Acid-sensing ion channel 1 is involved in both axonal injury and demyelination in multiple sclerosis and its animal model

Sandra Vergo,1 Matthew J. Craner,1 Ruth Etzensperger,1 Kathrine Attfield,1 Manuel A. Friese,1,2 Jia Newcombe,3 Margaret Esiri4 and Lars Fugger1

1 Department of Clinical Neurology, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DS, UK
2 Institut für Neuroimmunologie und Klinische MS-Forschung, Universitätsklinikum Hamburg-Eppendorf, Zentrum für Molekulare Neurobiologie Hamburg, 20251 Hamburg, Germany
3 NeuroResource, UCL Institute of Neurology, 1 Wakefield Street, London WC1N 1PJ, UK
4 Department of Neuropathology, Level 1, West Wing, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, UK

Correspondence to: Prof. Lars Fugger,
Department of Clinical Neurology,
Weatherall Institute of Molecular Medicine,
John Radcliffe Hospital,
University of Oxford,
Oxford OX3 9DS,
UK
E-mail: lars.fugger@imm.ox.ac.uk

Although there is growing evidence for a role of excess intracellular cations, particularly calcium ions, in neuronal and glial cell injury in multiple sclerosis, as well as in non-inflammatory neurological conditions, the molecular mechanisms involved are not fully determined. We previously showed that the acid-sensing ion channel 1 which, when activated under the acidotic tissue conditions found in inflammatory lesions opens to allow influx of sodium and calcium ions, contributes to axonal injury in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. However, the extent and cellular distribution of acid-sensing ion channel 1 expression in neurons and glia in inflammatory lesions is unknown and, crucially, acid-sensing ion channel 1 expression has not been determined in multiple sclerosis lesions. Here we studied acute and chronic experimental autoimmune encephalomyelitis and multiple sclerosis spinal cord and optic nerve tissues to describe in detail the distribution of acid-sensing ion channel 1 and its relationship with neuronal and glial damage. We also tested the effects of amiloride treatment on tissue damage in the mouse models. We found that acid-sensing ion channel 1 was upregulated in axons and oligodendrocytes within lesions from mice with acute experimental autoimmune encephalomyelitis and from patients with active multiple sclerosis. The expression of acid-sensing ion channel 1 was associated with axonal damage as indicated by co-localization with the axonal injury marker beta amyloid precursor protein. Moreover, blocking acid-sensing ion channel 1 with amiloride protected both myelin and neurons from damage in the acute model, and when given either at disease onset or, more clinically relevant, at first relapse, ameliorated disability in mice with chronic-relapsing experimental autoimmune encephalomyelitis. Together these findings suggest that blockade of acid-sensing ion channel 1 has the potential to provide both neuro- and myelo-protective benefits in multiple sclerosis.

Keywords: acid-sensing ion channel; multiple sclerosis; amiloride; neuroprotection; demyelination
Introduction

Multiple sclerosis is one of the most common causes of progressive disability affecting young people. Inflammatory mediated demyelination is a characteristic pathophysiological process and current disease-modifying therapies predominantly target inflammatory mechanisms. Although these therapies reduce MRI evidence of disease activity and relapse rates, they have, at best, shown only a modest effect on the development of disability. Moreover, it is the axonal degeneration and neuronal loss, rather than the inflammation per se, that is the pathophysiological substrate underlying permanent, clinical deficits (Bjartmar et al., 2000; Tallantyre et al., 2010). The mechanisms that contribute to this neurodegeneration, however, are not clearly delineated, and a greater understanding of these processes is critical to the development of realistic neuroprotective treatment strategies that will prevent progressive disability in patients.

The clinical sequelae of axonal loss are more apparent in the later stages of multiple sclerosis, but axonal injury occurs early in acute multiple sclerosis lesions (Bitsch et al., 2000; Kuhlmann et al., 2002). The mechanisms that contribute to the axonal injury are likely to be complex and involve a combination of inflammatory mediators, demyelination and loss of axonal trophic support. In addition, there are changes in neuronal ion channel expression and/or function that are now recognized to be of pathophysiological importance (Trapp and Stys, 2009), although it seems that chronic inflammation is a prerequisite for neurodegeneration at any stage of the disease (Frischer et al., 2009).

One of the central pathophysiological mechanisms leading to axonal and cellular injury is Na⁺/Ca²⁺ overload (Stys, 1998) to which neurons and oligodendrocytes demonstrate a selective vulnerability (Tekkok and Goldberg, 2001). Several ionic mechanisms that may contribute to toxic Na⁺/Ca²⁺ loading include gating of non-inactivating Na⁺ channels (Stys et al., 1993), reverse Na⁺/Ca²⁺ exchange (Stys et al., 1992; Kapoor et al., 2003) and voltage-gated Ca²⁺ channel activation (Brown et al., 2001). In addition, recent studies have demonstrated parallels between inflammation- and ischaemia-induced CNS injury (Aboul-Enein et al., 2003; Aboul-Enein and Lassmann, 2005) suggesting a similar mechanism. A common denominator in ischaemia and CNS inflammation is the resultant mitochondrial inhibition, failure of energy metabolism and lactate acidosis. Consistent with this we showed previously that inflammation leads to tissue acidosis in experimental autoimmune encephalomyelitis (EAE) (Friese et al., 2007), and that under these conditions acid-sensing ion channels (ASICs), a family of proton-gated cation channels widely expressed in the CNS, become active (Waldmann et al., 1997; Wemmie et al., 2003), suggesting that they could play an important role in neurodegeneration.

ASICs consist of several isoforms but the predominant functional ion channel subunit in the CNS is ASIC1a, which can flux both Na⁺ and Ca²⁺ (Wemmie et al., 2002; Xiong et al., 2008). Direct evidence implicating ASIC1a in neuronal injury has now been shown in experimental stroke (Xiong et al., 2004), neurodegenerative disease (Arias et al., 2008), cultured human cortical neurons (Li et al., 2010) as well as by our group in a multiple sclerosis model (Friese et al., 2007). In the latter study, we used C57BL/6 Asic1⁻/⁻ mice and wild-type mice immunized with myelin oligodendrocyte glycoprotein (MOG₃₅-₅₅). These mice develop a T cell-dependent acute monophasic disease with pathological features of demyelination and axonal degeneration (Black et al., 2006; Friese et al., 2007). Significantly lower disease severity and clinical deficit were observed in Asic1⁻/⁻ mice and in EAE mice treated with amiloride, apparently as a result of protection from axonal degeneration in the spinal cord. Furthermore while amiloride is a non-specific blocker of ASIC1, combinatorial treatment studies of amiloride in Asic1⁻/⁻ mice did not demonstrate any additional therapeutic benefit indicating that the effect of amiloride is mediated by ASIC1 blockade. The disease modifying effect of ASIC1 disruption or blockade by amiloride was shown not to involve changes in expression of essential immune activation markers on antigen presenting cells in vitro and to be independent of auto-reactive T cells (Friese et al., 2007). These findings support a non-immune modulatory role of ASIC1 blockade and suggest the effect is related to ASIC1 expression in the CNS. Recently, ASIC1 has been detected in oligodendrocytes (Feldman et al., 2008) and it is possible that upregulation of ASIC1 in these cells could influence myelin damage but the functional role of the ion channel and its expression pattern in CNS inflammation are unknown.

To better understand the role of ASIC1 in multiple sclerosis, we investigated whether ASIC1 is upregulated within inflammatory lesions in the CNS in EAE and, most importantly, in multiple sclerosis lesions, and whether ASIC1 expression is associated with demyelination and axonal injury. Having established the likely relevance of ASIC1 in multiple sclerosis, as well as in acute EAE, we tested the effects of ASIC1 blockade in a chronic relapsing-remitting EAE model that more faithfully reflects the relapsing-remitting disease course observed in the majority of patients with multiple sclerosis.

Materials and methods

Induction and assessment of experimental autoimmune encephalomyelitis in mice

C57BL/6 Asic1⁻/⁻ (Wemmie et al., 2002) and C57BL/6 (Harlan, The Netherlands) female mice aged 6–10 weeks were used for induction of acute EAE as previously described (Friese et al., 2007). Biozzi ABH (Harlan, The Netherlands) female mice aged 7–9 weeks exhibited a
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relapsing-remitting clinical phenotype when injected subcutaneously in the flank with 200 µl emulsion composed of 300 µg MOG35-55 peptide in 200 µl incomplete Freund’s adjuvant (Sigma-Aldrich, UK) supplemented with 5 mg/ml Mycobacterium tuberculosis H37 Ra (BD, UK) on Day 0 and in the other flank one week later. In addition, 400 ng pertussis toxin (Sigma-Aldrich) in phosphate-buffered saline was injected intravenously on the day of immunization and repeated 48 h later. Each animal had at least one relapse prior to sacrifice at 92 days post-immunization. Average length of disease was 67 ± 8 days.

We administered 10 mg/kg body weight amiloride (Sigma-Aldrich) in 100 µl distilled water, or the same volume of distilled water without drug, intraperitoneally daily, starting from five days post-immunization in C57BL/6 mice and at onset of disease (16 days post-immunization) or at the time of first relapse (30.5 ± 2.6 days post-immunization) in the Biozzi ABH mice and continued until sacrifice at 39 and 92 days post-immunization, respectively. Amloride treatment at time of first relapse was initiated individually to each mouse when weight loss together with a 0.5 change in clinical score was observed post first defined remission period. The mice were monitored daily for weight loss and scored on a 0–4 clinical scale with increasing score reflecting clinical worsening as follows: 1, flaccid tail; 2, abnormal righting reflex and/or abnormal gait in the absence of weakness; 3, partial hindlimb paralysis and 4, complete hindlimb paralysis. We applied the scale in 0.5 gradations for intermediate scores. To determine the clinical course of EAE, defined relapses and remissions were identified as described previously by Crainer et al. (2003).

All experiments were approved by the University of Oxford Clinical Medicine Ethical Review Committee and have been licensed under the Animals Scientific Procedures Act of the UK Home Office.

Immunohistochemistry

Animals for immunohistochemistry were perfused through the heart with a phosphate-buffered saline and then with 4% paraformaldehyde in 0.2 M phosphate buffer. Tissue was carefully excised, post-fixed for 40 min at 4°C, then cryoprotected in 30% sucrose in phosphate-buffered saline and frozen in Tissue-Tech O.C.T compound. Optic nerve or spinal cord was cryosectioned (8–12 µm) onto slides and desiccated overnight before continuing with immunohistochemistry.

Tissue sections were permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 before incubating in blocking solution (phosphate-buffered saline containing 5% normal goat serum and 3% bovine serum albumin). Anti-mouse/human polyclonal antiserum (MYT19) recognizing ASIC1 [Wemmie et al., (2003) and Supplementary Fig. 2] and other antibodies against phosphorylated neurofilament (SMI-31, Covance), non-phosphorylated neurofilament (SMI-32, Covance), β-amyloid precursor protein (β-APP, MAB348, Millipore), myelin basic protein (SMI-94, Covance), 2,3-cyclic nucleotide 3′-phosphodiesterase (CNPase, MAB326R, Millipore), CD45 (M0775, DAKO), glial fibrillary acidic protein (SMI-22R, Covance), CD68 (Abd Serotec, 5879-2008) and bixin-conjugated ricinus communis agglutinin-1 (RCA-1) (Vector Laboratories). When RCA-1 was used, a step to block endogenous tissue biotin and avidin was included after quenching endogenous peroxidase.

For ASIC1 immunohistochemistry the snap-frozen sections (10 µm) were fixed for 10 min in acetone before applying similar staining protocol as described above for mouse tissue. Primary antibodies were used against mouse/human polyclonal antiserum (MYT19) recognizing ASIC1, phosphorylated neurofilament (SMI-31, Covance), β-APP (MAB348, Millipore), myelin basic protein (SMI-94, Covance), CNPase (MAB326R, Millipore), MHC class II (M0775, DAKO), glial fibrillary acidic protein (SMI-22R, Covance), CD68 (Abd Serotec, 5879-2008) and bixin-conjugated ricinus communis agglutinin-1 (RCA-1) (Vector Laboratories). When RCA-1 was used, a step to block endogenous tissue biotin and avidin was included after quenching endogenous peroxidase activity.

In situ hybridization

Optic nerve tissue was fixed and cryoprotected as described above. Digoxigenin-labelled sense and antisense riboprobes that recognize Asic1 mRNA sequences 1091–1634 (GenBank accession number NM_009597.1) were generated by in vitro transcription (DIG RNA labelling kit, Roche) (Friese et al., 2007). Longitudinally cut optic nerve tissue sections (8 µm) were processed for in situ hybridization as previously described (Craner et al., 2003). Sense riboprobes gave no signal upon in situ hybridization.

Human tissue

Post-mortem spinal cord and optic nerve tissue, acquired via a rapid protocol from patients with acute multiple sclerosis (n = 9, 51 ± 4.8 years, mean disease duration 18 ± 3.3 years) and from controls (n = 7, 67 ± 6.4 years) with no CNS disease was obtained from the NeuroResource tissue bank, UCL Institute of Neurology, London, UK or the Thomas Willis Oxford Brain Collection, Oxford, UK. Tissue analysed for quantification was rapidly frozen (post-mortem delay 16 ± 1.7 hours) as 1 cm3 blocks on Tissue-Tek O.C.T mounting medium. Characterization of the lesions was performed using oil red O and haematoxylin staining to identify the lesion centre and border. Acute multiple sclerosis lesions with ongoing or recent demyelination were identified on the basis of the presence of substantial numbers of oil red O-positive macrophages containing neutral lipids resulting from myelin breakdown (Li et al., 1993). One multiple sclerosis case (MS/01-118, Supplementary Table 1) from a paraffin embedded tissue block was characterized using Luxol fast blue staining combined with anti-MHC II (M0775, DAKO) immunohistochemistry.

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**Tissue analysis**

Images for tissue analysis were captured either by Openlab software ver. 5.1 and Olympus BX60 microscope or Lasersharp software (Zeiss, UK) and a confocal (Radiance 2000; Bio-Rad Laboratories) system on a microscope (TE300; Nikon). Each system was coupled with a high-resolution digital camera.

For quantification of ASIC1 expression in spinal cord axons from C57BL/6 normal and EAE (15 and 30 days post-immunization) mice, neurofilament immunofluorescence (SMI31 and SMI32) of spinal cord sections (10 μm) was used to identify axons. Within preselected quadrants from each image, axons were then analysed for positive ASIC1 immunostaining (yellow colocalization) to sample a total field of 10^6 μm^2 per animal.

Longitudinal optic nerve tissue sections (10 μm) from C57BL/6 normal and EAE mice were used to identify ASIC1 expression in oligodendrocytes. CNPase^+veCD45^-ve cells were identified as oligodendrocytes to avoid false positive macrophages containing myelin/oligodendrocytes products in EAE. Using ImageJ (NIH) software, CNPase^+veCD45^-ve cells were manually outlined in the green channel (CNPase^+ve) in a blinded manner followed by calculation of mean fluorescence intensities from the red, green and blue channel separately within the outlined area. Positive immunostaining was seen as ×4 level of background (no primary slide) and 60–80 cells were examined per animal.

Longitudinal spinal cord tissue sections from normal control tissue donors (n = 4) and containing acute multiple sclerosis lesions (n = 5) were used to quantify numbers of ASIC1^+ve oligodendrocytes. To increase the likelihood of staining both myelin and the cell soma, a combination of CNPase and myelin basic protein immunofluorescence was used to identify oligodendrocytes, and RCA-1 staining was included to exclude potential false positive cells. ImageJ software was used as described above for the mouse tissue to quantify ASIC1^+veCNPase^+veRCA-1^-ve cells.

For quantification of ASIC1^+ve axons in spinal cord and optic nerve normal control tissue donors (n = 7) and multiple sclerosis active lesions (n = 8), multiple images were acquired. Images were analysed using ImageJ software utilizing thresholding to determine immunopositive staining as defined by ×3 level of background. Each image channel was pseudo-coloured on the basis of being immunopositive to facilitate objective counting of axons. The further application of a target grid extending across the width of the image and perpendicular to the axis of the nerve fibres was overlaid on randomly selected images (each 200 × 10 mm) per subject and axonal profiles with ASIC1 immunostaining within the grid were counted. This target grid approach was used in preference to whole field examination as it reduces the possibility of duplicating quantification of axons as they move in and out the tissue plane.

For quantification of axons in normal, chronic-relapsing EAE and amiloride-treated chronic-relapsing EAE, neurofilament immunofluorescence (SMI31 and SMI32) of spinal cord cross sections was used to identify total axons within preselected fields (2620 μm^2) at specific sites within the dorsal funiculus and dorsal corticospinal tract. ImageJ software and adapted ICTN plugin, which counted axonal profiles based on a minimum diameter of the axon, separation and threshold intensity, was used to count number of axons to sample a total field of 1.1 × 10^6 μm^2 per animal. Accuracy of this automated counting technique has previously been confirmed by manual counting (Friese et al., 2007).

Demyelination in optic nerve and spinal cord was identified using antibodies against myelin basic protein or CNPase or by using Luxol fast blue staining. Optic nerve sections were cut at different sites from the cutting surface with four sections per mouse. Myelin basic protein immunofluorescence intensity in those sections was used to represent the amount of myelin per mouse (mean fluorescence intensity from myelin basic protein immunostaining per 3250 μm^2 of optic nerve). The dorsal funiculus of the cervical spinal cord C5-C8 was examined for demyelination using Luxol fast blue staining and CNPase immunostaining of myelin. Again, four sections were stained per mouse of which the mean represents the amount of myelin for each animal. Microdensitometry was performed using ImageJ software.

The number of mice analysed for each condition was as follows: 15 days C57BL/6: normal = 4, EAE = 4; 30 days C57BL/6: normal = 4, EAE = 4; 92 days Biozzi ABH: normal = 6, chronic-relapsing EAE = 6-9, chronic-relapsing EAE +amiloride = 6.

**In vitro acidosis induced cell injury assay in murine primary oligodendrocyte enriched cell cultures**

Disassociated murine neonatal (P0-P1) cortices and corpus callosum from wild-type or Asic1^−/− C57BL/6 mice were cultured (37°C in 7.5% CO2) in Dulbecco’s modified Eagle’s medium with 10% horse serum on poly-d-lysine coated T75 tissue culture flasks with a density of two brains per flask. By Day 10, mixed glial cultures were obtained, consisting of oligodendrocyte precursor cells and microglia growing on an astrocyte monolayer. Purified oligodendrocyte precursor cells were isolated using a modification of the mechanical dissociation and differential adhesion method described by McCarthy and de Vellis (1980). Subcultures of mature oligodendrocytes were re-plated onto polyornithine-coated glass coverslips (9 mm, VWR) and differentiated for four days in vitro in astrocyte conditioned Dulbecco’s modified Eagle’s medium supplemented with bovine insulin (5 mg/ml), human transferrin (50 mg/ml), bovine serum albumin V (100 mg/ml), progesterone (6.2 ng/ml), putrescine (16 mg/ml), sodium selenite (5 ng/ml), T3 (400 ng/ml), T4 (400 ng/ml), l-glutamine (4 μM), penicillin and streptomycin and 0.5% horse serum.

For the acidosis induced injury cell assay wild-type or Asic1^−/− mature cultures were rinsed in phosphate-buffered saline, then incubated in buffer calibrated to pH 6.0 (140 mM NaCl, 5.4 mM KCl, 25 mM MES, 20 mM glucose, 1.3 mM CaCl_2, 1.0 mM MgCl_2, 15 mM NaHCO_3) or pH 7.4 (140 mM NaCl, 5.4 mM KCl, 25 mM HEPEs, 20 mM glucose, 1.3 mM CaCl_2, 1.0 mM MgCl_2, 15 mM NaHCO_3) for 4 h at 37°C. To test the effect of ASIC1 blockage, amiloride (100 μM; Sigma-Aldrich) or venom of the tarantula Psalmopoeus cambridgei containing psalmotoxin-1 (PtxT1) (100 ng/ml; Spiderpharm) was added in the incubation buffer calibrated to pH 6.0 or 7.4. Cells were then fixed in 4% paraformaldehyde/0.2 M phosphate buffer and processed for myelin basic protein immunocytochemistry. Results shown are from 2–3 independent experiments each with three replicates.

**Statistics**

All values are expressed as means ± standard error of the mean (SEM). Unless specifically indicated, we performed analyses of significance by the two-tailed Student’s t-test for two groups, or by ANOVA with post hoc multiple comparison analysis for multiple groups; Tukey test to compare all pairs of the group [GraphPad (San Diego, CA, USA) Prism Software 5.0]. All tests were classified as significant if the P-value was < 0.05.
**Results**

Our previous work demonstrated a role for ASIC1 in axonal injury in an acute EAE model but did not examine the distribution and expression of the channel in normal tissue and in active lesions, and its relationship with markers of cellular injury. First we investigated ASIC1 expression in acute EAE drawing parallels with that observed within multiple sclerosis lesions. To extend the relevance of our findings of ASIC1 expression in EAE and multiple sclerosis tissue and its association with cellular injury we tested the hypothesis that long-term amloride treatment would ameliorate disease severity in a chronic-relapsing model of multiple sclerosis.

**Increased acid-sensing ion channel 1 expression in axons within acute experimental autoimmune encephalomyelitis is associated with axonal injury**

First we confirmed a significant reduction in disease severity in amloride-treated EAE mice (1.7 ± 0.2, n = 10) compared with vehicle-treated EAE mice (2.5 ± 0.2, n = 6) (P < 0.001, repeated measures two way ANOVA with multiple regression; data shown in Supplementary Fig. 1), and subsequently used this model for all of the acute EAE experiments. In CNS neurons, ASIC1 is normally localized in the neuronal soma and dendritic spines (Zha et al., 2006), although in vitro there is some ASIC1 expression in axons (Zha et al., 2009). Accordingly we found very low levels of ASIC1 expression and few ASIC1 immunopositive (ASIC1+ve) axonal profiles in control white matter (<5%; Fig. 1A–C), but a significant increase in the proportion of ASIC1+ve axons (~40% at 15 days post-immunization and ~70% at 30 days post-immunization) in EAE lesions (Fig. 1A–C); ASIC1 immunofluorescence was mainly in small diameter axons (diameter <3 μm) in the spinal cord white matter tracts, while relatively few large diameter axons (diameter >3 μm) were positive. The majority of injured axons (β-APP+ve) were ASIC1+ve (Fig. 1F), and morphological features of axonal transection with terminal ovoid formation were associated with expression of ASIC1 in β-APP+ve axons (Fig. 1D, e–f). Together these observations are consistent with a role for abnormal ASIC1 expression contributing to axonal injury in EAE spinal cord.

**Acid-sensing ion channel 1 expression in oligodendrocytes in acute experimental autoimmune encephalomyelitis**

We next examined the role of ASIC1 expression in the oligodendrocytes, which are responsible for myelination. In longitudinal white matter tissue sections of acute EAE there was a significantly increased number of Asic1a messenger RNA positive and ASIC1+ve oligodendrocytes in EAE (~70% at 15 days post-immunization and ~80% at 30 days post-immunization) compared with control (~20%) (Fig. 2A–B).

Calpain I is a member of a family of Ca2+-dependent proteases and a recognized marker of oligodendrocyte injury that is upregulated in acute EAE as well as in multiple sclerosis tissue (Shields et al., 1999). With this marker there were few calpain I+ve cells in control mouse tissue but a significant increase in the number of cells in EAE (15 days post-immunization and 30 days post-immunization) (Fig. 2C–E). Moreover, ~66% of ASIC1+ve oligodendrocytes colocalized with calpain I at 15 days post-immunization suggesting that ASIC1 mediated Na+ and Ca2+ fluxes could contribute to oligodendrocyte injury. By 30 days post-immunization the number of ASIC1+ve/calpain I+ve oligodendrocytes had attenuated perhaps reflecting the reduced inflammatory infiltration and acidosis at this time. Overall, these findings suggest that the oligodendrocyte injury is associated with expression of ASIC1 in EAE at peak inflammation (15 days post-immunization).

**Acid-sensing ion channel 1 expression in acute multiple sclerosis lesions is also associated with demyelination and axonal injury**

ASIC1 expression has not been studied previously in normal control or multiple sclerosis CNS tissue. We focused on spinal cord and optic nerve acute multiple sclerosis lesions in which an inflammatory-induced energy failure and resultant acidosis facilitating ASIC1 activation is likely to be most evident. Figure 3A illustrates an example of an active lesion border shown by the patchy loss of Luxol fast blue staining recognizing myelin (left side) with a demyelinated lesion centre (right side). Interestingly, ASIC1 expression was higher in the areas of active demyelination (left side) compared with the fully demyelinated areas closer to the lesion centre (right side) (Fig. 3A–c), and was observed within both oligodendrocytes and axons. In parallel to our findings in EAE, we found a modest but significant increase in the number of ASIC1+ve oligodendrocytes in acute multiple sclerosis lesions with ongoing demyelination (Fig. 3B).

A xonal injury is commonly observed in acute multiple sclerosis lesions (Bitsch et al., 2000) and we demonstrate a significant reduction in the number of healthy axons, indicated by phosphorylated neurofilament in multiple sclerosis lesions compared with those of control tissue (Fig. 3D, histogram). In the inflamed lesion, border axons expressed ASIC1 and demonstrated terminal ovoid formation indicating axonal transection (Fig. 3A [insert] and 3C). There was a significant increase in the number of ASIC1 immunopositive axonal profiles lacking phosphorylated neurofilament in the active border of multiple sclerosis lesions (86.3 ± 8.5 per 1000 μm2) compared with control (8.5 ± 0.7 per 1000 μm2; Fig. 3D, histogram) suggesting that axonal expression of ASIC1 is associated with an abnormal state of neurofilament expression and the molecular signature of axonal injury. By contrast, the axonal expression of ASIC1 in healthy (phosphorylated neurofilament+ve) axons from either control or multiple sclerosis spinal cord was low (21.5 ± 1.5 per 1000 μm2) (Fig. 3D, histogram). In combination with the marker myelin basic protein we could not detect ASIC1 staining in myelinated healthy axons in control white matter.
However, ASIC1 expression was found in axons undergoing demyelination as well as in demyelinated axons in multiple sclerosis lesions (Supplementary Fig. 3) thereby exposing the extracellular component of the channel to inflammatory tissue conditions facilitating ASIC1 activation and contributing to axonal injury.

Consistent with previous studies (Trapp et al., 1998; Kuhlmann et al., 2002; Craner et al., 2004), we found evidence of axonal injury in acute multiple sclerosis lesions with an 8-fold increase in the number of β-APP+ve axons (Fig. 3E, histogram). In the EAE model, the majority of the ASIC1+ve axons observed in acute multiple sclerosis lesions (86.9 ± 8.7 per 1000 μm²) colocalized with β-APP (white arrow heads, e, f) in the presence of inflammatory infiltrate (CD45+ve cells). Injured small (<3 um) diameter axons expressed ASIC1 compared with the injured large (>3 um) diameter axons (14.3%; P < 0.001, Fishers exact test) suggesting that while ASIC1 contributes to injury in both large and small diameter axons, our findings parallel the predilection of injury to small diameter axons observed in multiple sclerosis (DeLuca et al., 2004).

Acidosis contributes to oligodendrocyte injury in vitro and demyelination in experimental autoimmune encephalomyelitis

To examine if acidosis-mediated ASIC1 function could induce oligodendrocyte injury independent of an inflammatory infiltrate we used murine primary oligodendrocyte enriched cell cultures prepared from...
post-natal (P0-P1) wild-type or Asic1−/− mouse pups. Mature oligodendrocytes, following in vitro differentiation, were identified by myelin basic protein immunofluorescence and showed well-defined processes at physiological pH (Fig. 4A-a, b). Under acidotic conditions there was a significant reduction in oligodendrocyte cell numbers as well as of myelin basic protein+ve oligodendrocyte processes (Fig. 4A-c) compared with findings in cell cultures kept at physiological pH 7.4. Consistent with a role of ASIC1 mediated acidosis-induced injury, oligodendrocytes were protected from acidosis-induced damage in the presence of either amiloride or the specific ASIC1 blocker, psalmotoxin-1, and also in Asic1−/− mice in vitro (Fig. 4A, d-f, histogram).

To see whether ASIC1 blockade could similarly prevent demyelination in vivo, we determined the efficacy of amiloride treatment in EAE on optic nerve demyelination. In wild-type EAE (30 days post-immunization, Fig. 3B) there was demyelination with a significant reduction in myelin basic protein+ve oligodendrocyte processes (Fig. 4A-c) compared with findings in cell cultures kept at physiological pH 7.4. Consistent with a role of ASIC1 mediated acidosis-induced injury, oligodendrocytes were protected from acidosis-induced damage in the presence of either amiloride or the specific ASIC1 blocker, psalmotoxin-1, and also in Asic1−/− mice in vitro (Fig. 4A, d-f, histogram).

Amiloride treatment reduces permanent disability in chronic-relapsing experimental autoimmune encephalomyelitis and is effective at later disease stages

We have shown data to suggest an important role for ASIC1 expression in neurons and oligodendrocytes in axonal damage in both multiple sclerosis and acute EAE lesions, and in EAE demyelination. The beneficial effect of amiloride has already been shown in the acute EAE model but 80% of patients with multiple sclerosis develop a relapsing-remitting disease that is usually followed by a chronic progressive phase; tissue damage, including axonal injury, occurs early and continues dependent on the balance between cell damage and repair processes. To determine if amiloride treatment could produce a sustained neuroprotective effect on established disease, we examined the chronic-relapsing model of EAE in Biozzi ABH mice, which shows a clinical progression comparable to that in multiple sclerosis (Jackson et al., 2009). Amiloride therapy significantly reduced disease severity in chronic-relapsing EAE compared with vehicle-treated chronic-relapsing EAE when treatment was started at disease onset (Fig. 5A), with a significant...
Figure 3 ASIC1 expression in acute multiple sclerosis lesions is associated with axonal injury and demyelination. (A) Longitudinal spinal cord section showing the active demyelinating lesion border with (a) Luxol fast blue staining and major histocompatibility complex class II (HLA-DR) immunocytochemistry to identify myelination and inflammation, respectively. ASIC1 expression is observed in both axonal and cellular profiles (b, c). Scale bar 50 μm. Inserts show ASIC1+ axonal profiles demonstrating a lack of phosphorylated neurofilament (P-NF), a marker of healthy axons, and abnormal morphology with terminal ovoids indicating axonal transection. (B) Representative images of ASIC1 expression in oligodendrocytes in normal control (a) and in the border of an active multiple sclerosis lesion with ongoing demyelination (b). Dot plot demonstrates increased ASIC1 expression in oligodendrocytes within acute multiple sclerosis (n = 5) compared with control (n = 4) (white arrow head indicate examples of ASIC1+ oligodendrocytes, while those marked with * are negative for ASIC1). * P < 0.05, Mann–Whitney test. Scale bar 10 μm. (C) A single axon in spinal cord tissue from control and within an acute multiple sclerosis lesion demonstrating that ASIC1 expression is associated with an abnormal axonal morphology and reduced expression of
reduction in mean maximal disease score (3.81 ± 0.08 versus 3.04 ± 0.27, Table 1) and in permanent disability score (2.76 ± 0.26 versus 1.54 ± 0.34, Table 1). Similar to our previous studies in acute EAE (Friese et al., 2007), we first examined the potential influence of ASIC1 on elements of the inflammatory process. Looking at the effect of long-term amiloride treatment on peripheral immune cells from chronic-relapsing-EAE mice (92 days post-immunization) we found no significant difference in proportions or absolute cell numbers of T cells, B cells and macrophages from spleen of untreated and amiloride-treated chronic-relapsing EAE mice. T cell proliferation and cytokine production were also not affected by amiloride treatment (Supplementary Fig. 4). Our studies in acute EAE have shown ASIC1 expression in some microglia/macrophages. We therefore undertook functional in vitro studies using primary microglia cells to test the effect of ASIC1 blockade by amiloride on expression of immune cell activation markers and phagocytosis after stimulation with interferon-γ. Using flow cytometric analysis, amiloride did not show any effect on upregulation of the cell surface markers MHC class II (I-A/I-E), CD80 and CD40 (Supplementary Fig. 5). In contrast, phagocytosis of fluorescent-labelled beads during acidosis by interferon-γ-stimulated microglia was modestly decreased after amiloride treatment. We confirmed this effect in microglia prepared from Asic1−/− mice suggesting an ASIC1-mediated mechanism (Supplementary Fig. 5). Taken together, these findings suggest only a modest influence on the macrophage/microglia mechanism (Supplementary Fig. 5). Taken together, these findings suggest only a modest influence on the macrophage/microglia compartment, which was selective to their phagocytic function. However, examining the neuropathological correlate of the disease-modifying effect, we found a highly significant neuroprotective benefit in amiloride treated chronic-relapsing EAE mice within both corticospinal tracts and dorsal columns compared with chronic-relapsing EAE (Fig. 5B–C). Moreover, there was a significant reduction in demyelination in amiloride-treated chronic-relapsing EAE compared with untreated chronic-relapsing EAE mice within the spinal cord dorsal funiculus (Fig. 5D–E). Importantly, and with direct relevance to translational studies, we also found improved outcomes when we started amiloride treatment at the onset of the first relapse (30.5 ± 2.6 days post-immunization) when mice with chronic-relapsing EAE experienced their second clinical episode (Fig. 5F–G, Table 1).

**Table 1** Incidence, clinical severity and termination rate of MOG35–55 induced chronic-relapsing-EAE in Biozzi ABH mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Incidence (RR-EAE) (%)</th>
<th>Day of disease onset/treatment</th>
<th>Maximal disease score</th>
<th>Non-remitting deficit score</th>
<th>Termination rate (%)</th>
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<tbody>
<tr>
<td>Disease onset</td>
<td></td>
<td></td>
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<tr>
<td>CR-EAE + water</td>
<td>18/19 (95)</td>
<td>16.7 ± 0.5</td>
<td>3.81 ± 0.08 (n = 18)</td>
<td>2.76 ± 0.26 (n = 17)</td>
<td>5/18 (27)</td>
</tr>
<tr>
<td>CR-EAE + amiloride</td>
<td>11/13 (85)</td>
<td>17 ± 0.3</td>
<td>3.04 ± 0.27 (n = 11)</td>
<td>1.54 ± 0.34 (n = 11)</td>
<td>2/11 (18)</td>
</tr>
<tr>
<td>First relapse</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CR-EAE + water</td>
<td>23/27 (85)</td>
<td>15.9 ± 0.9/30.5 ± 2.6</td>
<td>3.9 ± 0.08 (n = 23)</td>
<td>3.6 ± 0.18 (n = 23)</td>
<td>14/23 (61)</td>
</tr>
<tr>
<td>CR-EAE + amiloride</td>
<td>19/29 (66)</td>
<td>15.5 ± 0.6/30.5 ± 2.6</td>
<td>3.6 ± 0.11 (n = 19)</td>
<td>2.4 ± 0.29 (n = 19)</td>
<td>6/19 (31)</td>
</tr>
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</table>

Data shown are means ± SEM. CR = chronic-relapsing; RR = relapse-remitting.

a Incidence refers to mice with a relapse-remitting disease course.

b Maximal disease score = the maximum clinical score received at any time during the time of experiment.

c Non-remitting deficit score = clinical score on the final day of the experiment, with culled mice recorded as score 4.

d Termination rate = number of culled mice due to severe disease.

**Figure 3** Continued

phosphorylated neurofilament (a–d). Also shown is a high magnification image demonstrating a β-APP+ve ASIC1+ve axon undergoing phagocytic attack by a microglia/macrophage (*RCA-1+ve*) (e, f). Scale bar 5 μm. (D) Control spinal cord axons (a) demonstrate low levels of ASIC1 expression. In acute multiple sclerosis lesions, (b, c) frequent ASIC1+ve small diameter axonal profiles (P-NF+ve) (1–3 μm) are observed (white arrows, b). Occasional P-NF+ve axons express high levels of ASIC1 and then usually having a small diameter (<3 μm, examples indicated by yellow arrowheads, b, c). Large diameter axons (>3 μm) are very rarely ASIC1 positive (examples indicated by white arrowheads, c). Scale bar 10 μm. Histogram demonstrates a significant increased number of ASIC1+ve axons in acute multiple sclerosis (n = 8) compared with control (n = 7). (E) In acute multiple sclerosis lesions small diameter axons express high levels of ASIC1 and are associated with β-APP (yellow arrow heads indicate examples of ASIC1+ve/β-APP+ve axons while white arrowhead shows an example of an infrequent β-APP+ve larger diameter axon negative for ASIC1, d). Scale bar 10 μm. The histogram demonstrates that the majority of β-APP+ve axons in multiple sclerosis lesions are expressing ASIC1 (multiple sclerosis n = 8, control n = 7). Results are presented as means ± SEM, *P < 0.05. MBP = myelin basic protein; MS = multiple sclerosis.

**Discussion**

ASIC1 has been shown to contribute to neuronal injury within a variety of CNS injury models but there have been no previous studies examining the contribution of ASIC1 to axonal loss and demyelination in multiple sclerosis or its animal model, EAE. Our results implicate ASIC1 upregulation and function as important molecular mechanisms in the axonal and myelin damage of multiple sclerosis as well as in the acute and chronic-relapsing EAE. Moreover we demonstrate a neuro- and myelo-protective action of amiloride, a licensed drug that inhibits ASIC1, not only in acute EAE but also when applied at and after onset of disease in the chronic-relapsing EAE model.
These findings suggest that ASIC1 represents a strong potential therapeutic target in multiple sclerosis as the incumbent tissue state induced by CNS inflammation may lead to excessive ASIC1 activation and intracellular flux of Na\(^+\) and Ca\(^{2+}\) causing cellular injury. It is established that molecular mechanisms that lead to excessive intracellular Ca\(^{2+}\) and activation of Ca\(^{2+}\)-inducible degradative pathways are likely to be important in multiple sclerosis (Trapp and Stys, 2009). As well as causing axonal injury, convergent pathways in hypoxic-ischaemic damage may also lead to injury of mature oligodendrocytes and their processes responsible for myelination via Ca\(^{2+}\)-dependent mechanisms (Karadottir et al., 2005; Micu et al., 2006). Furthermore, hypoxia-like metabolic injury has been suggested as a contributory pathogenic component in multiple sclerosis lesions (Lassmann, 2003; Lassmann et al., 2003). In multiple sclerosis lesions, mitochondrial dysfunction with reduced ATP production suggests the existence of energy failure (Dutta et al., 2006) and while this may independently lead to cellular injury, other metabolic consequences such as acidosis are also likely to be important. Indeed, elevated lactate levels as measured by magnetic resonance spectroscopy, have been shown to correlate with the degree of inflammation in multiple sclerosis lesions (Bitsch et al., 1999), and it is within acute inflammatory multiple sclerosis lesions that the extent of axonal injury is greatest (Kuhlmann et al., 2002). Moreover, it seems that the presence of inflammation is a prerequisite for neurodegeneration at all stages of multiple sclerosis (Frischer et al., 2009).

Consistent with a role of ASIC1 in axonal injury within CNS inflammation we found a significant increase in the number of axons expressing ASIC1 in EAE and, moreover, the majority of these ASIC1\(^{+ve}\) axons was positive for \(\beta\)-APP, a marker of axonal injury, and/or demonstrated morphological features of axonal injury. These findings were paralleled in multiple sclerosis tissue, as the majority of injured axons expressing ASIC1 were of small diameter (< 3 \(\mu\)m). This is of particular interest since neuropathological studies of the corticospinal tract in patients with

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**Figure 4** Acidosis contributes to oligodendrocyte injury *in vitro* and ASIC1 blockade ameliorates demyelination in acute EAE. (A) Mature oligodendrocytes (MBP\(^{+ve}\)) demonstrate defined processes at physiological pH (a, b). Presence of injury, demonstrated by reduced number and loss of processes when exposed to low pH (c), was reduced by Asic1\(^{−/−}\) and pharmacological ASIC1 blockade (d-f). The histogram demonstrates a significant protective effect of pharmacological and genetic blockade of ASIC1 in oligodendrocytes exposed to acidosis. Data presented are normalized to internal control [pH 7.4 wild-type (WT) and Asic1\(^{−/−}\)] with each bar representing myelin basic protein \(^{+ve}\) cells from 6–9 coverslips from 2–3 independent experiments (***\(P < 0.001\) to wild-type pH 6.0). (B) Demyelination in EAE is ameliorated by Asic1\(^{−/−}\) and amiloride treatment in EAE. Representative images of optic nerve cross section processed for myelin basic protein immunostaining and quantitative histogram demonstrating loss of myelin in wild-type EAE compared with control and relative preservation of myelin in optic nerves of amiloride-treated and Asic1\(^{−/−}\) EAE mice. Each bar represents mean myelin basic protein fluorescence intensity and results are shown as means ± SEM (***\(P < 0.01\), **\(P < 0.001\) to wild-type-EAE). Scale bars 20 \(\mu\)m. MBP = myelin basic protein; PcTx1 = *P. cambridgei* psalmotoxin-1.
Figure 5  Amiloride treatment initiated at disease onset or at first relapse reduces disability in chronic-relapsing (CR) EAE. (A) Disease scores (mean ± SEM) are shown for untreated chronic-relapsing EAE (grey squares, n = 18) and for amiloride-treated chronic-relapsing EAE (black triangles, n = 11) groups of Biozzi ABH mice from Day 0–92 post-MOG 35-55 injection. Amiloride treatment reduced disease progression illustrated by lower maximal disease score and non-remitting deficit. (B, C) Representative sections and histogram demonstrate that the number of neurofilament immunostained axons is reduced in untreated mice with chronic-relapsing EAE (n = 6) in both the corticospinal tract (CST) and dorsal column (DC) in comparison to control mice (n = 6), but amiloride treated EAE mice exhibit protection of axons compared with untreated mice with chronic-relapsing EAE. Scale bars 5 μm. (D, E) Representative images of Luxol fast blue (LFB) (continued)
multiple sclerosis have demonstrated a selective dropout of small diameter fibres (<3 μm) (DeLuca et al., 2004), suggesting that abnormal ASIC1 expression within this subgroup contributes to their selective injury compared with the preservation of larger diameter axons in multiple sclerosis. Interestingly, we noted the highest expression of ASIC1 was found at the active lesion border, characterized by the presence of inflammatory cells, patchy demyelination and morphological signs of acute axonal damage (Fig. 2). Only low ASIC1 expression was observed towards the centre of the lesion suggesting that local factors present in the pathogenic environment of the lesion border may influence upstream ASIC1 transcripts. Previous microarray studies have demonstrated a number of transcripts that show differential expression between the border and centre of active lesions (Mycko et al., 2003) and cytokines have been shown to have a modulatory effect on ion channel expression (Kochukov et al., 2009). For instance, one potential candidate that could influence ASIC1 expression is nerve growth factor, which has been shown to increase acid-sensing ion channel transcripts in the peripheral nervous system (Mamet et al., 2002) following inflammation. Nerve growth factor levels are elevated in multiple sclerosis lesions (Micera et al., 1999) as well as in the cerebrospinal fluid (Laudiero et al., 1992) from patients with multiple sclerosis. Nerve growth factor has also been shown to influence the expression of voltage gated sodium channels (Leffler et al., 2002), which are abnormally expressed in axons within EAE and multiple sclerosis and are associated with axonal injury (Crane et al., 2004). In combination with our studies, this suggests potential synergistic mechanisms leading to Na+ influx and axonal Ca2+ overload, which is supported by studies utilizing in vitro ischaemic axonal injury models (Nikolaeva et al., 2005). In addition, astrocytes known to express functional voltage gated sodium channels (Sontheimer and Waxman, 1992) have recently been demonstrated to show selective upregulation of the sodium channel Na+,1.5 in multiple sclerosis lesions (Black et al., 2010). This reflects a differential pattern of sodium channel expression of both isoform and cell type and raises the question whether ASIC1 expression is selective to cell type or part of a pan cellular upregulation programme. Therefore, we undertook immunohistochemical studies to examine a potential upregulation of ASIC1 in astrocytes in multiple sclerosis lesions using an antibody against glial fibrillary acidic protein as an astrocytic marker. Rarely astrocytes and their processes demonstrated ASIC1 expression in multiple sclerosis lesions, while the majority of astrocytes both in control and multiple sclerosis tissue were negative for ASIC1 (Supplementary Fig. 6A). These findings suggest that the abnormal expression of ASIC1 observed in CNS inflammatory disease is predominantly selective to neurons and oligodendrocytes.

We have demonstrated increased ASIC1 expression in combination with cellular injury markers in EAE and multiple sclerosis. This suggests that ASIC1 expression provides a likely candidate for Ca2+ overload and one that would contribute mainly under the conditions of inflammation or ischaemia where acidosis is present. However, our results do not exclude a role for other inflammatory mediators of ASIC1 function such as nitric oxide, which is increased in multiple sclerosis lesions (Smith and Lassmann, 2002) and inhibits mitochondrial respiration (Jekabson et al., 2003). Intriguingly, nitric oxide has been shown to oxidize cysteine residues in the extracellular loop of the ASIC1 subunit resulting in increased channel opening and subsequent Na+ and Ca2+ fluxes (Cadiou et al., 2007) and may represent another component of the inflammatory milieu that contributes to abnormal ASIC1 function in multiple sclerosis. In addition, serine proteases released during inflammatory cellular damage have also been shown to modulate the function of ASIC1 (Poirot et al., 2004), preventing desensitization of the channel under conditions of persistent extra-cellular acidification. Thus it appears that several mediators may be present in the pathogenic environment of CNS inflammatory lesions capable of potentiating ASIC1 activation and contributing to the Ca2+-dependent pathophysiological mechanisms that result in axonal loss and demyelination.

The novel finding of ASIC1 expression in both axons and oligodendrocytes is of interest as both components have a synergistic relationship for CNS tissue function. The presence of myelin around axons has been demonstrated not only to be important for efficient conduction of electrical signals but also to provide important trophic support for axonal survival and integrity (Li et al., 1994). Chronic demyelination of an axon is likely to eventually result in axonal degeneration (Trapp and Stys, 2009). Further evidence of the complex relationship between the axon and oligodendrocyte is demonstrated in Wallerian degeneration, an important contributor to axonal pathology in multiple sclerosis (Dziedzic et al., 2010), and also associated with oligodendrocyte injury (Abe et al., 1999). While multiple mechanisms are likely to be responsible for oligodendrocyte injury and/or apoptosis in CNS inflammatory disease, our results indicate that ASIC1 expression in mature oligodendrocytes and their processes contributes to acidosis-induced oligodendrocyte injury in vitro and demyelination during in vivo CNS inflammation. Moreover, therapeutic blockade of ASIC1 is myelo-protective in both acute and chronic relapsing-remitting models of multiple sclerosis.
Ion channel expression is not exclusive to neurons and oligodendrocytes, but has also been found in microglia contributing to activation and migration as well as regulation of phagocytosis by these cells in the CNS (Craner et al., 2005; Black et al., 2009). In diseased states, increased ion flux contributing to cellular activation may lead to indirect mechanisms of cellular injury such as release of nitric oxide, cytokines and reactive oxygen species and increased phagocytic activity (Block et al., 2007; Friese and Fugger, 2007; Kigerl et al., 2009). Although we have observed microglial/macrophage expression of ASIC1, the ion channel was only found in a small proportion of this cell type in acute multiple sclerosis lesions. Furthermore, functional studies of upregulation of immune cell activation marker expression and phagocytosis in primary microglia cultures after stimulation with interferon-γ demonstrate only a modest effect of ASIC1 on microglia that is restricted to phagocytosis.

Our previous studies demonstrated acidosis mediated axonal injury that can be ameliorated by genetic and pharmacological blockade of ASIC1 (Friese et al., 2007). However, multiple sclerosis is a chronic disease that in the early stages is usually characterized by a relapsing-remitting pattern that over time leads to accumulated permanent clinical deficits. To begin to examine the critical question of long-term benefit of amiloride treatment in CNS inflammatory disease we utilized a chronic relapsing-remitting model of multiple sclerosis. We demonstrate not only a benefit of commencing amiloride treatment at disease onset but also treating from the time of the first relapse. Moreover we observe that predominant clinical efficacy of amiloride is on the reduction of permanent disability, which has been shown to correlate with axonal loss in multiple sclerosis. These findings, along with the observed reduction in axonal loss and demyelination in chronic-relapsing EAE, suggest that blockade of ASIC1 is not only neuro- but also myelo-protective, and therefore could form the basis for a new treatment of established CNS inflammatory disease.

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Supplementary material
Supplementary material is available at Brain online.

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Friese MA, Craner MJ, Etzensperger R, Vergo S, Wemmie JA, Welsh MJ, et al. Acid-sensing ion channel-1 contributes to axonal degeneration in...


