Co-localization of sodium channel \( \text{Na}_v1.6 \) and the sodium–calcium exchanger at sites of axonal injury in the spinal cord in EAE

Matthew J. Craner, Bryan C. Hains, Albert C. Lo, Joel A. Black and Stephen G. Waxman

Department of Neurology and PVA/EPVA Center for Neuroscience Research, Yale University School of Medicine, New Haven, and Rehabilitation Research Center, VA Connecticut Healthcare System, West Haven, Connecticut, USA

Correspondence to: Stephen G. Waxman MD, PhD, Department of Neurology, Yale School of Medicine, 333 Cedar Street, LCI-707, New Haven, CT 06510, USA
E-mail: stephen.waxman@yale.edu

Summary
Axonal degeneration contributes to the development of non-remitting neurological deficits and disability in multiple sclerosis, but the molecular mechanisms that underlie axonal loss in multiple sclerosis are not clearly understood. Studies of white matter axonal injury have demonstrated that voltage-gated sodium channels can provide a route for sodium influx into axons that triggers reverse operation of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) and subsequent influx of damaging levels of intra-axonal calcium. The molecular identities of the involved sodium channels have, however, not been determined. We have previously demonstrated extensive regions of diffuse expression of Na\(_v\)1.6 and Na\(_v\)1.2 sodium channels along demyelinated axons in experimental allergic encephalomyelitis (EAE). Based on the hypothesis that the co-localization of Na\(_v\)1.6 and NCX along extensive regions of demyelinated axons may predispose these axons to injury, we examined the expression of myelin basic protein, Na\(_v\)1.2, Na\(_v\)1.6, NCX and \( \beta \)-amyloid precursor protein (\( \beta \)-APP), a marker of axonal injury, in the spinal cord dorsal columns of mice with EAE. We demonstrate a significant increase in the number of demyelinated axons demonstrating diffuse Na\(_v\)1.6 and Na\(_v\)1.2 sodium channel immunoreactivity in EAE (92.2 \pm 2.1% of \( \beta \)-APP positive axons were Na\(_v\)1.6-positive). Only 38.0 \pm 2.9% of \( \beta \)-APP positive axons were Na\(_v\)1.2 positive, and 95% of these co-expressed Na\(_v\)1.6 together with Na\(_v\)1.2. Using triple-labelled fluorescent immunohistochemistry, we demonstrate that 73.5 \pm 4.3% of \( \beta \)-APP positive axons co-express Na\(_v\)1.6 and NCX, compared with 4.4 \pm 1.0% in \( \beta \)-APP negative axons. Our results indicate that co-expression of Na\(_v\)1.6 and NCX is associated with axonal injury in the spinal cord in EAE.

Keywords: sodium channel; axonal injury; multiple sclerosis; sodium/calcium exchanger; experimental allergic encephalomyelitis

Abbreviations: \( \beta \)-APP = \( \beta \)-amyloid precursor protein; EAE = experimental allergic encephalomyelitis; MBP = myelin basic protein; MOG = myelin oligodendrocyte glycoprotein; NCX = sodium/calcium exchanger; NO = nitric oxide; TTX = tetrodotoxin.

Introduction
Although demyelination and inflammation have classically been considered to be the histopathological hallmarks of multiple sclerosis, axonal pathology in multiple sclerosis has been recognized for over a century (Charcot, 1868) and it has recently been demonstrated that axonal degeneration underlies the development of non-remitting deficits in multiple sclerosis (Davie \( \text{et al} \), 1995; Ganter \( \text{et al} \), 1999; Bjartmar \( \text{et al} \), 2000; Lovas \( \text{et al} \), 2000; Wujek \( \text{et al} \), 2002). The molecular mechanisms that result in axonal degeneration in multiple sclerosis have, however, not been delineated.

Evidence suggesting that voltage-gated sodium channels and the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) might collaborate in triggering degeneration of white matter axons was provided by early studies that demonstrated that sodium channels can participate in the production of calcium-mediated axonal degeneration of axons within the anoxic optic nerve (Stys
Sodium channels, NCX and axonal injury in EAE

et al., 1992a). Sodium channels can provide a route for persistent sodium current that drives reverse operation of NCX, which results in a damaging inflow of calcium ions. Studies by Imaizumi et al. (1998) in the spinal cord dorsal columns provided further evidence for the involvement of sodium channels and NCX in axonal degeneration following exposure to anoxia. More recently, a role of sodium channels and NCX in axonal degeneration in neuroinflammatory disorders has been suggested by the protective effect of low doses of the sodium channel blockers lidocaine and flecainide (Kapoor et al., 2003) and tetrodotoxin (TTX) (Garthwaite et al., 2002) and of the NCX blocker bepridil (Kapoor et al., 2003) on axonal injury triggered by nitric oxide (NO). NO is present at elevated levels in multiple sclerosis lesions (Bo et al., 1994; Brosnan et al., 1994; Smith et al., 1999) and, like anoxia, it can trigger mitochondrial failure with resultant energy depletion and an increase in intra-axonal sodium (Brown et al., 1995; Bolanos et al., 1997; Kapoor et al., 2003). Also consistent with a role of sodium channels in axonal degeneration, treatment with phenytoin (Lo et al., 2002, 2003) and with flecainide (Bechtold et al., 2002) has a neuroprotective effect, preventing axonal degeneration in experimental allergic encephalomyelitis (EAE). However, the molecular identity of the sodium channels involved in the cascade that leads to axonal degeneration in neuroinflammatory disorders has not been determined.

Na1.6 and Na1.2 channels are two of at least nine molecularly distinct subtypes of sodium channel that are expressed in mammals (Goldin et al., 2000). Na1.6 is a TTX-sensitive sodium channel that produces both transient and persistent currents (Raman and Bean, 1997; Tanaka et al., 1999; Herzog et al., 2003). Na1.6 is the major sodium channel at nodes of Ranvier (Caldwell et al., 2000) and, while also found in unmyelinated axons (Black et al., 2002), it is not detectable in the internodal region of myelinated axons. Recently, we have demonstrated the development of continuous Na1.6 or Na1.2 immunoreactivity along extended lengths (tens of microns) of demyelinated axons in the optic nerve in EAE (Craner et al., 2003a). Although the development of a diffuse distribution of Na1.6 throughout long regions of demyelinated axons may facilitate restoration of action potential propagation (Bostock and Sears, 1976, 1978; Foster et al., 1980; Black et al., 2002), ectopic expression of Na1.6 along the axonal membrane might also have a deleterious effect on axons, especially if Na1.6 and NCX are co-localized. We explored this hypothesis by examining the spinal cord of mice with EAE to determine whether there is a correlation between the expression of diffuse Na1.6 sodium channel axonal immunoreactivity with the expression of NCX, and of immunoreactivity to β-amyloid precursor protein (β-APP), a well-established marker of axonal injury (Cochran et al., 1991; Trapp et al., 1998; Bitsch et al., 2000; Kuhlmann et al., 2002).

Here we report, in a model of EAE that exhibits axonal degeneration, a significant increase in the number of axons that display extended regions of sodium channel immuno-reactivity for Na1.6 and Na1.2 in the spinal cord of mice with EAE. We demonstrate that Na1.6 is preferentially expressed in β-APP positive axons. Moreover, we show that Na1.6 is co-localized with NCX in β-APP positive axons, suggesting that co-incident distribution of Na1.6 and NCX along demyelinated axons may contribute to the development of axonal degeneration in EAE.

Methods

Induction of EAE

Animal protocols followed guidelines established by the NIH and were approved by the Yale University Institutional Animal Care and Use Committee. Biozzi mice (Harlan Sera-Lab Limited, Loughborough, UK) aged 6–10 weeks were injected in the flank with 200 µl of an emulsion composed of 300 µg myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (rat origin, synthesized by the W.M. Keck Biotechnology Resource Center, Yale University, CT, USA) in incomplete Freund’s adjuvant (IFA) (Sigma, St Louis, MO, USA) supplemented with 500 µg of Mycobacterium (8:1 ratio of tuberculosis and butyricum) (Difco, Detroit, MI, USA). The MOG injection, with mycobacterium-supplemented IFA, was repeated in the alternate flank one week later. In addition, 500 ng pertussis toxin in 200 µl phosphate-buffered saline (PBS) was administered intraperitoneally (i.p.) to each mouse coincident with the first MOG injection and repeated 48 h later. Age- and sex-matched Biozzi mice served as controls.

All animals induced with MOG developed a relapsing–remitting clinical phenotype, with each animal having at least two relapses prior to sacrifice at 54–141 days post-injection (average length of disease of 93 ± 10 days) (Craner et al., 2003b). Staining of spinal cord sections for all animals demonstrated patchy loss of myelin basic protein (MBP) immunoreactivity, suggesting the presence of demyelination that extended throughout the spinal cord. There was no apparent oedema within EAE spinal cord. Spinal cords of mice with experimental allergic encephalomyelitis (EAE) (n = 12) were compared with spinal cords from aged-matched Biozzi controls (n = 7).

Immunocytochemistry

Mice were anaesthetized with ketamine/xylazine (80/5 mg/kg, i.p.) and perfused with 4% paraformaldehyde in 0.14M PBS. Spinal cords were post-fixed for 30 min in 4% paraformaldehyde in 0.14M PBS, and cryoprotected overnight at 4°C in 30% sucrose in 0.14M PBS. To confirm the presence of axonal degeneration within this model of EAE, lumbar spinal cords were transversely sectioned at 12 µm onto slides and desiccated overnight. To identify all axons, sections were incubated with antibodies against both phosphorylated neurofilaments (SMI-31, 1:20 000; Sternberger Monoclonals Inc., Lutherville, MD, USA) and non-phosphorylated neurofilaments (SMI-32, 1:20 000; Sternberger Monoclonals Inc.) as described previously by Lo et al. (2002). Image acquisition and analysis for counting of profiles was performed as previously described (Lo et al., 2003).

To localize Na1.6, Na1.2 and NCX after flat embedding in rectangular moulds in OCT medium, lumbar spinal cords were longitudinally sectioned at 8 µm onto serial slides and desiccated overnight prior to processing. Tissue sections were processed for immunocytochemistry as described previously (Black et al., 1999).
Fig. 1 Sodium channel immunostaining along extensive domains of demyelinated axons in EAE. Representative images of spinal cord stained for myelin basic protein (MBP, green) and Na_{1.6} or Na_{1.2} (red) in controls (A, B) and EAE (C, D). In the controls (A, B), well-defined MBP immunostaining extends along axons (yellow arrow heads), while sodium channel immunostaining is confined to nodes of Ranvier and does not extend for more than a few microns (indicative of myelinated fibres). The inset in (A) shows focal Na_{1.6} immunostaining at a node of Ranvier. In EAE spinal cord (C, D), there is a significant increase in the number of axonal profiles demonstrating continuous Na_{1.6} or Na_{1.2} immunostaining extending for tens of microns along the fibre axis. In axons displaying extensive continuous sodium channel immunostaining, MBP immunostaining tended to be absent (yellow arrows, C, D) or markedly attenuated (white arrow, D), indicating ongoing demyelination or remyelination. (×1000).

Briefly, sections were incubated simultaneously with monoclonal antibodies to MBP (1:4000; Sternberger Monoclonals Inc.), β-APP (1:100; Chemicon, Temecula, CA, USA) or Na_{1.2}/Ca_{2+} exchanger [NCX1 isoform, shown to be expressed in white matter axons (Steffensen et al., 1997)] (1:200; RDI, Flanders NJ, USA) and polyclonal antibodies to Na_{1.6} (residues 1042–1061; 1:100; Alomone) or Na_{1.2} (residues 467–485; 1:100; Alomone). Sections were then washed in PBS and incubated with appropriate secondary antibodies comprising goat anti-rabbit IgG-Cy3 (1:2000; Amersham, Piscataway, NJ, USA), goat anti-mouse IgG-Alexa Fluor 488 (1:1000; Molecular Probes, Eugene, OR, USA), goat anti-mouse IgM-Alexa Fluor 488 (1:1000; Molecular Probes) and goat anti-mouse IgG-Cy5 (1:200, Rockland, Gilbertsville, PA, USA) in blocking solution for 3 h, washed in PBS and mounted. Control experiments that included the omission of primary or secondary antibodies showed no staining (data not shown).

To examine the co-localization of Na_{1.6} and Na_{1.2} with β-APP in spinal cord axons, sections were incubated sequentially in: anti-Na_{1.6}, goat anti-rabbit IgG-Cy3, anti-Na_{1.2} and β-APP followed by goat anti-rabbit IgG-Cy2 and goat anti-mouse IgG-Cy5. Control experiments in which the second polyclonal antibody incubation was omitted exhibited no Cy2 fluorescence.

**Tissue analysis**

Multiple representative images from lumbar (L4-5) spinal cord, from which random samples were selected for analysis, were accrued by confocal microscopy with a NIKON Eclipse E600 microscope. Analysis was confined to images in which axons were sectioned longitudinally as evident from the presence of linear profiles of MBP immunostaining, or of linear (presumably demyelinated) axonal profiles with diffuse sodium channel immunoreactivity, running for >20–30 μm within the plane of single sections. The dorsal funiculus was examined preferentially in this study, although qualitatively there were no detectable differences in the distribution of linear axonal profiles with diffuse sodium channel immunoreactivity within both lateral and ventral funiculi.

Within the spinal cord sections, we identified extended regions of diffuse Na_{1.2} and/or Na_{1.6} sodium channel immunoreactivity along demyelinated (demonstrated by the loss MBP immunostaining) linear axonal profiles (Fig. 1). As an index of the frequency of these profiles, we counted the number of axonal profiles that displayed diffuse regions of immunostaining to either Na_{1.2} or Na_{1.6}, extending >8 μm in length (therefore excluding nodal clusters of immunostaining) as described previously (Craner et al., 2003a). To facilitate quantification, a target line (six 100 μm increments for total length of 600 μm) perpendicular to the axis of the nerve fibres was overlaid on randomly selected images and axonal profiles (>8 μm length) with sodium channel immunostaining that intersected the target line were counted. The data presented represent the mean number of axonal profiles with diffuse Na_{1.2} or Na_{1.6} immunostaining ± SEM per 600 μm of target in control and EAE spinal cord. Due to substantial changes in EAE (see Results), it was not possible to blind the observer as to whether a section was from EAE or control spinal cord. To validate the scoring of axonal profiles with diffuse sodium channel immunopositivity, two observers independently evaluated five mice each and differed by <9% with respect to the number of profiles in control compared with EAE.

To determine the relationship between diffuse axonal Na_{1.6} and Na_{1.2} sodium channel immunoreactivity and axonal injury, a subset of EAE (n = 6) and control mice (n = 4) were examined using antibodies to the Na_{1.2}/Ca_{2+} exchanger and β-APP. To facilitate the identification of immunopositive profiles, quantitative microdensitometry of immunostaining signal was performed using IPLab Scientific Image Processing software (Scanalytics Inc., Fairfax, VA, USA) (Craner et al., 2003a). Signal intensities were obtained by manually outlining profiles (10–15 μm in length) and using the IPLab integrated densitometry function to calculate mean signal intensities for the outlined areas. A profile was identified as a detectable linear outline extending for >8 μm within the plane of section, and as immunopositive if it displayed at an optical intensity at least twice that of background levels. Statistical analysis was performed using Student’s t-test and χ² test. Data are presented as mean ± SEM.

**Results**

**Most dorsal column axons are myelinated and express Na_{1.6} sodium channels at nodes**

Na_{1.6} immunostaining was evident [consistent with previous reports (Caldwell et al., 2000; Arroyo et al., 2002)] at almost all nodes of Ranvier within the dorsal columns of control mice and well-defined MBP immunostaining, which bounded the nodes, was present over extensive regions (Fig. 1A and B). Na_{1.2} was only very rarely (<5%) observed at nodes of Ranvier in control tissue. Control spinal cords showed only infrequent diffuse non-nodal (>8 μm length) Na_{1.6} and Na_{1.2} immunolabelling of axons; this was consistent with previous studies indicating that the majority of fibres in the dorsal funiculus are myelinated (Chung et al., 1987). In control tissue, there were only 9.4 ± 2.4 axon
profiles per 600 μm target with diffuse Na1.2 immunostaining and 5.1 ± 1.6 axon profiles per 600 μm target with diffuse Na1.6 immunostaining (Fig. 2), which we interpret as sodium channel immunolabelling along unmyelinated fibres.

Demyelinated axons display diffuse expression of Na1.6 and Na1.2 sodium channels in EAE
In contrast to control spinal cords, there was a significant increase in the number of axon profiles in the spinal cords of mice with EAE which demonstrated diffuse Na1.6 or Na1.2 immunostaining extending for >8 μm along the fibre axis, consistent with previous findings in optic nerve, another white matter tract (Craner et al., 2003a). The number of axons with extensive (>8 μm) diffuse Na1.6 immunostaining (26.9 ± 6.9 per 600 μm target, P < 0.001 compared with controls) was increased five-fold in EAE, while the number of axons displaying extensive diffuse regions of Na1.2 immunostaining (35.5 ± 4.7, P < 0.001 compared with controls) was increased more than threefold (Fig. 2). In double labelling experiments with antibodies to MBP (a marker of myelination) and to sodium channels Na1.6 or Na1.2, we confirmed that, in the majority of axon profiles with diffuse sodium channel immunoreactivity, MBP immunostaining was absent (Fig. 1C and D, yellow arrows) or attenuated (Fig. 1D, white arrow), supporting the suggestion that the majority of the axonal profiles with extended sodium channel immunostaining represent demyelinated axons.

Na1.6 is associated with axonal injury
We assessed the extent of axonal degeneration in MOG-induced EAE by counting lumbar dorsal column spinal cord axons using a combination of antibodies directed towards both phosphorylated and non-phosphorylated neurofilaments (Lo et al., 2003). This analysis demonstrated a significant loss of axons within the dorsal columns of EAE, with a decrease in the density of axons labelled with neurofilament antibodies from 54.5 ± 3.4 per 500 μm² (n = 3) in controls to 24.1 ± 3.8 per 500 μm² (n = 4; P < 0.005) in EAE, representing a 56% dropout of axons (Fig. 3).

β-APP immunostaining, which provides a marker of axonal injury (Trapp et al., 1998; Bitsch et al., 2000; Kuhlmann et al., 2002) demonstrated robust axonal injury in EAE. There were only 2.6 ± 0.8 β-APP positive axon profiles per 600 μm of target in control tissue, but there were 38.5 ± 4.1 β-APP positive axon profiles per 600 μm of target in EAE.

To test the hypothesis that Na1.6, compared with Na1.2, is more closely associated with axonal injury, we next assessed whether β-APP positive axons tend to express extensive (>8 μm) Na1.6 or Na1.2 immunostaining. We observed that 94.0 ± 1.8% of β-APP positive axons express either one or both of the isoforms of sodium channel examined and that a significantly larger proportion of β-APP positive axons are Na1.6 immunopositive (56.0 ± 3.1%) over extensive (>8 μm regions) or Na1.6 and Na1.2 immunopositive (i.e. co-express both Na1.6 and Na1.2) (36.2 ± 2.7%), compared with only a very small percentage of β-APP positive axons that are Na1.2 immunopositive (1.8 ± 0.4%, n = 222; P < 0.001) (Fig. 4A and B). These findings suggest that Na1.6, and not Na1.2, is preferentially associated with axonal injury in EAE. We therefore extended our analysis by examining the co-localization of Na1.6 and the Na+/Ca2+ exchanger (NCX) in β-APP positive axons.

NCX is co-localized with Na1.6 within β-APP positive axons in EAE
Because physiological studies indicate that NCX, triggered by a persistent sodium current, can operate in a reverse mode to import a deleterious accumulation of calcium that results in...
degeneration of myelinated axons within white matter (Stys et al., 1992a), we asked whether NCX is present along extensive regions of spinal cord axons in EAE and, if so, whether NCX is co-localized with Nav1.6 and is associated with degenerating axons. As shown in Fig. 5, the percentage of axons displaying extensive (>8 μm) NCX-positive immunostaining, as a function of all axonal profiles, is increased in EAE (41.6 ± 7.4%, n = 1155; P < 0.01) compared with control (6.5 ± 0.1%; n = 126) mice spinal cords. We utilized triple-labelled fluorescent immunohistochemistry to co-localize β-APP with Na,1.6/NCX-positive immunostaining (i.e. profiles of axons co-expressing Na,1.6 and NCX). A representative field is illustrated in Fig. 6, which shows co-localization of Na,1.6 (Fig. 6B) and NCX (Fig. 6C) along extensive regions of β-APP positive axons. The percentage of β-APP positive axons that displayed extensive regions of Na,1.6/NCX immunolabelling (i.e. that displayed both Na,1.6 and NCX) (73.5 ± 4.3%; n = 287) was significantly greater than the percentage of β-APP negative axons that displayed Na,1.6/NCX immunolabelling (4.4% ± 1.0%; n = 318; P < 0.001) (Fig. 7). These data demonstrate that the majority of β-APP positive axons in EAE display extensive regions where both Na,1.6 and NCX are present.

Discussion

In this study, we used subtype-specific sodium channel antibodies to examine the distribution of Na,1.6 and Na,1.2.
Sodium channels, NCX and axonal injury in EAE

in the dorsal columns of the spinal cord. We demonstrate an increase in the number of axons with extensive regions of diffuse Na\textsubscript{v}1.6 and Na\textsubscript{v}1.2 sodium channel immunostaining and lack of MBP immunostaining, which is consistent with demyelination, in chronic-relapsing EAE. Na\textsubscript{v}1.6 was present over extensive regions of >92% of β-APP positive axons. Moreover, we identified a strong association between the expression of both NCX and Na\textsubscript{v}1.6 over extensive regions, and expression of β-APP, a marker of axonal injury, in demyelinated axons within EAE dorsal columns.

The presence of myelin or myelin-forming cells and their axo-glial junctions can influence the distribution of sodium channels within the axon membrane (Rosenbluth, 1988; Kaplan et al., 1997; Rios et al., 2003). Similar to previous descriptions in models of genetic dysmyelination (Boiko et al., 2001), experimental allergic neuritis (Novakovic et al., 1998) and doxorubicin-induced demyelination (England et al., 1990), we observed diffuse sodium channel immunostaining that extended in a relatively non-focal manner along axon profiles with absent or markedly attenuated MBP immunostaining, indicating the presence of demyelination. Our use of subtype-specific antibodies permitted us to identify Na\textsubscript{v}1.6 and Na\textsubscript{v}1.2 expression that extended diffusely for at least 8 μm along demyelinated axons. Na\textsubscript{v}1.2 (Westenbroek et al., 1989; Gong et al., 1999; Whitaker et al., 2000) and Na\textsubscript{v}1.6 (Black et al., 2002) channels are known to be present along non-myelinated axons within the CNS, and Na\textsubscript{v}1.6 has been shown to contribute to action potential conduction along non-myelinated axons (Black et al., 2002). However, the specific contributions of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.2 to conduction along demyelinated axons have not been studied. The relatively non-focal distribution of Na\textsubscript{v}1.6 and/or Na\textsubscript{v}1.2 channels may provide a substrate for continuous (Bostock and Sears, 1976, 1978) or discontinuous (Smith et al., 1982) impulse conduction, both of which have been observed in demyelinated axons.

Although the non-nodal axonal distribution of sodium channels along demyelinated axons may subserve restoration of impulse conduction, it is also possible that these diffusely distributed channels may have a deleterious effect. Intraxonal accumulation of Ca\textsuperscript{2+} has been implicated as an activator of a set of deleterious events, including protease activation and mitochondrial failure in CNS injury (Banik et al., 1987; Young, 1992; Buki et al., 2000). Voltage-gated sodium channels have been established to play an important role in CNS white matter injury within the optic nerve and dorsal columns in anoxic and crush injury models (Stys et al., 1991, 1992a; Agrawal and Fehlings, 1996; Imaizumi et al., 1998). Moreover, the administration of TTX or the removal of Na\textsuperscript{+} from the perfusate provides nearly complete protection in these models of axonal injury, demonstrating a link between Na\textsuperscript{+} influx and axonal degeneration. It has been proposed that sodium influx into injured axons attenuates the normal steep sodium gradient across the axon membrane (on which the NCX is dependent for forward functioning, with resultant removal of Ca\textsuperscript{2+} from the cytoplasm), so that with sustained activation of sodium channels, there is reverse operation of NCX and the accumulation of intra-axonal calcium (Stys et al., 1991, 1992a; Stys and Lopachin, 1998). The molecular identity of the sodium channel that drives the reverse mode of NCX in injured axons has not been determined. Stys et al. (1992a, 1993) showed that a non-inactivating sodium conductance plays a prominent role in this process. Baker and Bostock (1997) demonstrated a low-threshold persistent sodium current within large diameter dorsal root ganglion neurons, which give rise to myelinated axons. Na\textsubscript{v}1.6 is expressed at high levels within this class of neurons (Black et al., 1996). Supporting a role for Na\textsubscript{v}1.6 in triggering Ca\textsuperscript{2+}-mediated injury of axons, Na\textsubscript{v}1.6 channels can produce a persistent current (Raman and Bean, 1997; Tanaka et al., 1999; Herzog et al., 2003), which becomes larger with depolarization and which is not observed for Na\textsubscript{v}1.1 or Na\textsubscript{v}1.2 channels (Smith et al., 1998). There is also evidence that Na\textsubscript{v}1.2 can produce a persistent current when co-expressed with βγ subunits of G protein (Ma et al., 1997). However, it appears that, in many cell types, Na\textsubscript{v}1.6 is responsible for the majority of persistent current (Maurice et al., 2001).

Nitric oxide is present at increased concentrations within multiple sclerosis lesions (Bo et al., 1994; Brosnan et al., 1994) and can produce reversible conduction block along CNS (Redford et al., 1997) and PNS (Shrager et al., 1998) axons. Demyelinated axons are particularly sensitive to NO (Redford et al., 1997). At higher concentrations (Kapoor et al., 1999) or in axons that have been physiologically active (Smith et al., 2001), NO can provoke persistent conduction block and axonal degeneration. Kapoor et al. (2003) showed that sodium channel blockers can protect axons from NO-induced degeneration. They speculate that NO injures axons by inhibiting mitochondrial respiration, thus producing an increase in the intra-axonal Na\textsuperscript{+} due to energy failure. Their experiments extend studies in white matter axons subjected to energy depletion by hypoxia; these demonstrated that Na\textsuperscript{+} influx via sodium channels drives NCX to import calcium.

![Fig. 5](http://brain.oxfordjournals.org/) Increased percentage of axons expressing extensive NCX-positive immunostaining in EAE. The histogram illustrates a significant increase in the percentage of axonal profiles that are NCX-positive over extensive (>8 mm) regions in EAE, compared with control.
into the axoplasm (Stys et al., 1992a) and that white matter axons can be protected from anoxic injury by tertiary and quaternary anaesthetics blocking sodium channels (Stys et al., 1992b) and by the sodium channel blockers phenytoin, carbamazepine (Fern et al., 1993) and mexilitine (Stys and Lesiuk, 1996). Kapoor et al. (2003) showed that partial blockade of sodium channels with flecaainide and lidocaine has a protective effect on axons exposed to NO. In addition, TTX protects optic nerve axons from NO-induced injury (Garthwaite et al., 2002).

We found that 52% of β-APP positive axons expressed extensive regions of Na,1.6 alone, and 36% of β-APP positive axons expressed extensive regions of Na,1.6 together with Na,1.2. In contrast, <2% of β-APP positive axons expressed Na,1.2 alone. These findings, in conjunction with a 56% reduction in dorsal column axonal density and the co-localization of extensive regions displaying NCX and Na,1.6 in β-APP positive axons compared with β-APP negative axons, suggest a relationship between NCX and Na,1.6 expression and axonal degeneration in spinal cord axons in EAE. A role for NCX in the degeneration of dorsal column axons is supported by studies demonstrating that dorsal column axons can be partially protected from anoxic injury not only by sodium channel blockers, but also by benzamil and bepridil, which are blockers of NCX (Imaizumi et al., 1998), and from NO-induced injury by treatment with bepridil (Kapoor et al., 2003). Recently, Lo et al. (2002, 2003) and Bechtold et al. (2002) demonstrated axonal preservation following administration of sodium channel blockers that are used in the clinical setting (phenytoin,

Fig. 6 β-APP positive spinal cord axons co-express NCX and Na,1.6 over extensive regions. The digital images demonstrate axons in EAE spinal cord immunostained for β-APP (blue; A, E), sodium channel Nav1.6 (red; B) or Na,1.2 (red; F) and NCX (green; C, G). (D) and (H) correspond to merged images (white). A–D show co-expression of Na,1.6, NCX and β-APP, a marker of axonal injury. In contrast, E–H demonstrate β-APP/NCX positive profiles but absence of Na,1.2 immunostaining (×1800).
Sodium channels, NCX and axonal injury in EAE

Acknowledgements

We wish to thank Lynda Tyrell, Bart Toftness and Pam Zwinger for excellent technical assistance. This work was supported in part by grants from the National Multiple Sclerosis Society (RG-1912) and the Rehabilitation Research Service and Medical Research Service, Department of Veterans Affairs. The authors also wish to thank the Paralyzed Veterans of America, the Eastern Paralyzed Veterans Association and the Nancy Davis Foundation for support. M.J.C. thanks the Medical Director General, UK for support.

References


Brown GC, Bolanos JP, Heales SJ, Clark JB. Nitric oxide produced by...


Whitaker WR, Clare JJ, Powell AJ, Chen YH, Faull RL, Emson PC.

