesthesia, presumably due to circulation of small amounts of injectorate to the brain. Seizures were treated with an additional 10 minutes of isofluorane anesthesia, and if necessary, with additional i.p. injection of 125 µg diazepam. 24 hours after injection, mice were sacrificed and perfused by intracardial injection of saline followed by 4% paraformaldehyde,. The spinal cord was removed, postfixed in paraformaldehyde for 24 hours, cryoprotected in sucrose and subsequently embedded in OCT.

Electron microscopy -

Mice were perfused and fixed overnight in 4% paraformaldehyde/2.5% glutaraldehyde/1% tannic acid in 0.1 molar sodium cacodylate buffer. Samples were postfixed in 2% osmium tetroxide in 0.1M cacodylate buffer. Samples were then dehydrated from 50% to absolute ethanol, changed to propylene oxide and then embedded in epon. Epon blocks were sectioned on a Leica EM UC6 ultramicrotome. Thick sections were stained with 1% toluidine blue and ultrathin sections were stained with uranyl acetate and lead citrate. Thin sections were viewed in a Hitachi Transmission Electron Microscope using an AMT digital camera system for micrographs.

Quantification of axonal damage in YFP⁺ axons -

Spinal cords were sectioned longitudinally in 10 µm sections. All sections containing dorsal columns were assessed by conventional fluorescence microscopy. For quantification, images of dorsal columns (magnification 20x) from the whole section were digitally assembled to generate a montage of the dorsal columns. A grid overlay was superimposed on the resulting image and each grid box was scored by a blinded observer for the presence of axon damage at various distances from the injection site using the following system: 0, no damage; 1, axon swelling and/or beading; 2, axon fragmentation (McCarran and Goldberg, 2007).

Gait assessment of mice -

Hindlimb movements were examined 24 hours after injection and were graded categorically as either normal, mild to moderately affected (weak hindlimbs), or severely affected (hindlimb paralysis).

Immunocytochemistry -

Spinal cords embedded in OCT were serially cut into 10 μ m sections. Sections were permeabilized with ice cold acetone, quenched with hydrogen peroxide and incubated overnight at 4°C with the following primary antibodies: GluR1 (1:200), GluR2/3 (1:200), GluR4 (1:200, all: Upstate Biotechnology, Lake Placid, N.Y.), GFAP and SMI 312 (1:500, Covance, USA). Biotin-labeled secondary antibodies were applied and detected using TSA amplification technology (Perkin-Elmer Life Science Products, Boston, USA). Negative controls with isotype-matched irrelevant antibodies were performed for each of the primary antibodies. Double immunofluorescence labeling was carried out with primary antibodies applied sequentially. Images were acquired either on a Nikon Eclipse TE 300 microscope or a Zeiss LSM 5 Pascal inverted laser scanning confocal microscope using Zeiss LSM imaging software.

Rotarod assay -

Rotarod testing was performed using a motor-driven rotating cylinder with a rough surface flanked by two larger plates (Rotamex, Columbus Instruments, Columbus, OH). Mice were placed on a rotating cylinder that accelerated at a set rate. Each mouse was trained in the three days preceding spinal cord injection in two sessions daily. 24 hours after injection, each animal was tested in a series of three consecutive trials separated by 5 minute intervals. Time until the animal dropped from the rod was determined and the best time of the three trials was counted.

Statistical analysis -

Linear repeated measures mixed models were used to describe the effect of study group (control vs. shiverer), AMPA dose and distance from injection site on axon counts per unit area. Study group, dose and distance were fixed by design and represent the range about which conclusions will be drawn, so all were treated as fixed effects. Axon counts were analyzed on a square root scale in order to more closely approximate a Gaussian distribution. All two-way interactions were examined. Linear contrasts used to test for the presence of linear and quadratic trends by dose, as well as to compare outcomes by between study groups at each dose and between pairs of doses within each study group. The models were also used to find adjusted estimates of mean axon counts by study group and dose. All analyses were carried out using SAS v.9.1.

Results

Dorsal columns lack myelin in mice homozygous (shi/shi) but not heterozygous for the shiverer mutation (+/shi)

We analyzed dorsal column axons from shi/shi mice, shi/+ and WT (+/+) mice by electron microscopy. As described before (Readhead and Hood, 1990), axons in shiverer mice were severely lacking in myelin. A large proportion of axons were devoid of myelin and when present, myelin was not well compacted and lacked the major dense line. In contrast, myelin in heterozygous shi/+ mice appeared compact, structurally normal and comparable in thickness to that in WT mice (Figure 1, upper panel), as reported before (Cammer et al.,

1984). Therefore, we used heterozygous littermates of shiverer mice in addition to WT mice backcrossed to contain the *neuron-specific* YFP as controls.

Visualization of axons in spinal cord dorsal columns in YFP⁺ shiverer and control mice

thy1-YFP-H mice express yellow fluorescent protein (YFP) in a subset of neurons including cortical layer 5 neurons (Feng et al., 2000) resulting in YFP labeling of corticospinal tract axons within the dorsal columns. Figure 2 (upper panel) shows well-preserved YFP⁺ axons in the dorsal columns of naïve mice. Individual axons are clearly distinguishable and visible over long distances allowing tracing of axonal morphology in fine detail. Fluorescence intensities in the dorsal columns were comparable in shiverers and controls. We detected occasional focal swelling in YFP⁺ axons of shiverer mice (Fig. 2b, inset). These axonal swellings increased in number with increasing age and may be indicative of axonal degeneration. Similarly, accumulation of β -APP in shiverer axons has been reported before (Loers et al., 2004).

Visualization of axon damage in shiverer and control mice after S-AMPA injection

Acute axonal pathology in spinal cord dorsal columns after S-AMPA injection was evaluated by electron and light microscopy. Electron micrographs of the ventral-most portion of the dorsal columns in the vicinity of the injection site showed dystrophic axons that were edematous, lacked organelles and cytoskeleton components as well as dissolution of axoplasmic membranes within 24 hours after injection of 20 mM S-AMPA in both shiverer and control mice (Fig. 1, lower row). Using light microscopy, we detected distinct beading and fragmentation of YFP⁺ axons in both shiverer and control mice (Figure 2, lower row). These morphologic features of YFP⁺ axon degeneration have been described in detail in *in vitro* and *in vivo* models of ischemia, peripheral nerve injury and spinal cord injury (Kerschensteiner et al., 2005; McCarran and Goldberg, 2007; Underhill and Goldberg, 2007), and were found to be sensitive markers correlating closely with injury as assessed by conventional light and electron microscopy (Beirowski et al., 2004).

Dysmyelinated axons are more vulnerable to AMPA-mediated excitotoxicity than myelinated axons

Next, we set out to quantify and compare AMPA receptor-mediated axonal degeneration in shiverer and control mice. Injection of vehicle resulted in minimal local damage to axons at the injection site (Fig 3a). Injection of S-AMPA caused dose-dependent, prominent and widespread axonal damage with maximum intensity around the injection site. Low and medium S-AMPA concentrations (10 and 20mM) caused significantly more axonal damage in shiverer compared to WT mice. Moreover, axonal damage was incremental at 10 and 20 mM S-AMPA in shiverer mice while axons in myelinated control mice sustained comparable, low damage at both concentrations. Damage was similar in both groups at high concentration (30 mM S-AMPA; Fig. 3b). Therefore, the threshold to reach significant excitotoxic axonal damage is lower in shiverer than in WT mice.

Behavioral deficits after S-AMPA-injection are more pronounced in shiverer than control mice

Parallel to the histological assessment of axonal damage after S-AMPA injection, we assessed behavioral changes in shiverer mice and controls. Of note, seizures observed after S-AMPA were more frequent and of longer duration in shiverer mice compared to WT or heterozygous controls. S-AMPA injection induced hind limb and tail paresis in shiverer mice more so than in control mice. 10 mM of S-AMPA induced complete paralysis in 4 of 6 shiverer mice while control mice showed no (4/6) or only mild paraparesis (2/6). Injection of 20 mM S-AMPA resulted in complete paralysis in 5 of 7 and mild-moderate paraparesis in 2 of 7 shiverer mice. Only 1 control mouse of 6 was fully paralyzed, the remainder showed mild-moderate paraparesis. 30 mM S-AMPA induced paralysis in all mice injected.

To better quantify these behavioral changes, we used the rotarod assay, a forced activity that provides an objective and quantitative measure of motor control (Kuhn et al., 1995). Animals were acclimated to the assay for two days before injection. At baseline, shiverer mice had significantly impaired motor coordination and performed poorly compared to control mice at a standard setting of 2 rpm starting speed and acceleration of 1rpm/10sec. Similar findings with this assessment paradigm in shiverer mice were reported by Kuhn et al. (Kuhn et al., 1995). Due to the large baseline difference, we applied differing speed and acceleration settings in shiverer mice in order to increase sensitivity of rotarod testing. Shiverer mice were started at 1 rpm and accelerated at 0.3 rpm/10 sec resulting in similar latency on the rotarod in both groups prior to injection. Rotarod scores after S-AMPA injections were expressed as percentage of performance after vehicle injection. Increasing concentrations of S-AMPA resulted in a relative decrease in performance in both groups (Fig. 4). Shiverer mice performed consistently worse than heterozygous and WT controls with a statistically significant difference at 20 mM S-AMPA.

Activation of NMDA receptors does not result in axonal injury in shiverer or control mice

Three recent studies have shown that oligodendrocytes express NMDA receptors on myelin and on oligodendrocyte processes (Karadottir et al., 2005; Micu et al., 2006; Salter and Fern, 2005). To determine whether direct activation of NMDA receptors mediates excitotoxicity-induced axonal damage, we injected high concentrations (up to 100 mM) of NMDA into lumbar dorsal columns of WT and shiverer *thy1-YFP2.2* mice. As noted in the methods, brief seizure activity was observed in the majority of mice after recovery from anesthesia, confirming a biological effect of circulating NMDA. However, NMDA did not result in hind limb paresis in any of the injected animals or impaired performance on rota-rod testing after 24 hrs or 72 hrs. (Fig. 5a, b) Histological analysis of the dorsal columns showed morphologically intact axons in both groups (Fig. 5c). Thus, direct activation of NMDA receptors did not result in either morphological or functional evidence of axonal matter damage in myelinated or unmyelinated axons.

Shiverer mice express more GluR1 than control mice

To investigate the mechanism of increased axonal sensitivity to S-AMPA-induced excitotoxicity in shiverer mice, we studied AMPA receptor expression in naïve shiverer and control mice. AMPA receptors are heteromeric complexes composed of four separate

subunits, GluR1 through GluR4 (Talos et al., 2006). The expression of different GluR subunits is developmentally regulated and dictates the functional properties of the AMPA receptor complex. The presence of the subunit GluR2 prevents permeability for Ca^{2+} and is therefore less conducive to excitotoxicity (Jonas and Burnashev, 1995).

We determined expression of the AMPA receptor subunits by immunolabeling in spinal cord white matter of shiverer and control mice (Fig. 6a). Expression of GluR1 was greatly increased in shiverer mice. Double labeling that this was present on astrocytic cell bodies and on long GFAP-positive processes that ran in parallel to axons (Fig. 6b). In contrast, immunolabeling with GluR1 in control mouse white matter was less intense and confined to cell bodies. In shiverer mice, GluR1 did not appear to be upregulated in oligodendrocytes compared to wild type mice. Similarly, immunoreactivity for GluR2/3 and GluR4 showed no noticeable difference between shiverer and control white matter. In both groups, none of the glutamate receptor subtypes appeared to localize to axons.

Discussion

This study shows that excitotoxic damage to central axons *in vivo* does not require presence of myelin. For our study, we developed a novel *in vivo* model of excitotoxicity, injection of glutamate agonists into the dorsal columns of mice, allowing for histological and functional assessment of axonal damage. To accurately quantify axonal pathology, we evaluated morphological changes in *thy1-YFP2.2* transgenic mice expressing fluorescent protein in axons, an approach superior to immunohistochemical axonal markers (Bannerman and Hahn, 2007; McCarran and Goldberg, 2007). Functional deficits, i.e. hind limb paresis, were evaluated 24 hours after injection. To compare fully myelinated and unmyelinated axons, we chose a genetic approach, shiverer mice lacking compact myelin, as a prototypic model for dysmyelination. One potential limitation of shiverer mice is that developmental hypomyelination may differ from demyelination acquired under neurological conditions such as MS or spinal cord injury. However, this genetic model provides uniform and widespread absence of spinal cord myelin, an important experimental advantage for studies of injury induced by microstereotaxic injection. Models of acquired demyelination induced by ethidium bromide or lyssolecithin proved less suitable because of significant inflammation and presence of oligodendrocyte precursor cells (data not shown) that would have introduced new variables to these experiments (Woodruff and Franklin, 1999).

It has been demonstrated previously that S-AMPA injection into cerebral white matter and striatum results in damage to axons (Fowler et al., 2003; Fowler et al., 2006). Moreover, axons are protected by the AMPA/kainate receptor antagonist, NBQX, in *in vivo* models of experimental allergic encephalomyelitis (EAE) (Pitt et al., 2000; Smith et al., 2000), spinal cord injury (Rosenberg et al., 1999; Wrathall et al., 1994) and models of white matter ischemia (Kanellopoulos et al., 2000; Tekkok and Goldberg, 2001; Tekkok et al., 2005). Excitotoxic injury to axons could be transmitted through oligodendrocytes and/or myelin, structures that are highly sensitive to glutamate toxicity. This pathway has been suggested previously by us (McCarran and Goldberg, 2007; Tekkok and Goldberg, 2001) and Fowler and colleagues (Fowler et al., 2006) who observed that axons in *Plp* knockout mice were less susceptible to AMPA toxicity. *Plp* null mice lack the myelin integral membrane protein,

proteolipid protein (PLP), but produce normally compacted myelin (Griffiths et al., 1998). Therefore, myelin may interact with axons through PLP to induce excitotoxic damage. The putative pathway of axonal excitotoxicity mediated through myelin is the subject of the present study.

We report that injection of S-AMPA but not NMDA caused dose-dependent, prominent and widespread axonal damage in dorsal column white matter. Unmyelinated axons in shiverer mice showed increased sensitivity to S-AMPA compared to myelinated axons suggesting that myelin is not required for excitotoxic injury to axons. The histological findings of axonal pathology corresponded with behavioral changes in shiverer mice that were more severe than in controls. At a dose of 30mM S-AMPA, behavioral deficits and axonal damage were equal in shiverer and wildtype mice, presumably because at this concentration, maximum activation of AMPA/Kainate receptors is reached. Putative causes of increased axonal vulnerability in shiverer mice include changes in the axonal microenvironment, lack of trophic support or lack of barrier function of myelin to toxic substances (Edgar and Garbern, 2004; Irvine and Blakemore, 2008).

NMDA receptors are expressed on oligodendroglial processes and oligodendrocyte precursors. In ischemia, NMDA receptors raise calcium within myelin and contribute to injury in myelin (Micu et al., 2006; Salter and Fern, 2005) and myelinated axons (Karadottir et al., 2005; Micu et al., 2006; Salter and Fern, 2005) *in vitro*. In contrast, in previous studies from our laboratory and others NMDA receptor blockade failed to prevent ischemic damage (Baltan et al., 2008; Tekkok and Goldberg, 2001, {Tekkok, 2007 #147; Tekkok et al., 2007). In our model, high concentrations of NMDA (50 – 100 mM) did not induce histological changes in shiverer or control white matter. In separate experiments, application of AMPA but not NMDA caused oligodendrocyte loss and myelin injury in transgenic mice expressing green fluorescent protein in oligodendrocytes (data not shown). Similarly, behavioral deficits on rotarod testing were absent in both groups. Thus, direct activation of white matter NMDA receptors does not cause axonal damage in this model. For this reason, we did not examine the expression of NMDA receptors in shiverer vs. wildtype mice.

If myelin is not essential for axonal damage, how is excitotoxicity signaled to axons? A possible pathway is that of direct injury through axonal glutamate receptors. This mechanism has not been demonstrated experimentally; isolated axons in culture are not injured by direct application of glutamate agonists, and are not protected from energy deprivation by glutamate receptor blockade (Underhill and Goldberg, 2007). Several careful studies failed to observe glutamate receptors in white matter axons *in vivo* (Brand-Schieber and Werner, 2003a; Garcia-Barcina and Matute, 1998; Petralia and Wenthold, 1992). Stys and colleagues demonstrated immunoreactivity for glutamate receptor subtypes GluR3 and 4 in myelinated axons (Li and Stys, 2000).

In more recent, elegant studies by Stys, axolemmal expression of functional glutamate receptor subtypes (GluR4, -5 and -6) on central, myelinated axons was demonstrated (Ouardouz et al., 2009a; Ouardouz et al., 2009b). Moreover, two separate glutamate receptor-dependent signaling pathways were identified that both lead to increase in intra-axonal Ca^{2+} . This suggests that excitotoxic damage to axons is a direct response to

glutamate receptor activation and is mediated by localized Ca^{2+} release. This is an intriguing possibility although it has not yet been demonstrated that these receptors are present in sufficient

This study does not exclude a role for excitotoxicity mediated indirectly through activation of glutamate receptors on white matter glial cells. Although shiverer mice lack most myelin, oligodendrocytes are still present, and in higher numbers than control animals (Bu et al., 2004). In cell culture, excitotoxicity to oligodendroglia may cause secondary damage to axons without direct contact through myelin (Underhill and Goldberg, 2002). Therefore, excitotoxicity mediated by oligodendrocytes and/or astrocytes could contribute to vulnerability of both myelinated and demyelinated axons, e.g. through release of reactive oxygen species or other toxic factors that can damage axons (Matute, 2007). The increased number of oligodendrocytes in shiverer mice could then contribute to the heightened axonal susceptibility in shiverer mice. An unexpected finding in this study was that of markedly increased expression of GluR1 in white matter astrocytes of shiverer mice, providing another possible pathway for enhanced white matter vulnerability specific to these mice, i.e. through activation of astrocytic glutamate receptors resulting in indirect toxicity to axons.

Results from ischemia models demonstrate both ionic and excitotoxic mechanisms of axonal injury under conditions of energy deprivation (Stys, 2004; Tekkok and Goldberg, 2001; Tekkok et al., 2007). Similar pathways of axonal degeneration are likely relevant to other white matter diseases. We and others have proposed that excitotoxicity plays a role in axonal damage in MS, based on animal studies in which AMPA/kainate antagonists prevented both axonal and oligodendroglial loss (Pitt et al., 2000; Smith et al., 2000). Furthermore, we demonstrated that glutamate uptake mechanisms are permanently disrupted in MS (Pitt et al., 2003; Werner et al., 2001), suggesting focal glutamate overload in MS lesions.

The present study suggests that excitotoxicity affects also non-myelinated axons. Although the mechanisms of this damage are not yet understood, our findings could be relevant to diseases of chronic demyelination such as multiple sclerosis, spinal cord injury, periventricular leukomalacia, and vascular dementia. Absence of myelin could render axons highly sensitive to glutamate toxicity and facilitate progressive axonal loss. If confirmed, our findings would provide a rationale for treatment aimed at continuous suppression of excitotoxicity in these conditions.

Acknowledgements:

We thank Dr. Kathryn Trinkaus, Division of Biostatistics, Washington University for assistance with statistical analyses. This work was supported by the Frala Osherow Fund for MS research, a pilot award from the National MS Society USA and the Barnes-Jewish Hospital Foundation, NIH Neuroscience Blueprint Center Core Grant P30 NS057105 to Washington University, P01 NS032636 (MPG), and R01 NS36265 (MPG). D.P. was a fellow of the National MS Society (FG # NMSS 1666-A-1). AHC was supported in part by the Manny and Rosalyn Rosenthal - Dr. John L. Trotter MS Center Chair in Neuroimmunology.

References

Agrawal SK, Fehlings MG, 1997 Role of NMDA and non-NMDA ionotropic glutamate receptors in traumatic spinal cord axonal injury. *J Neurosci.* 17, 1055–63. [PubMed: 8994060]

- Bakiri Y, Hamilton NB, Karadottir R, Attwell D, 2008 Testing NMDA receptor block as a therapeutic strategy for reducing ischaemic damage to CNS white matter. *Glia*. 56, 233–40. [PubMed: 18046734]
- Baltan S, Besancon EF, Mbow B, Ye Z, Hamner MA, Ransom BR, 2008 White matter vulnerability to ischemic injury increases with age because of enhanced excitotoxicity. *J Neurosci*. 28, 1479–89. [PubMed: 18256269]
- Bannerman PG, Hahn A, 2007 Enhanced visualization of axonopathy in EAE using thy1-YFP transgenic mice. *J Neurol Sci*. 260, 23–32. [PubMed: 17493638]
- Beirowski B, Berek L, Adalbert R, Wagner D, Grumme DS, Addicks K, Ribchester RR, Coleman MP, 2004 Quantitative and qualitative analysis of Wallerian degeneration using restricted axonal labelling in YFP-H mice. *J Neurosci Methods*. 134, 23–35. [PubMed: 15102500]
- Brand-Schieber E, Werner P, 2003a (+/-)-Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid and kainate receptor subunit expression in mouse versus rat spinal cord white matter: similarities in astrocytes but differences in oligodendrocytes. *Neurosci Lett*. 345, 126–30. [PubMed: 12821187]
- Brand-Schieber E, Werner P, 2003b AMPA/kainate receptors in mouse spinal cord cell-specific display of receptor subunits by oligodendrocytes and astrocytes and at the nodes of Ranvier. *Glia*. 42, 12–24. [PubMed: 12594733]
- Bu J, Banki A, Wu Q, Nishiyama A, 2004 Increased NG2(+) glial cell proliferation and oligodendrocyte generation in the hypomyelinating mutant shiverer. *Glia*. 48, 51–63. [PubMed: 15326615]
- Cammer W, Kahn S, Zimmerman T, 1984 Biochemical abnormalities in spinal cord myelin and CNS homogenates in heterozygotes affected by the shiverer mutation. *J Neurochem*. 42, 1372–8. [PubMed: 6200571]
- David JC, Yamada KA, Bagwe MR, Goldberg MP, 1996 AMPA receptor activation is rapidly toxic to cortical astrocytes when desensitization is blocked. *J Neurosci*. 16, 200–9. [PubMed: 8613786]
- Edgar JM, Garbern J, 2004 The myelinated axon is dependent on the myelinating cell for support and maintenance: molecules involved. *J Neurosci Res*. 76, 593–8. [PubMed: 15139018]
- Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR, 2000 Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron*. 28, 41–51. [PubMed: 11086982]
- Fowler JH, McCracken E, Dewar D, McCulloch J, 2003 Intracerebral injection of AMPA causes axonal damage in vivo. *Brain Res*. 991, 104–12. [PubMed: 14575882]
- Fowler JH, Edgar JM, Pringle A, McLaughlin M, McCulloch J, Griffiths IR, Garbern JY, Nave KA, Dewar D, 2006 Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid-mediated excitotoxic axonal damage is attenuated in the absence of myelin proteolipid protein. *J Neurosci Res*. 84, 68–77. [PubMed: 16625661]
- Garcia-Barcina JM, Matute C, 1996 Expression of kainate-selective glutamate receptor subunits in glial cells of the adult bovine white matter. *Eur J Neurosci*. 8, 2379–87. [PubMed: 9064077]
- Garcia-Barcina JM, Matute C, 1998 AMPA-selective glutamate receptor subunits in glial cells of the adult bovine white matter. *Brain Res Mol Brain Res*. 53, 270–6. [PubMed: 9473692]
- Griffiths I, Klugmann M, Anderson T, Yool D, Thomson C, Schwab MH, Schneider A, Zimmermann F, McCulloch M, Nadon N, Nave KA, 1998 Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science*. 280, 1610–3. [PubMed: 9616125]
- Irvine KA, Blakemore WF, 2008 Remyelination protects axons from demyelination-associated axon degeneration. *Brain*. 131, 1464–77. [PubMed: 18490361]
- Jonas P, Burnashev N, 1995 Molecular mechanisms controlling calcium entry through AMPA-type glutamate receptor channels. *Neuron*. 15, 987–90. [PubMed: 7576666]
- Kanellopoulos GK, Xu XM, Hsu CY, Lu X, Sundt TM, Kouchoukos NT, 2000 White matter injury in spinal cord ischemia: protection by AMPA/kainate glutamate receptor antagonism. *Stroke*. 31, 1945–52. [PubMed: 10926962]
- Karadottir R, Cavalier P, Bergersen LH, Attwell D, 2005 NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. *Nature*. 438, 1162–6. [PubMed: 16372011]

- Kerschensteiner M, Schwab ME, Lichtman JW, Misgeld T, 2005 In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. *Nat Med.* 11, 572–7. [PubMed: 15821747]
- Kuhn PL, Petroulakis E, Zazanis GA, McKinnon RD, 1995 Motor function analysis of myelin mutant mice using a rotarod. *Int J Dev Neurosci.* 13, 715–22. [PubMed: 8787862]
- Li S, Mealing GA, Morley P, Stys PK, 1999 Novel injury mechanism in anoxia and trauma of spinal cord white matter: glutamate release via reverse Na⁺-dependent glutamate transport. *J Neurosci.* 19, RC16. [PubMed: 10407058]
- Li S, Stys PK, 2000 Mechanisms of ionotropic glutamate receptor-mediated excitotoxicity in isolated spinal cord white matter. *J Neurosci.* 20, 1190–8. [PubMed: 10648723]
- Loers G, Aboul-Enein F, Bartsch U, Lassmann H, Schachner M, 2004 Comparison of myelin, axon, lipid, and immunopathology in the central nervous system of differentially myelin-compromised mutant mice: a morphological and biochemical study. *Mol Cell Neurosci.* 27, 175–89. [PubMed: 15485773]
- Matute C, Sanchez-Gomez MV, Martinez-Millan L, Miledi R, 1997 Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc Natl Acad Sci U S A.* 94, 8830–5. [PubMed: 9238063]
- Matute C, 2007 Interaction between glutamate signalling and immune attack in damaging oligodendrocytes. *Neuron Glia Biol.* 3, 281–5. [PubMed: 18634560]
- McCarran WJ, Goldberg MP, 2007 White matter axon vulnerability to AMPA/kainate receptor-mediated ischemic injury is developmentally regulated. *J Neurosci.* 27, 4220–9. [PubMed: 17429000]
- McDonald JW, Althomsons SP, Hyrc KL, Choi DW, Goldberg MP, 1998 Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. *Nat Med.* 4, 291–7. [PubMed: 9500601]
- Micu I, Jiang Q, Coderre E, Ridsdale A, Zhang L, Woulfe J, Yin X, Trapp BD, McRory JE, Rehak R, Zamponi GW, Wang W, Stys PK, 2006 NMDA receptors mediate calcium accumulation in myelin during chemical ischaemia. *Nature.* 439, 988–92. [PubMed: 16372019]
- Ouardouz M, Coderre E, Basak A, Chen A, Zamponi GW, Hameed S, Rehak R, Yin X, Trapp BD, Stys PK, 2009a Glutamate receptors on myelinated spinal cord axons: I. GluR6 kainate receptors. *Ann Neurol.* 65, 151–9. [PubMed: 19224535]
- Ouardouz M, Coderre E, Zamponi GW, Hameed S, Yin X, Trapp BD, Stys PK, 2009b Glutamate receptors on myelinated spinal cord axons: II. AMPA and GluR5 receptors. *Ann Neurol.* 65, 160–6. [PubMed: 19224531]
- Petralia RS, Wenthold RJ, 1992 Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. *J Comp Neurol.* 318, 329–54. [PubMed: 1374769]
- Pitt D, Werner P, Raine CS, 2000 Glutamate excitotoxicity in a model of multiple sclerosis. *Nat Med.* 6, 67–70. [PubMed: 10613826]
- Pitt D, Nagelmeier IE, Wilson HC, Raine CS, 2003 Glutamate uptake by oligodendrocytes: Implications for excitotoxicity in multiple sclerosis. *Neurology.* 61, 1113–20. [PubMed: 14581674]
- Readhead C, Hood L, 1990 The dysmyelinating mouse mutations shiverer (shi) and myelin deficient (shimld). *Behav Genet.* 20, 213–34. [PubMed: 1693848]
- Rosenberg LJ, Teng YD, Wrathall JR, 1999 2,3-Dihydroxy-6-nitro-7-sulfamoyl benzo(f)quinoxaline reduces glial loss and acute white matter pathology after experimental spinal cord contusion. *J Neurosci.* 19, 464–75. [PubMed: 9870974]
- Salter MG, Fern R, 2005 NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. *Nature.* 438, 1167–71. [PubMed: 16372012]
- Smith T, Groom A, Zhu B, Turski L, 2000 Autoimmune encephalomyelitis ameliorated by AMPA antagonists. *Nat Med.* 6, 62–6. [PubMed: 10613825]
- Stys PK, 2004 Axonal degeneration in multiple sclerosis: is it time for neuroprotective strategies? *Ann Neurol.* 55, 601–3. [PubMed: 15122698]
- Talos DM, Fishman RE, Park H, Folkerth RD, Follett PL, Volpe JJ, Jensen FE, 2006 Developmental regulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor subunit

- expression in forebrain and relationship to regional susceptibility to hypoxic/ischemic injury. I. Rodent cerebral white matter and cortex. *J Comp Neurol.* 497, 42–60. [PubMed: 16680782]
- Tekkok SB, Goldberg MP, 2001 Ampa/kainate receptor activation mediates hypoxic oligodendrocyte death and axonal injury in cerebral white matter. *J Neurosci.* 21, 4237–48. [PubMed: 11404409]
- Tekkok SB, Faddis BT, Goldberg MP, 2005 AMPA/kainate receptors mediate axonal morphological disruption in hypoxic white matter. *Neurosci Lett.* 382, 275–9. [PubMed: 15925103]
- Tekkok SB, Ye Z, Ransom BR, 2007 Excitotoxic mechanisms of ischemic injury in myelinated white matter. *J Cereb Blood Flow Metab.* 27, 1540–52. [PubMed: 17299453]
- Underhill SM, Goldberg MP, 2002 Cell culture models of white matter vulnerability to excitotoxicity. *Glia.* Supplement 1, 247.
- Underhill SM, Goldberg MP, 2007 Hypoxic injury of isolated axons is independent of ionotropic glutamate receptors. *Neurobiol Dis.* 25, 284–90. [PubMed: 17071096]
- Werner P, Pitt D, Raine CS, 2001 Multiple sclerosis: altered glutamate homeostasis in lesions correlates with oligodendrocyte and axonal damage. *Ann Neurol.* 50, 169–80. [PubMed: 11506399]
- Woodruff RH, Franklin RJ, 1999 Demyelination and remyelination of the caudal cerebellar peduncle of adult rats following stereotaxic injections of lysolecithin, ethidium bromide, and complement/anti-galactocerebroside: a comparative study. *Glia.* 25, 216–28. [PubMed: 9932868]
- Wrathall JR, Choiniere D, Teng YD, 1994 Dose-dependent reduction of tissue loss and functional impairment after spinal cord trauma with the AMPA/kainate antagonist NBQX. *J Neurosci.* 14, 6598–607. [PubMed: 7965063]
- Yamaya Y, Yoshioka A, Saiki S, Yuki N, Hirose G, Pleasure D, 2002 Type-2 astrocyte-like cells are more resistant than oligodendrocyte-like cells against non-N-methyl-D-aspartate glutamate receptor-mediated excitotoxicity. *J Neurosci Res.* 70, 588–98. [PubMed: 12404513]
- Yoshioka A, Hardy M, Younkin DP, Grinspan JB, Stern JL, Pleasure D, 1995 Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors mediate excitotoxicity in the oligodendroglial lineage. *J Neurochem.* 64, 2442–8. [PubMed: 7539052]

Figure 1.

AMPA-induced axon injury in shiverer mice. **(a–c)** Electron micrographs of dorsal column white matter in untreated mice. Axons are unmyelinated or severely hypomyelinated in homozygous shiverer (*shi/shi*) mice (a). In contrast, myelin in heterozygous *+/shi* mice is compact (b) and appears similar to wild type mice (c). **(d, e)** Dorsal column axons after injection with S-AMPA 20 mM in shiverer mice (d) and heterozygous controls (e) displaying axonal edema and disintegration of organelles and cytoskeletal filaments (calibration bar, 1 μ m).

Figure 2. AMPA induced injury of YFP+ axons in shiverer mice and controls. **(a, b)** Longitudinal sections of dorsal columns from uninjected *thy1-YFP2.2* control (a) and *thy1-YFP2.2* shiverer mice (b). Axonal distentions (torpedos) are seen in shiverer mice (inset). **(c, d)** Axonal injury 24 hours after S-AMPA injection (20 mM) characterized by beading and fragmentation in control (c) and shiverer mice (d).

Figure 3.

Dose-dependence of S-AMPA induced axonal injury. (a) YFP+ axon morphology was scored in longitudinal sections 24 hours after AMPA injection in shiverer or control animals at various rostral-caudal distances from the injection site. Injection with vehicle resulted in minimal damage at the injection site. Increasing concentrations of S-AMPA resulted in increasing and widespread axonal damage ($n=5$ or 6 per concentration and group). (b) Axonal damage differed significantly between shiverers and control at S-AMPA concentrations of 10 mM and 20 mM but not 30 mM or vehicle. All S-AMPA concentrations produced significant damage compared to vehicle injections in both groups. Linear repeated measures mixed models were used to describe effects of study group, concentration and distance from injection site on axon counts per unit area. (Data are mean $\pm$ SEM. * $p<0.002$, ** $p<0.0001$)

Figure 4.

Rotarod performance of shiverer and control mice after S-AMPA injection. Performance (time on rotarod) is expressed as percentage of performance prior to injection. S-AMPA injection resulted in both groups in significant drop of performance at all concentrations compared to vehicle. Rotarod performance differed significantly between groups only at a S-AMPA concentration of 20 mM. Values shown are the mean $\pm$ SEM from two independent experiments with a total of 6 or 7 mice per data point. Asterisk indicates $p < 0.01$, two-way ANOVA with Bonferroni post-tests.

Figure 5.

Injection of NMDA does not damage axons. (b, c) Injection of dorsal columns with NMDA (80 mM) did not induce axonal damage in *thy1-YFP2.2* shiverer (b) or control mice (c). Injection sites are labeled with microcruby (red) that was co-injected with NMDA. (a) No behavioral deficits were noted on rotarod testing in either group. Data are mean $\pm$ SEM, n = 5 per group.

Figure 6.

Upregulation of GluR1 in shiverer white matter. (a) Fluorescence micrographs show anti-glutamate receptor 1–4 antibodies (green) and nuclear stain (DAPI, blue) in spinal cord dorsal columns from shiverer and control mice. (a) Labeling for GluR1 but not GluR2/3 or GluR4 was markedly more intense in shiverer. Bright signal was apparent on cell bodies and elongated processes running in parallel with axons. (b) GluR1 (red) is expressed in close proximity to axons labeled by the pan-axonal marker SMI 312 (green, top) and colocalizes with the astrocytic marker GFAP (blue, bottom).