A role for galanin in human and experimental inflammatory demyelination

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The neuropeptide galanin is widely expressed by many differing subsets of neurons in the nervous system. There is a marked upregulation in the levels of the peptide in a variety of nerve injury models and in the basal forebrain of humans with Alzheimer's disease. Here we demonstrate that galanin expression is specifically and markedly upregulated in microglia both in multiple sclerosis (MS) lesions and shadow plaques. Galanin expression is also upregulated in the experimental autoimmune encephalomyelitis (EAE) model of MS, although solely in oligodendrocytes. To study whether the observed increase in expression of galanin in inflammatory demyelination might modulate disease activity, we applied the EAE model to a panel of galanin transgenic lines. Over-expression of galanin in transgenic mice (Gal-OE) abolishes disease in the EAE model, whilst loss-of-function mutations in galanin or galanin receptor-2 (GalR2) increase disease severity. The pronounced effects of altered endogenous galanin or GalR2 expression on EAE disease activity may reflect a direct neuroprotective effect of the neuropeptide via activation of GalR2, similar to that previously described in a number of neuronal injury paradigms. Irrespective of the mechanism(s) by which galanin alters EAE disease activity, our findings imply that galanin/GalR2 agonists may have future therapeutic implications for MS.

EAE | GalR2 | Glia | multiple sclerosis

The neuropeptide galanin (1) is widely, but by no means ubiquitously, expressed in the adult brain (2–5), and following injury there is a dramatic increase in the levels of the peptide in many neuronal subpopulations (6–8) and in the basal forebrain of patients with Alzheimer's disease (9, 10). In addition to the neuronal expression of galanin, a small number of reports have shown that the neuropeptide is also expressed by oligodendrocyte or microglial cells following cortical spreading depression (11), transient forebrain ischaemia (12) or colchicine treatment (13, 14). Of note, the adult Gal-OE mice used in this study have a marked increase in neuronal galanin expression that is predominantly in the terminal fields of the hippocampus (15). Before the current study there was no evidence that galanin was expressed in non-neuronal cells in these mice.

We have previously shown that kainic acid-induced cell death in the CA1 and CA3 regions of the hippocampus is markedly reduced in Gal-OE mice compared to WT controls (15). Similarly, in vitro treatment with staurosporine or glutamate induced significantly less cell death in hippocampal cultures from Gal-OE animals than in WT controls (15). In contrast, Gal-KO mice demonstrated more hippocampal cell death following in vivo and in vitro neuronal damage than WT controls (15). Furthermore the addition of exogenous galanin peptide or the GalR2/3-specific agonist Gal2–11 reduced the amount of cell death when co-administered with either glutamate or staurosporine in WT primary hippocampal cultures (15). We have recently extended these findings by demonstrating that organotypic hippocampal cultures from mice with a loss-of-function mutation in GalR2 (GalR2-MUT) treated with glutamate show more cell death than WT controls and this cannot be rescued with the addition of either exogenous galanin or Gal2–11 (16). In support of these findings, studies by Pirondi and colleagues have shown that glutamate induced upregulation of *c-fos* can be reversed using galanin or Gal2–11 (17). Similarly, in vivo studies using an adeno-associated virus expressing the coding sequence for the galanin peptide reduced kainic acid-induced hippocampal cell death in rats (18), whilst addition of galanin or Gal2–11 in vitro protected basal forebrain cultures from A-beta-induced neurotoxicity (19). In summary, these results demonstrate that galanin plays a neuroprotective role in a variety of injury paradigms, and its effects are principally mediated via activation of GalR2. We therefore hypothesized that galanin expression might also be upregulated in the brains of patients with MS and the neuropeptide might play an analogous neuroprotective role in the mouse EAE model of MS.

Results

We first studied post mortem brain tissue from chronic MS sufferers and found that galanin was markedly upregulated in 25–45% of cells in MS lesions including shadow plaques (Fig. 1 *A*, *B* and *E*). Little or no detectable galanin staining was observed in normal appearing white matter (NAWM) from patients with MS, and none in post mortem brain tissue from normal individuals (Fig. 1*E*). Galanin-positive cells had a ramified morphology suggestive of microglia (Fig. 1 *A* and *B*), confirmed by double immunofluorescence staining with CD45, although not all microglia were galanin-positive (Fig. 1 *C* and *D*). In contrast, none of the galanin-positive cells in MS lesions expressed neuronal, astrocyte, or oligodendrocyte markers. Galanin was also upregulated in the CNS of mice with acute EAE, although the neuropeptide was solely expressed in oligodendrocytes with no detectable expression in neurons or microglia (Fig. 2).

We then asked whether the observed increase in galanin in inflammatory demyelination might modulate disease activity, by studying the development of EAE in a panel of previously described and complementary galanin transgenic lines. Gal-OE mice (20), which fully recapitulate the endogenous expression pattern of galanin in the intact animal and inducibly overexpress the peptide following axotomy (21), were compared with lines carrying loss-of-function mutations in the galanin peptide (22) (Gal-KO) and GalR2 (23) (GalR2-MUT). Gal-OE (line OE2) mice (20) were completely resistant to the development of any clinical disease in the EAE model, compared to strain-, age-, and

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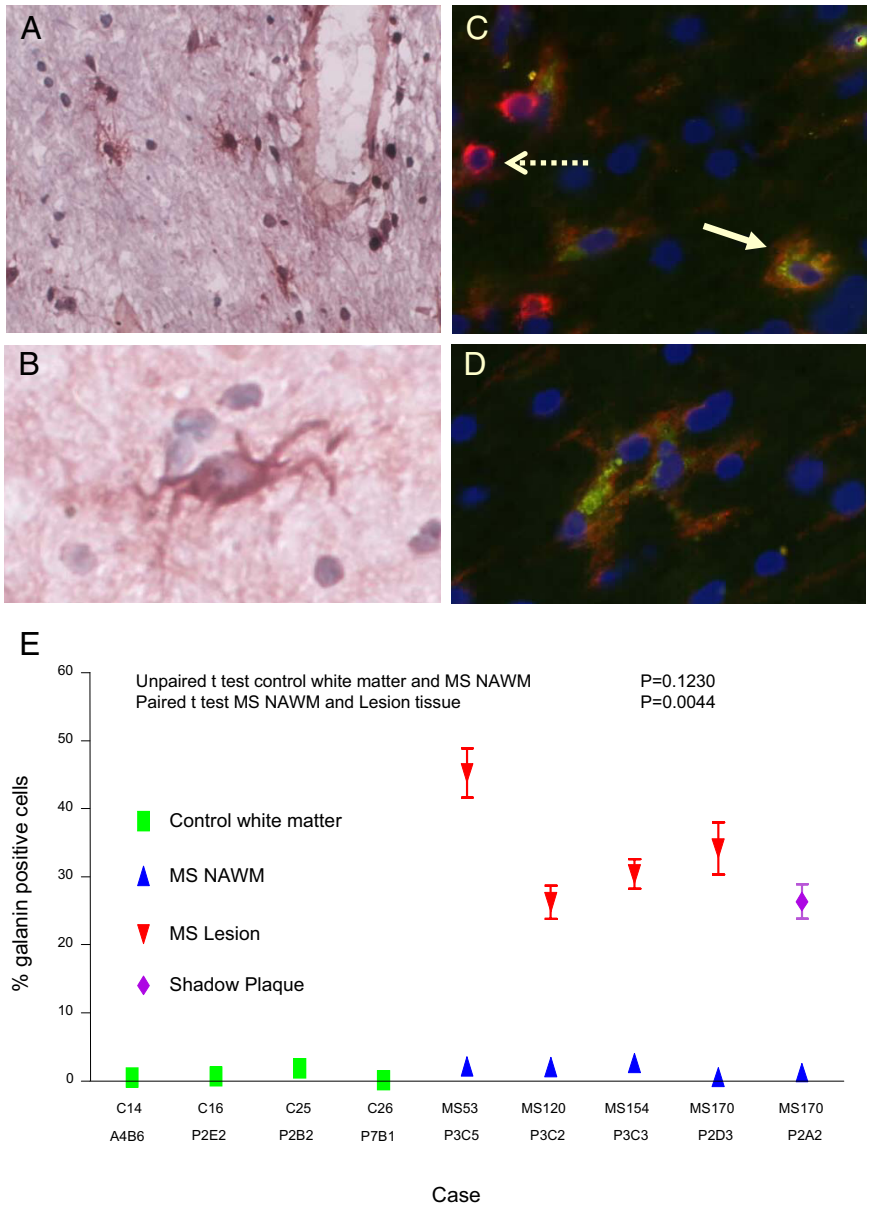

Fig. 1. Galanin immunostaining (DAB) of (*A*) an MS lesion within the parietal lobe white matter, revealing numerous galanin-positive cells, and (*B*) high power view of a galanin-positive cell in a shadow plaque. In both cases, the ramified morphology of the galanin-positive cells is suggestive of microglia. (*C*) Double immunofluorescence staining in an MS lesion confirms that the galanin-positive cells (AlexaFluor 488 nm; green) colocalise with the microglial marker CD45 (AlexaFluor 546 nm; red, solid arrow). Of note, not all microglia in MS lesions are galanin-positive (broken arrow). (*D*) High power image of a galanin and CD45 double-positive cell, with Hoechst nuclear staining (blue). (*E*) Quantification of galanin-expressing cells in cortical MS lesions, shadow plaques, NAWM and non-MS control tissue, demonstrating 30–50% of MS lesions and shadow plaques are positive for galanin, whilst in contrast <2% of control white matter or NAWM express galanin. Cases C16, C25, MS53, and MS170 were male, and C14, C26, MS120, and MS154 were female.

sex-matched WT controls, with cumulative total EAE scores (equivalent to the area under the curve (AUC) of the mean EAE clinical score against time) of 0.1 ± 0.1 vs. 26.1 ± 1.9, $P < 0.001$ (Fig. 3*A*). Identical findings were observed in line OE46, which was generated from the same transgene (20). In contrast, Gal-KO mice developed clinical disease earlier than strain-matched WT controls (time to peak EAE clinical score 14 ± 0.6 vs. 20 ± 0.8 days respectively, $P < 0.01$, Fig. 3*B*), whilst the GalR2-MUT mice developed more severe disease (AUC 28.6 ± 1.7 vs. 18.6 ± 1.3, $P < 0.05$) and at an earlier time point (time to peak EAE clinical score 20 ± 0.7 vs. 24 ± 0.9 days respectively, $P < 0.05$) than strain-matched WT animals (Fig. 3*C*). At the end of the 28-day study period, spinal cords were harvested from each animal and scored pathologically. The results paralleled the clinical scores with no observable disease in the Gal-OE group, and the highest score in the GalR2-MUT mice (Figs. 3*D* and 4).

Discussion

Our data demonstrate that galanin is an important component of the response to central demyelination. Importantly, our analysis of mice with experimental autoimmune encephalomyelitis, the most commonly studied animal model of central demyelination, provides compelling evidence that galanin exerts an important functional role to limit disease activity in this context: overexpression of galanin abrogates the development and severity of disease, whereas loss-of-function mutations in the galanin or GalR2 genes, results in its exacerbation.

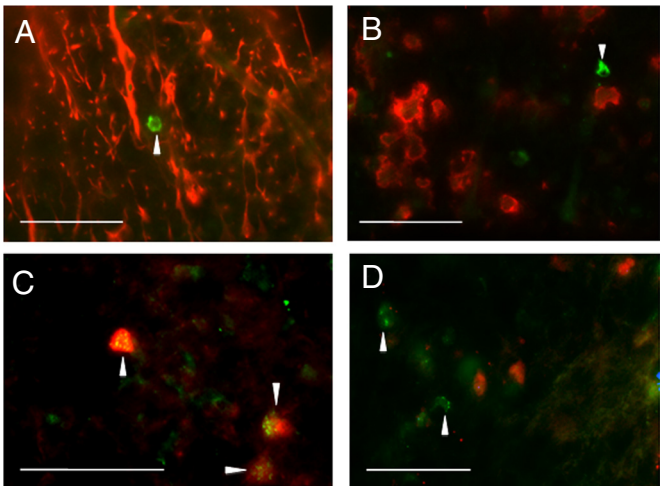

Fig. 2. Galanin expression in the mouse spinal cord after EAE. Galanin-expressing cells (green) are indicated by arrowheads in all images. Galanin staining is not observed in (*A*) GFAP-positive astrocytes (red) or (*B*) CD11b-positive microglia (red). (*C*) Many PLPdsRED-positive oligodendrocytes express galanin, but (*D*) not in naive healthy (no EAE induction) spinal cord from PLPdsRED transgenic mice. (Scale bars, 50 μm.)

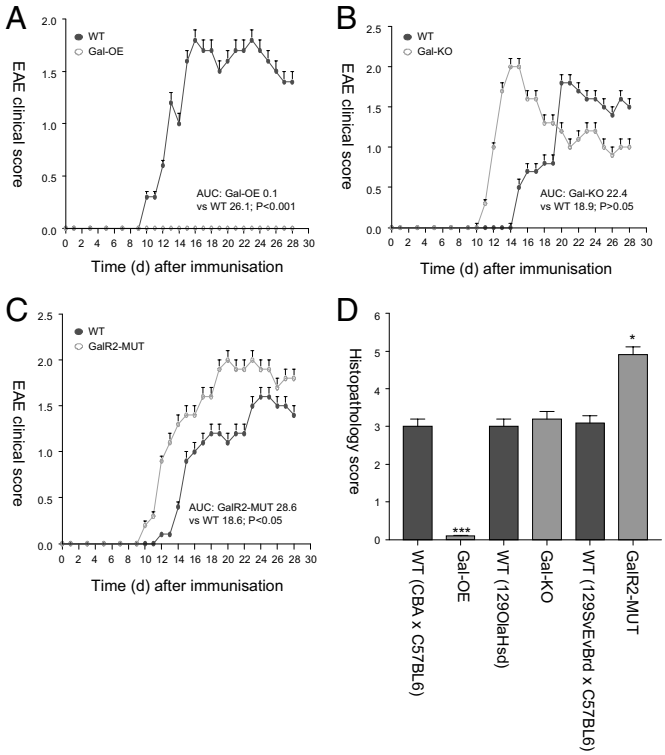

Fig. 3. (*A*) EAE clinical scores for groups of WT (CBA × C57BL6, $n = 10$) and Gal-OE ($n = 10$) mice after immunization with $MOG_{35–55}$, demonstrating an absence of disease in the Gal-OE mice, associated with a significant reduction in AUC. (*B*) EAE clinical scores for groups of WT (129OlaHsd, $n = 10$) and Gal-KO ($n = 10$) mice after immunization with $MOG_{35–55}$, demonstrating a significant reduction to the time of peak disease in the Gal-KO mice. (*C*) EAE clinical scores for groups of WT (129SvEvBrd × C57BL6, $n = 10$) and GalR2-MUT ($n = 10$) mice after immunization with $MOG_{35–55}$, demonstrating a significant reduction in the time of peak disease and a significant increase in disease activity in the GalR2-MUT mice, assessed by AUC. (*D*) Combined inflammation and demyelination scores for the spinal cord for each genotype and strain-matched WT animals 28 days after the induction of EAE, demonstrating an almost complete absence of disease in the Gal-OE mice and a significant increase in the GalR2-MUT mice. Data are presented as mean ± SEM and statistical significance is denoted by $*P < 0.05$, $***P < 0.001$.

Our data also indicate that galanin expression is markedly upregulated in both the brains of patients with MS and mice with EAE. Importantly, it is now the consensus view that the clinical severity of inflammatory demyelination, in both mouse and man, is likely to be a consequence of the degree of axonal degeneration (24–26). It is therefore of considerable interest that our prior work indicates that galanin exerts neuroprotective effects after injury (15) and that these activities have been shown to be principally mediated via neuronally expressed GalR2 (16). In this study, we have also identified that in the context of central demyelination in patients with MS, galanin was expressed not by neurons, as had been identified in other contexts such as kainate-induced neuronal injury, but by microglia, probably reflecting the predominant white matter focus of pathology in this instance (27). We did consider the possibilities that galanin expression in MS lesions was a consequence of microglial phagocytosis of galanin-positive cells (e.g., neurons or oligodendrocytes) and/or that expressing cells were infiltrating monocytes/macrophages. However, we did not observe significant galanin expression in other local (neuronal) cells, which makes this possibility less likely. Equally importantly, the galanin-expressing cells often had a ramified, process-bearing morphology, which is very different from the spheroid morphology either of microglia engaged in phagocytosis or of infiltrating monocytes/macrophages.

It might initially appear that the expression profiles of galanin within human MS tissue and in EAE are incongruous, given that we have shown that the neuropeptide is expressed by microglia in the human disease but by oligodendrocytes in mice. It should be noted, however, that by necessity, these analyses have been undertaken in different contexts: chronic disease that has been extant for many years in man and acute disease in the mouse. Oligodendrocytopathy is now recognized as an early key component of the central demyelination that occurs in MS (28). It is therefore possible that the oligodendrocyte specific expression profile seen in acute EAE is a reflection of this targeting, such that the production of galanin is a direct response to acute oligodendrocyte injury. It will therefore be important in future studies to undertake analyses of hyperacute MS tissue to determine whether the galanin expression profile mirrors that seen in acute EAE. On the other hand, it has also been suggested that in chronic MS there is a fundamental shift in the balance of immune activation, such that the focus shifts from a T cell to an innate immune response (29). Our data would suggest that part of this innate response involves the production of galanin by microglia, which might ultimately serve to reduce the extent of secondary axonal injury. It is of note that previous work in rodents has also provided some evidence for expression of galanin in microglia in various paradigms including transient forebrain ischaemia (12) and colchicine treatment (13).

The physiological actions of galanin are mediated by three G protein coupled galanin receptor subtypes, designated GalR1, GalR2, and GalR3. All three subtypes are expressed by differing neuronal populations in the brain [reviewed in (30, 31)], and in addition Su and colleagues have shown that GalR2 (but not GalR1 or GalR3) is expressed by rat primary microglia and the murine BV2 and rat HAPI glial clonal cell lines (32). Consistent with a neuroprotective role, GalR2 (unlike GalR1 or GalR3) is known to signal via $G_{q/11}$ to activate phospholipase C and protein kinase C (33, 34) and hence ERK (16). Previous studies have also shown that hippocampal neuroprotection is dependent in part upon activation of ERK (35, 36) and ERK KO mice have increased susceptibility to EAE (37). Our data do not exclude the

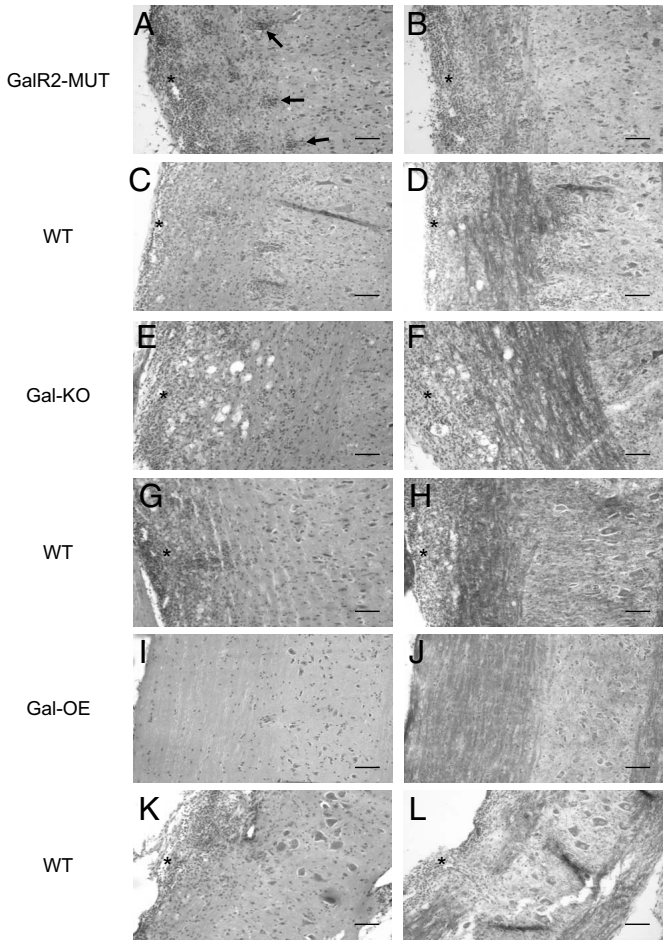

Fig. 4. Representative histopathological changes in the spinal cord of mice from each of the experimental groups described in Fig. 3, harvested at day 28. Serial longitudinal sections of spinal cord are stained by haematoxylin and eosin (*A*, *C*, *E*, *G*, *I*, *K*) and luxol fast blue (*B*, *D*, *F*, *H*, *J*, *L*). There is an increase is the severity of disease in the GalR2-MUT mice (*A* and *B*) compared to strain-matched WT mice (*C* and *D*). The pathology is similar in severity and nature in Gal-KO (*E* and *F*) compared to strain-matched WT mice (*G* and *H*). In each case there is a plaque of granulomatous inflammation over the peripheral spinal cord white matter which focally extends into the underlying parenchyma (marked with an asterisk). The areas of inflammatory change show complete demyelination. Distinct perivascular cuffs of mononuclear inflammatory cells are located deeper in the white matter parenchyma of GalR2-MUT mice (*A*, marked by arrows). By contrast, the spinal cord from Gal-OE mice (*I* and *J*) is histologically normal with no evidence of inflammation or demyelination, compared to strain-matched WT mice (K and *L*). (Scale bar, 200 μm).

possibility that galanin might also exert immunomodulatory effects.

Irrespective of the mechanism(s) by which galanin alters EAE disease activity, our findings imply that galanin/GalR2 agonists could have future therapeutic implications for MS. At present, this hypothesis cannot be directly tested since the existing GalR2/3 peptide agonist Gal2–11 would be expected to have a half-life in vivo of $<$10 min [similar to the full length galanin peptide (38, 39)], precluding its systemic administration. Once GalR2-specific drug-like agonists are identified and characterized then preclinical proof-of-concept studies using the EAE model will be undertaken.

Materials and Methods

Animals. All animals were fed standard chow and water ad libitum. Animal care and procedures were performed within the United Kingdom Home Office protocols and guidelines.

Galanin Over Expressing (Gal-OE) Mice. Details of the strain and breeding history have been previously described (20, 21). In brief, galanin over expressing mice, bred to homozygosity, were generated using a ≈25-kb transgene containing the entire murine galanin coding region and 19.9kb of upstream sequence. The transgene was excised by restriction digest and microinjected into fertilised oocytes. Two transgenic lines, denoted OE2 and OE46, were then bred and characterized, see Bacon et al. for further details (20). Both transgenic lines have remained inbred on the CBA $\times$ C57BL6 F1 hybrid background. The majority of the studies present here used OE2 mice, although for confirmatory purposes (and to exclude the possibility that the transgene insertion site is in an MHC locus) identical experiments were also undertaken in the OE46 line. Strain-, age-, and sex-matched WT mice were used as controls in all experiments.

Galanin Knockout (Gal-KO) Mice. Details of the strain and breeding history have been previously described (22). In brief, mice homozygous for a targeted mutation in the galanin gene were generated using the E14 cell line. A PGK-Neo cassette in reverse orientation was used to replace exons 1–5, and the mutation was bred to homozygosity and has remained inbred on the 129OlaHsd strain. Strain-, age-, and sex-matched WT mice were used as controls in all experiments.

GalR2 Mutant (GalR2-MUT) Mice. Details of the strain and breeding history have been previously described (23). In brief, mice deficient for the *GalR2* gene were generated and licensed from Lexicon Genetics. The 5.17-kb gene-trap vector VICTR48 (VIral Construct for TRapping) was inserted within the single intron of the murine GalR2 gene in a 129Sv/EvBrd ES cell line clone (40). Omnibank clone OST105469 was used to obtain germ-line transmission of the disrupted *GalR2* allele. Heterozygote pairs on the 129SvEvBrd $\times$ C57BL6 background were transferred to the University of Bristol and then bred to homozygosity and have been maintained on that background. Strain-, age-, and sex-matched WT mice were used as controls in all experiments.

EAE. EAE was induced with $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) obtained from GLBiochem as previously described (41). Mice (8–10 weeks old) were primed s.c. at the base of tail with 100 μg of MOG in 0.1 mL of an emulsion containing an equal volume of CFA and PBS containing heat-killed *Mycobacterium tuberculosis*, H37 Ra (Difco) at 4 mg/mL. On days 0 and 2 postimmunization, 200 ng of pertussis toxin (Sigma) was administered i.p. in 0.5 mL PBS. Mice were monitored daily for disease and EAE grades were scored as follows: (1) flaccid tail, (2) partial hind-limb paralysis and impaired righting reflex, (3) total hind-limb paralysis, (4) fore- and hind-limb paralysis, and (5) moribund. Any animal judged grade 4 or more after 24 h was culled immediately.

Histopathology Scores. A combined inflammation and demyelination score was generated as previously described (42), using paraformaldehyde fixed spinal cord stained with haematoxylin and eosin for the inflammatory scoring (0: no inflammatory cells; 1: a few scattered inflammatory cells; 2: organization of inflammatory infiltrates around blood vessels; 3: extensive perivascular cuffing with extension into adjacent parenchyma, or parenchymal infiltration without obvious cuffing) and luxol fast blue/cresyl violet staining for demyelination scoring (0: no demyelination; 1: a few, scattered naked axons; 2: small groups of naked axons; 3: large groups of naked axons; 4: confluent foci of demyelination) as originally described by Racke et al. (43). The two scores were generated by a trained, board-certified histopathologist blind to the genotype of the mice (n = 10), and then summed.

Human Tissue Samples. Five postmortem cerebrum samples from four patients with clinically definite MS were obtained from the Multiple Sclerosis Society Tissue Bank. The mean age of the patients (two male, two female) was 57 years; range 35 to 72. Lesions were classified by Dr Federico Roncaroli (neuropathologist at the MS Tissue Bank). Four of the cerebrum samples contained lesions and the remaining samples contained shadow plaques; all samples contained areas of normal appearing white matter (NAWM). Four brain samples from four control cases (mean age 67 years, range 35–92 years; two male, two female) were also studied. Causes of death were cardiac failure (2), tongue carcinoma, and myeloid leukaemia.

Immunohistochemistry

Human. Cryostat sections (7 μm) were cut from all nine samples (MS and control) and then processed for immunohistochemistry as previously described (44). In brief, sections were left to air dry for 48 h and then stored at

minus 80 °C until required. Immunoperoxidase staining for galanin was carried out on three sections from each sample on separate occasions. Sections incubated without primary antibody or with a rabbit IgG isotype control antibody acted as negative controls. After fixing in acetone, sections were washed in PBS (PBS) and permeabilized in methanol at −20 °C for 10 min. Sections were then immersed in 6% hydrogen peroxide/methanol for 30 min to reduce autologous staining and washed in running water for 10 min. The sections were blocked for 20 min with normal blocking serum (Vector universal elite ABC Kit, Vector Laboratories) and incubated with the primary antibody overnight. A previously validated rabbit polyclonal anti-galanin antibody (21) (Affiniti Research Products Ltd Biomol) was used at a dilution of 1:800 in PBS. The following day, sections were washed in PBS and incubated with biotinylated secondary antibody solution (Vector universal elite ABC Kit) for 20 min, then washed in PBS and incubated with ABC reagent (Vector universal elite ABC Kit) for 20 min. The sections were washed again before being incubated with diaminobenzidine (DAB) solution for 10 min. The DAB solution was made up in 5 mL deionized water using reagents from the DAB substrate kit, purchased from Vector Laboratories (100 μL Solution Buffer, 200 μL DAB reagent, 100 μL H_2O_2). Sections were washed in cold running water for 3–5 min, treated with Cupric Copper Sulfate DAB Enhancer for 4 min (2 g $CuSO_4.5H_2O$/3.6 g NaCl/500 mL deionized water) to enhance staining and then washed in cold running water for 5 min. Sections were counterstained with Harris' Haematoxylin (BDH) for 30 s under cold running water for 3 min. Sections were dehydrated, cleared with xylene, and mounted in DPX.

For immunofluorescence double labeling, sections were washed in PBS and permeabilized in methanol at −20 °C for 10 min after fixation in acetone. Sections were then washed in PBS and incubated for 1 h with 10% normal goat serum (NGS)/PBS to block nonspecific binding. Primary antibodies were then added to the sections and incubated at 4 °C overnight. Primary antibodies including mouse anti-CD45 at 1 in 50 (Dako UK Ltd), mouse anti-βIII microtubulin at 1 in 50 (Cymbus Biosciences), and mouse anti-GFAP at 1 in 400 (Dako UK Ltd) and were diluted in a solution of rabbit polyclonal anti-galanin at 1 in 800 (see above) in 10% NGS. After incubation with primary antibody, sections were washed in PBS and incubated with secondary antibodies for 45 min in the dark. Secondary antibodies were goat anti-rabbit IgG conjugated to Alexa Fluor 488 nm and goat anti-mouse IgG Alexa Fluor 546 nm (both from Cambridge Bioscience; diluted 1:2,000 in 10% NGS). Sections were then washed in PBS and incubated with 10% NGS for 5 min. Nuclei were counterstained with 1 μg/mL Hoechst 33342 for 3 min. Sections had a final wash in PBS and were then mounted in Vectashield aqueous mountant (Vector Laboratories).

Galanin-stained tissue sections were viewed by light microscopy at ×400 magnification. In control or NAWM samples three fields were selected at random from each slide. For samples containing lesion tissue or shadow plaques, three fields within the lesioned or remyelinated area were selected. A total cell count and a count of galanin positive cells were taken for each field. To account for variation in cell number between individual patients, galanin immunoreactive cell numbers were calculated as a percentage of the total cell count.

Mouse. EAE (as described above) was induced in 8-week-old male C57Bl6 mice and the previously described transgenic PLP-dsRED mice maintained on the C57Bl6 background (45), in which expression of the red fluorescent coral protein dsRed is driven by the promoter region of the murine proteolipid protein (PLP) gene. At day 18 postinduction, mice were killed using sodium pentobarbitone injection and perfused with 20 mL PBS followed by 4% paraformaldehyde in PBS. Spinal cords were dissected and postfixed in 4% paraformaldehyde for 1 h at room temperature, then cryoprotected in 20% sucrose/PBS overnight at 4 °C and frozen, and 10-μm sections were cut and collected on slides (Superfrost plus). Sections were blocked and permeabilised in 10% normal goat serum in PBS/0.3% Triton X-100 (blocking solution) for 1 h at room temperature then incubated in rabbit polyclonal antibody to galanin (Santa Cruz H-80 1:100 dilution in blocking solution) overnight at 4 °C. They were also simultaneously incubated with either mouse monoclonal anti-GFAP Ab (1/500, Chemicon) or rat anti-mouse monoclonal antiCD11b-Ab (1/500, BD PharMingen, 553308). Sections were washed thrice in PBS and then incubated with secondary Ab at 1/500 (Goat anti-rabbit FITC, Goat anti-mouse TRITC, Goat anti-rat TRITC, from Jackson Laboratories) at room temperature for 1 h, washed thrice in PBS, and coverslipped using Moviol. Photographs were taken on a Zeiss Axioplan 2 fluorescent microscope and images imported into Photoshop (Adobe Systems) for sizing.

Statistics. Data are presented as means ± SEM. Data were analyzed as appropriate by Student's *t* test or by one-way analysis of variance (ANOVA) using GraphPad Prism version 4. A P value of <0.05 was taken to be significant.

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