

# Co-localization of sodium channel Na<sub>v</sub>1.6 and the sodium–calcium exchanger at sites of axonal injury in the spinal cord in EAE

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## 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<sup>+</sup>/Ca<sup>2+</sup> 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<sub>v</sub>1.6 and Na<sub>v</sub>1.2 sodium channels along demyelinated axons in experimental allergic encephalomyelitis (EAE). Based on the hypothesis that the co-localization of Na<sub>v</sub>1.6 and NCX along extensive regions of demyelinated axons may pre-

dispose these axons to injury, we examined the expression of myelin basic protein, Na<sub>v</sub>1.2, Na<sub>v</sub>1.6, NCX and β-amyloid precursor protein (β-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<sub>v</sub>1.6 and Na<sub>v</sub>1.2 sodium channel immunoreactivity in EAE (92.2 ± 2.1% of β-APP positive axons were Na<sub>v</sub>1.6-positive). Only 38.0 ± 2.9% of β-APP positive axons were Na<sub>v</sub>1.2 positive, and 95% of these co-expressed Na<sub>v</sub>1.6 together with Na<sub>v</sub>1.2. Using triple-labelled fluorescent immunohistochemistry, we demonstrate that 73.5 ± 4.3% of β-APP positive axons co-express Na<sub>v</sub>1.6 and NCX, compared with 4.4 ± 1.0% in β-APP negative axons. Our results indicate that co-expression of Na<sub>v</sub>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:** β-APP = β-amyloid precursor protein; EAE = experimental allergic encephalomyelitis; MBP = myelin basic protein; MOG = myelin oligodendrocyte glycoprotein; NCX = sodium/calcium exchanger; NO = nitric oxide; TTX = tetrodotoxin.

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## 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 *et al.*, 1995; Ganter *et al.*, 1999; Bjartmar *et al.*, 2000; Lovas *et al.*, 2000; Wujek *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<sup>+</sup>/Ca<sup>2+</sup> 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

*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.

Na<sub>v</sub>1.6 and Na<sub>v</sub>1.2 channels are two of at least nine molecularly distinct subtypes of sodium channel that are expressed in mammals (Goldin *et al.*, 2000). Na<sub>v</sub>1.6 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). Na<sub>v</sub>1.6 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 Na<sub>v</sub>1.6 or Na<sub>v</sub>1.2 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 Na<sub>v</sub>1.6 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 Na<sub>v</sub>1.6 along the axonal membrane might also have a deleterious effect on axons, especially if Na<sub>v</sub>1.6 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 Na<sub>v</sub>1.6 sodium channel axonal immunoreactivity with the expression of NCX, and of immunoreactivity to  $\beta$ -amyloid precursor protein ( $\beta$ -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 Na<sub>v</sub>1.6 and Na<sub>v</sub>1.2 in the spinal cord of mice with EAE. We demonstrate that Na<sub>v</sub>1.6 is preferentially expressed in  $\beta$ -APP positive axons. Moreover, we show that Na<sub>v</sub>1.6 is co-localized with NCX in  $\beta$ -APP positive axons, suggesting that co-incident distribution of Na<sub>v</sub>1.6 and NCX along demyelinated axons may contribute to the development of axonal degeneration in EAE.

## Methods

### Induction of EAE

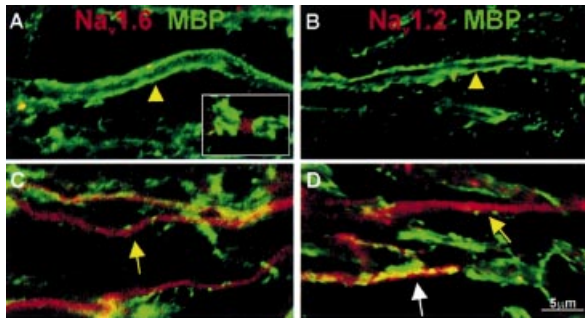
Animal protocols followed guidelines established by the NIH and were approved by the Yale University Institutional Animal Care and Use Committee. Biozzi mice (Harlan Sera-Lab Limited, Loughborough, UK) aged 6–10 weeks were injected in the flank with 200  $\mu$ l of an emulsion composed of 300  $\mu$ g 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  $\mu$ g 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  $\mu$ l 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  $93 \pm 10$  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  $\mu$ m 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 Na<sub>v</sub>1.6, Na<sub>v</sub>1.2 and NCX after flat embedding in rectangular moulds in OCT medium, lumbar spinal cords were longitudinally sectioned at 8  $\mu$ m 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  $\text{Na}_v1.6$  or  $\text{Na}_v1.2$  (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  $\text{Na}_v1.6$  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  $\text{Na}_v1.6$  or  $\text{Na}_v1.2$  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. ( $\times 1000$ ).

Briefly, sections were incubated simultaneously with monoclonal antibodies to MBP (1:4000; Sternberger Monoclonals Inc.),  $\beta$ -APP (1:100; Chemicon, Temecula, CA, USA) or  $\text{Na}^+/\text{Ca}^{2+}$  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  $\text{Na}_v1.6$  (residues 1042–1061; 1:100; Alomone) or  $\text{Na}_v1.2$  (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  $\text{Na}_v1.6$  and  $\text{Na}_v1.2$  with  $\beta$ -APP in spinal cord axons, sections were incubated sequentially in: anti- $\text{Na}_v1.6$ , goat anti-rabbit IgG-Cy3, anti- $\text{Na}_v1.2$  and  $\beta$ -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  $>20$ – $30 \mu\text{m}$  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  $\text{Na}_v1.2$  and/or  $\text{Na}_v1.6$  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  $\text{Na}_v1.2$  or  $\text{Na}_v1.6$ , extending  $>8 \mu\text{m}$  in length (therefore excluding nodal clusters of immunostaining) as described previously (Craner *et al.*, 2003a). To facilitate quantification, a target line (six 100  $\mu\text{m}$  increments for total length of 600  $\mu\text{m}$ ) perpendicular to the axis of the nerve fibres was overlaid on randomly selected images and axonal profiles ( $>8 \mu\text{m}$  length) with sodium channel immunostaining that intersected the target line were counted. The data presented represent the mean number of axonal profiles with diffuse  $\text{Na}_v1.2$  or  $\text{Na}_v1.6$  immunostaining  $\pm$  SEM per 600  $\mu\text{m}$  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.

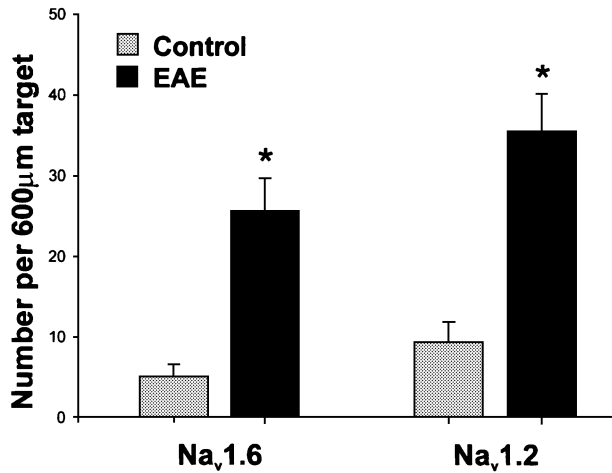
To determine the relationship between diffuse axonal  $\text{Na}_v1.6$  and  $\text{Na}_v1.2$  sodium channel immunoreactivity and axonal injury, a subset of EAE ( $n = 6$ ) and control mice ( $n = 4$ ) were examined using antibodies to the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and  $\beta$ -APP. 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  $\mu\text{m}$  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  $>8 \mu\text{m}$  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  $\chi^2$  test. Data are presented as mean  $\pm$  SEM.

## Results

### Most dorsal column axons are myelinated and express $\text{Na}_v1.6$ sodium channels at nodes

$\text{Na}_v1.6$  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).  $\text{Na}_v1.2$  was only very rarely ( $<5\%$ ) observed at nodes of Ranvier in control tissue. Control spinal cords showed only infrequent diffuse non-nodal ( $>8 \mu\text{m}$  length)  $\text{Na}_v1.6$  and  $\text{Na}_v1.2$  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  $9.4 \pm 2.4$  axon





**Fig. 2** Increased number of axons with extensive Na<sub>v</sub>1.2 and Na<sub>v</sub>1.6 immunostaining in EAE spinal cord. The histogram demonstrates a significant increase in number of axons displaying diffuse sodium channel immunostaining extending >8 mm along the fibre axis in EAE. \* =  $P < 0.001$  compared with controls.

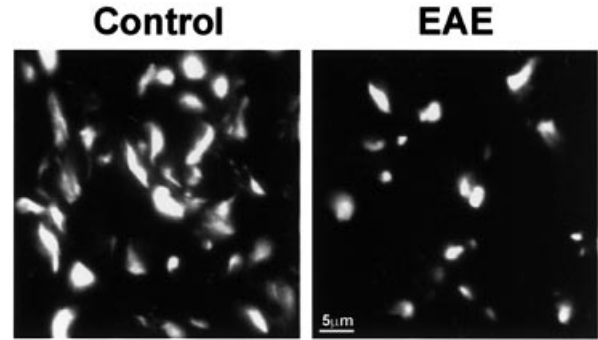
profiles per 600  $\mu\text{m}$  target with diffuse Na<sub>v</sub>1.2 immunostaining and  $5.1 \pm 1.6$  axon profiles per 600  $\mu\text{m}$  target with diffuse Na<sub>v</sub>1.6 immunostaining (Fig. 2), which we interpret as sodium channel immunolabelling along unmyelinated fibres.

### ***Demyelinated axons display diffuse expression of Na<sub>v</sub>1.6 and Na<sub>v</sub>1.2 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 Na<sub>v</sub>1.6 or Na<sub>v</sub>1.2 immunostaining extending for >8  $\mu\text{m}$  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  $\mu\text{m}$ ) diffuse Na<sub>v</sub>1.6 immunostaining ( $26.9 \pm 6.9$  per 600  $\mu\text{m}$  target,  $P < 0.001$  compared with controls) was increased five-fold in EAE, while the number of axons displaying extensive diffuse regions of Na<sub>v</sub>1.2 immunostaining ( $35.5 \pm 4.7$ ,  $P < 0.001$  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 Na<sub>v</sub>1.6 or Na<sub>v</sub>1.2, 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.

### ***Na<sub>v</sub>1.6 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



**Fig. 3** Axonal degeneration in the spinal cord in MOG-induced EAE. Neurofilament immunostaining of dorsal column axons (spinal cord cut in cross-section) demonstrating a significant reduction in the density of axons in EAE (right panel) compared with control (left panel).

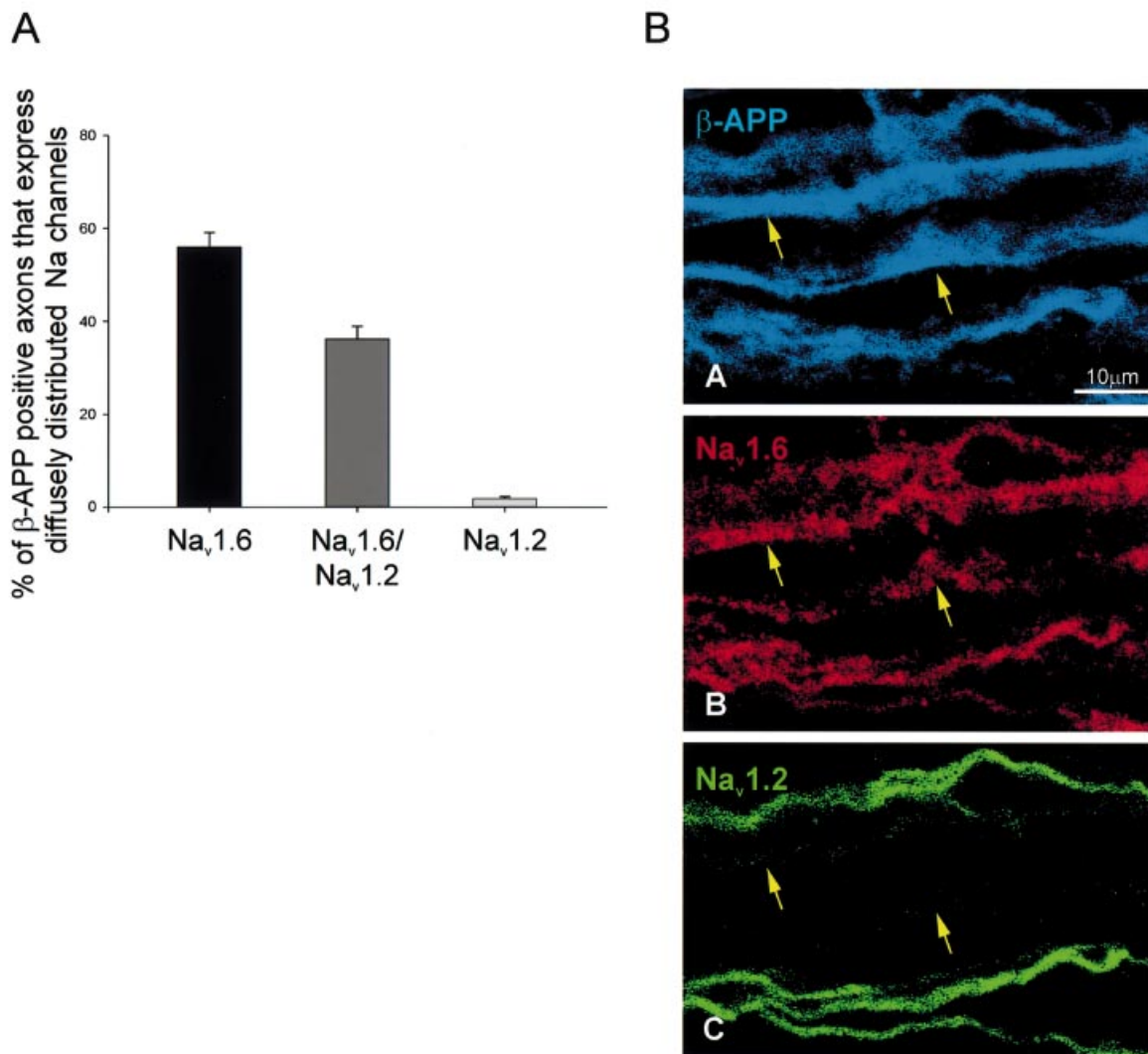
both phosphorylated and non-phosphorylated neurofilaments (Lo *et al.*, 2003). This analysis demonstrated a significant loss of axons within the dorsal columns of EAE, with a decrease in the density of axons labelled with neurofilament antibodies from  $54.5 \pm 3.4$  per 500  $\mu\text{m}^2$  ( $n = 3$ ) in controls to  $24.1 \pm 3.8$  per 500  $\mu\text{m}^2$  ( $n = 4$ ;  $P < 0.005$ ) in EAE, representing a 56% dropout of axons (Fig. 3).

$\beta$ -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  $2.6 \pm 0.8$   $\beta$ -APP positive axon profiles per 600  $\mu\text{m}$  of target in control tissue, but there were  $38.5 \pm 4.1$   $\beta$ -APP positive axon profiles per 600  $\mu\text{m}$  of target in EAE.

To test the hypothesis that Na<sub>v</sub>1.6, compared with Na<sub>v</sub>1.2, is more closely associated with axonal injury, we next assessed whether  $\beta$ -APP positive axons tend to express extensive (>8  $\mu\text{m}$ ) Na<sub>v</sub>1.6 or Na<sub>v</sub>1.2 immunostaining. We observed that  $94.0 \pm 1.8\%$  of  $\beta$ -APP positive axons express either one or both of the isoforms of sodium channel examined and that a significantly larger proportion of  $\beta$ -APP positive axons are Na<sub>v</sub>1.6 immunopositive ( $56.0 \pm 3.1\%$ ) over extensive (>8  $\mu\text{m}$  regions) or Na<sub>v</sub>1.6 and Na<sub>v</sub>1.2 immunopositive (i.e. co-express both Na<sub>v</sub>1.6 and Na<sub>v</sub>1.2) ( $36.2 \pm 2.7\%$ ), compared with only a very small percentage of  $\beta$ -APP positive axons that are Na<sub>v</sub>1.2 immunopositive ( $1.8 \pm 0.4\%$ ,  $n = 222$ ;  $P < 0.001$ ) (Fig. 4A and B). These findings suggest that Na<sub>v</sub>1.6, and not Na<sub>v</sub>1.2, is preferentially associated with axonal injury in EAE. We therefore extended our analysis by examining the co-localization of Na<sub>v</sub>1.6 and the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX) in  $\beta$ -APP positive axons.

### ***NCX is co-localized with Na<sub>v</sub>1.6 within $\beta$ -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



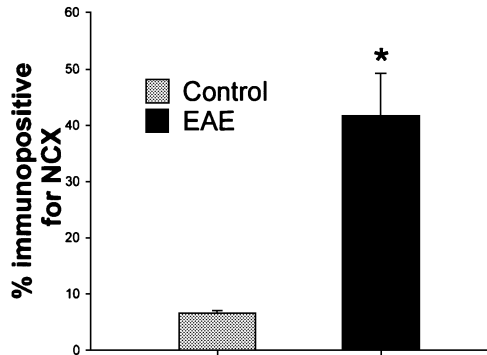
**Fig. 4**  $\text{Na}_v1.6$  is expressed along extensive regions of most  $\beta$ -APP immunopositive axons. **(A)** Histogram demonstrating the percentage of  $\beta$ -APP positive axons that are  $\text{Na}_v1.6$  positive over extensive ( $>8$  mm) regions,  $\text{Na}_v1.6/\text{Na}_v1.2$ -positive (i.e. that co-express both  $\text{Na}_v1.2$  and  $\text{Na}_v1.6$ ), or  $\text{Na}_v1.2$ -positive in EAE (grey bar). **(B)** Digital images of a representative field demonstrating axonal profiles in EAE spinal cord immunostained for  $\beta$ -APP (blue; top panel),  $\text{Na}_v1.6$  (red; middle panel) and  $\text{Na}_v1.2$  (green; bottom panel). Note that, among  $\beta$ -APP positive axons (blue, top panel), a greater proportion are  $\text{Na}_v1.6$  positive (red, middle panel) than are  $\text{Na}_v1.2$  positive (green, bottom panel). Arrows point to  $\beta$ -APP positive and  $\text{Na}_v1.6$  positive axons that did not display  $\text{Na}_v1.2$  immunostaining ( $\times 1000$ ).

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  $\text{Na}_v1.6$  and is associated with degenerating axons. As shown in Fig. 5, the percentage of axons displaying extensive ( $>8 \mu\text{m}$ ) NCX-positive immunostaining, as a function of all axonal profiles, is increased in EAE ( $41.6 \pm 7.4\%$ ,  $n = 1155$ ;  $P < 0.01$ ) compared with control ( $6.5 \pm 0.1\%$ ;  $n = 126$ ) mice spinal cords. We utilized triple-labelled fluorescent immunohistochemistry to co-localize  $\beta$ -APP with  $\text{Na}_v1.6/\text{NCX}$ -positive immunostaining (i.e. profiles of axons co-expressing  $\text{Na}_v1.6$  and NCX). A representative field is illustrated in Fig. 6, which shows co-localization of  $\text{Na}_v1.6$  (Fig. 6B) and NCX (Fig. 6C) along extensive regions of  $\beta$ -APP positive axons. The

percentage of  $\beta$ -APP positive axons that displayed extensive regions of  $\text{Na}_v1.6/\text{NCX}$  immunolabelling (i.e. that displayed both  $\text{Na}_v1.6$  and NCX) ( $73.5 \pm 4.3\%$ ;  $n = 287$ ) was significantly greater than the percentage of  $\beta$ -APP negative axons that displayed  $\text{Na}_v1.6/\text{NCX}$  immunolabelling ( $4.4\% \pm 1.0\%$ ,  $n = 318$ ;  $P < 0.001$ ) (Fig. 7). These data demonstrate that the majority of  $\beta$ -APP positive axons in EAE display extensive regions where both  $\text{Na}_v1.6$  and NCX are present.

## Discussion

In this study, we used subtype-specific sodium channel antibodies to examine the distribution of  $\text{Na}_v1.6$  and  $\text{Na}_v1.2$



**Fig. 5** 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.

in the dorsal columns of the spinal cord. We demonstrate an increase in the number of axons with extensive regions of diffuse  $\text{Na}_v1.6$  and  $\text{Na}_v1.2$  sodium channel immunostaining and lack of MBP immunostaining, which is consistent with demyelination, in chronic-relapsing EAE.  $\text{Na}_v1.6$  was present over extensive regions of >92% of  $\beta$ -APP positive axons. Moreover, we identified a strong association between the expression of both NCX and  $\text{Na}_v1.6$  over extensive regions, and expression of  $\beta$ -APP, a marker of axonal injury, in demyelinated axons within EAE dorsal columns.

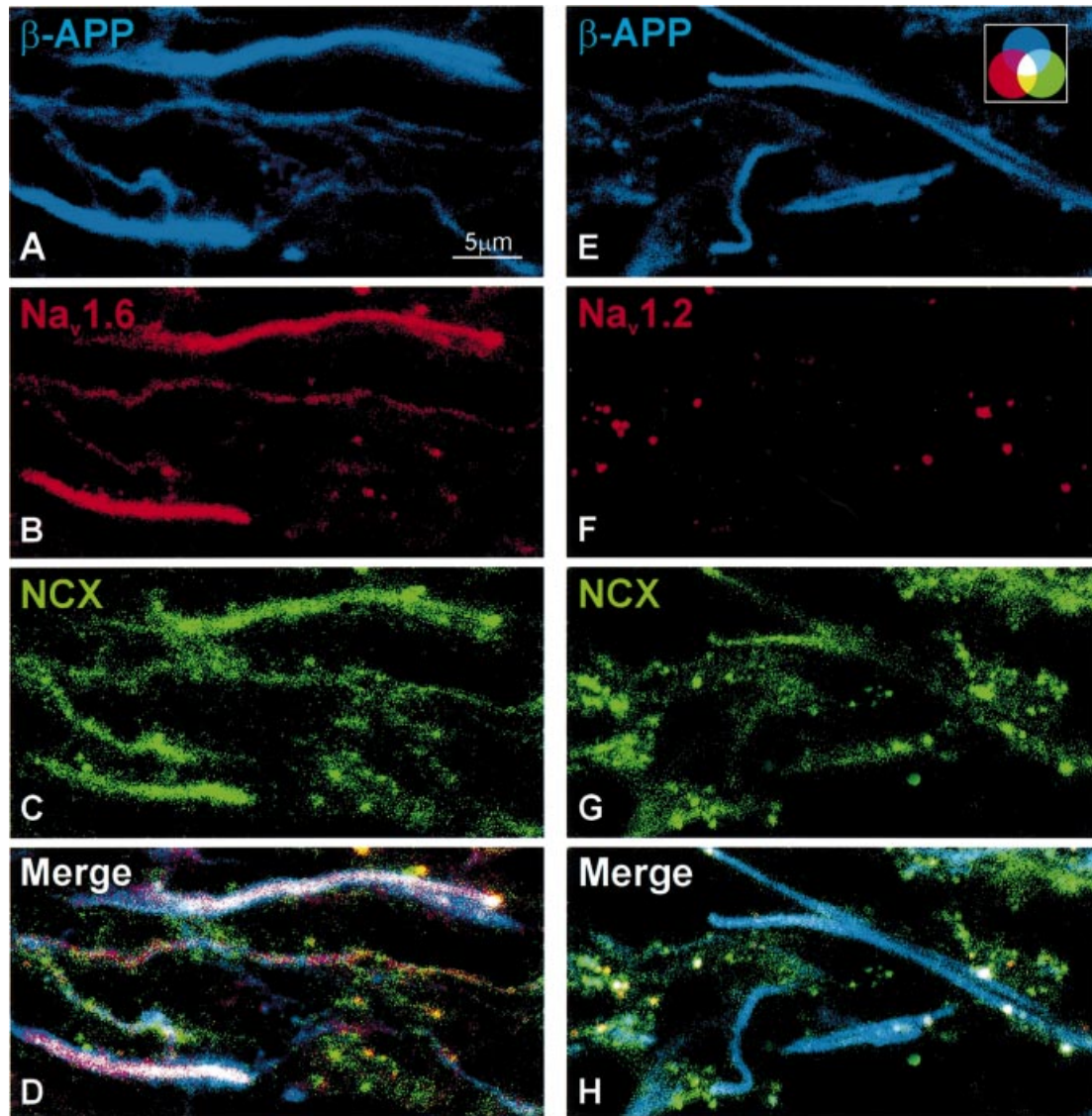
The presence of myelin or myelin-forming cells and their axo-glial junctions can influence the distribution of sodium channels within the axon membrane (Rosenbluth, 1988; Kaplan *et al.*, 1997; Rios *et al.*, 2003). Similar to previous descriptions in models of genetic dysmyelination (Boiko *et al.*, 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  $\text{Na}_v1.6$  and  $\text{Na}_v1.2$  expression that extended diffusely for at least 8  $\mu\text{m}$  along demyelinated axons.  $\text{Na}_v1.2$  (Westenbroek *et al.*, 1989; Gong *et al.*, 1999; Whitaker *et al.*, 2000) and  $\text{Na}_v1.6$  (Black *et al.*, 2002) channels are known to be present along non-myelinated axons within the CNS, and  $\text{Na}_v1.6$  has been shown to contribute to action potential conduction along non-myelinated axons (Black *et al.*, 2002). However, the specific contributions of  $\text{Na}_v1.6$  and  $\text{Na}_v1.2$  to conduction along demyelinated axons have not been studied. The relatively non-focal distribution of  $\text{Na}_v1.6$  and/or  $\text{Na}_v1.2$  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. Intra-axonal accumulation of  $\text{Ca}^{2+}$  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  $\text{Na}^+$  from the perfusate provides nearly complete protection in these models of axonal injury, demonstrating a link between  $\text{Na}^+$  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  $\text{Ca}^{2+}$  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.  $\text{Na}_v1.6$  is expressed at high levels within this class of neurons (Black *et al.*, 1996). Supporting a role for  $\text{Na}_v1.6$  in triggering  $\text{Ca}^{2+}$ -mediated injury of axons,  $\text{Na}_v1.6$  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  $\text{Na}_v1.1$  or  $\text{Na}_v1.2$  channels (Smith *et al.*, 1998). There is also evidence that  $\text{Na}_v1.2$  can produce a persistent current when co-expressed with  $\beta\gamma$  subunits of G protein (Ma *et al.*, 1997). However, it appears that, in many cell types,  $\text{Na}_v1.6$  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  $\text{Na}^+$  due to energy failure. Their experiments extend studies in white matter axons subjected to energy depletion by hypoxia; these demonstrated that  $\text{Na}^+$  influx via sodium channels drives NCX to import calcium



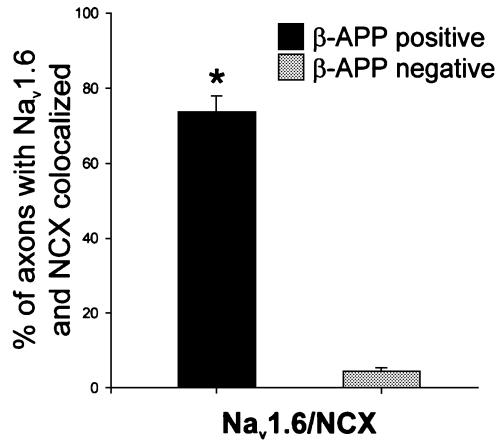


**Fig. 6**  $\beta$ -APP positive spinal cord axons co-express NCX and  $\text{Na}_v1.6$  over extensive regions. The digital images demonstrate axons in EAE spinal cord immunostained for  $\beta$ -APP (blue; **A**, **E**), sodium channel  $\text{Na}_v1.6$  (red; **B**) or  $\text{Na}_v1.2$  (red; **F**) and NCX (green; **C**, **G**). (**D**) and (**H**) correspond to merged images (white). **A–D** show co-expression of  $\text{Na}_v1.6$ , NCX and  $\beta$ -APP, a marker of axonal injury. In contrast, **E–H** demonstrate  $\beta$ -APP/NCX positive profiles but absence of  $\text{Na}_v1.2$  immunostaining ( $\times 1800$ ).

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 flecainide 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  $\beta$ -APP positive axons expressed extensive regions of  $\text{Na}_v1.6$  alone, and 36% of  $\beta$ -APP positive axons expressed extensive regions of  $\text{Na}_v1.6$  together with  $\text{Na}_v1.2$ . In contrast, <2% of  $\beta$ -APP positive axons expressed  $\text{Na}_v1.2$  alone. These findings, in conjunction with a

56% reduction in dorsal column axonal density and the colocalization of extensive regions displaying NCX and  $\text{Na}_v1.6$  in  $\beta$ -APP positive axons compared with  $\beta$ -APP negative axons, suggest a relationship between NCX and  $\text{Na}_v1.6$  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. 7** NCX and Na<sub>v</sub>1.6 are co-expressed in β-APP positive axons in EAE. Triple immunolabelling was used to determine the proportion of β-APP positive axons and β-APP negative axons that co-express NCX and Na<sub>v</sub>1.6 over extensive regions. The proportion of axons that co-express Na<sub>v</sub>1.6 and NCX is significantly higher in β-APP positive axons (filled bar, left) compared with β-APP negative axons (grey bar, right). \* =  $P < 0.001$ .

lidocaine, flecainide) to mice and rats with EAE, suggesting that neuroprotection of axons via pharmacological blockade of sodium channels might provide a useful therapeutic approach in neuroinflammatory disorders.

While we observed extensive regions of expression of both Na<sub>v</sub>1.6 and NCX in 73.5% of β-APP positive axons in EAE, we were unable to detect these extensive regions of co-expression of Na<sub>v</sub>1.6 and NCX in about one quarter of β-APP positive axons. We are unable to explain this apparent discrepancy. It is possible that, as axonal degeneration proceeds and axonal protein molecules are degraded, Na<sub>v</sub>1.6 and NCX levels fall below detectable levels. Alternatively, cytoskeletal molecules that anchor Na<sub>v</sub>1.6 and NCX in the axon membrane may be degraded as axons degenerate. Further work is needed to examine these hypotheses. Irrespective of this, however, our results colocalize Na<sub>v</sub>1.6 and NCX over extensive regions in nearly three-quarters of axons that are β-APP positive (a marker of injury in EAE), while these molecules are co-expressed in only 4% of β-APP negative axons.

In summary, our results demonstrate an association between the co-expression of diffusely distributed Na<sub>v</sub>1.6 and NCX, and β-APP, a marker of axonal injury, in dorsal column axons in EAE. These findings, in combination with a significant reduction in the density of dorsal column axons, are consistent with the hypothesis that Na<sub>v</sub>1.6 and NCX participate in a cascade that contributes to axonal degeneration in EAE. Physiological and pharmacological studies on dorsal column axons will be needed to confirm this role. Additional immunocytochemical studies will be needed to determine whether the association between Na<sub>v</sub>1.6, NCX and axonal injury is present in other tracts in EAE and in human neuroinflammatory disorders.

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