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Inhibition of CXCR2 signaling promotes recovery in models of Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a neurodegenerative disease characterized by demyelination/remyelination episodes that ultimately fail. Chemokines and their receptors have been implicated in both myelination and remyelination failure. Chemokines regulate migration, proliferation and differentiation of immune and neural cells during development and pathology. Previous studies have demonstrated that the absence of the chemokine receptor CXCR2 results in both disruption of early oligodendrocyte development and long term structural alterations in myelination. Histological studies suggest CXCL1, the primary ligand for CXCR2, is upregulated around the peripheral areas of demyelination suggesting this receptor/ligand combination modulates responses to injury. Here we show that in focal LPC induced demyelinating lesions, localized inhibition of CXCR2 signaling reduced lesion size and enhanced remyelination while systemic treatments were relatively less effective. Treatment of spinal cord cultures with CXCR2 antagonists reduced CXCL1 induced A2B5 + cell proliferation and increased differentiation of myelin producing cells. More critically, treatment of myelin oligodendrocyte glycoprotein peptide 35-55-induced EAE mice, an animal model of multiple sclerosis, with small molecule antagonists against CXCR2 results in increased functionality, decreased lesion load, and enhanced remyelination. Our findings demonstrate the importance of antagonizing CXCR2 in enhancing myelin repair by reducing lesion load and functionality in models of multiple sclerosis and thus provide a therapeutic target for demyelinating diseases.

Keywords

oligodendrocyte; myelin repair; spinal cord; Multiple Sclerosis; remyelination; neuroinflammation

INTRODUCTION

Multiple sclerosis (MS) is a devastating heterogeneous inflammatory demyelinating disorder of the central nervous system (CNS) affecting young adults (Davis, 1970; Antel, 1999; Compston and Coles, 2002; Benedict and Bobholz, 2007). Hallmarks of the disease include recurrent demyelinating episodes that result in the progression of neurological deficits due to the slowing and ultimately failure of axonal conduction (Davis, 1970; Waxman, 1977; Blakemore et al., 2000; Baumann and Pham-Dinh, 2001; Nashmi and Fehlings, 2001; Bjartmar

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et al., 2003). Remyelination frequently occurs during early stages of disease, however this fails during later disease stages and the number of persistent demyelinating lesions increases (Chari, 2007).

The majority of current therapies for MS target the inflammatory response that is distinctive of the disease (Miller et al., 1998; Hohol et al., 1999; Miller et al., 1999; McFarland and Martin, 2007; Trapp and Nave, 2008). Although active inflammation predominates in the initial stages of the disease, the neurological disabilities associated with the chronic disease accumulates independent of inflammatory mediators (McFarland and Martin, 2007; Frohman et al., 2008) and long-term recovery requires myelin repair. Models of MS include the experimental autoimmune encephalomyelitis (EAE) (Cross et al., 1991), where widespread demyelination is largely a consequence of immunological attack on resident CNS myelin following immunization by specific myelin peptides. Focal demyelinated lesions can be induced by direct injection of lysolecithin (LPC) (Ousman and David, 2000; Bambakidis et al., 2003; Mi et al., 2005) where demyelination is largely a consequence of chemical dissolution of myelin sheaths. Finally, cuprizone intoxication (Zatta et al., 2005) results in demyelination largely as a consequence of metabolic perturbations. In the present study, we utilize lysolecithin lesions and MOG_{35-55} peptide induced EAE to assess the efficacy of inhibiting the CXCR2 chemokine receptor in mediating demyelination and remyelination.

Chemokines, or chemoattractant cytokines, comprise a family of inducible secreted molecules of small molecular weight (8-10kDa) (Hesselgesser and Horuk, 1999; Fernandez and Lolis, 2002; Laing and Secombes, 2004), that function as activators of leukocytes, wound healing, modulators of angiogenesis, and tumorigenesis (Robinson et al., 2001; Smith et al., 2005; Charo and Ransohoff, 2006). The functions of chemokines have been primarily implicated in modulation of immune coordination and inflammation (Fernandez and Lolis, 2002; Flynn et al., 2003; Laing and Secombes, 2004; Charo and Ransohoff, 2006) where they contribute to the localization of specific cells with fine spatio-temporal precision (Charo and Ransohoff, 2006). Chemokine receptors are G-protein coupled receptors (GPCRs) usually linked to pertussis toxin sensitive Gi proteins (Bajetto et al., 2001). The receptor CXCR2 is the primary mediator of signaling by the chemokine CXCL1 (Katancik et al., 2000), a soluble secreted chemoattractive molecule of the ELR family of CXC chemokines. Their interaction initiates intracellular processes that modulate a number of cellular events, including migration, proliferation, and differentiation (Bajetto et al., 2001; Miller, 2002; Tsai et al., 2002). Several studies have characterized the expression and function of chemokine receptors on neural cells in the vertebrate CNS. For example, the CXCR4 receptor and its cognate ligand SDF1 have been implicated in the control of neuronal precursors in the hippocampus and cortex (Imitola et al., 2004). During oligodendrocyte development, signaling through CXCR2 in combination with platelet derived growth factor AA promotes oligodendrocyte precursor proliferation (Robinson et al., 1998; Robinson et al., 2001; Woodruff et al., 2004). Furthermore, the CXCR2 signaling pathway contributes to the correct localization of oligodendrocytes in white matter tracts of the spinal cord (Miller, 2002; Tsai et al., 2002; Padovani-Claudio et al., 2006).

Elevated levels of CXC chemokine expression are associated with a range of pathological CNS insults including infection, tumors, ischemia and demyelination (Liu et al., 1993; Spanaus et al., 1997; Filipovic et al., 2003; Keane et al., 2004; Sue et al., 2004; Omari et al., 2006; Valles et al., 2006) and this may reflect ingress of immune cells in response to local expression. For example, overexpression of CXCL1 in oligodendrocytes induces neutrophil invasion and astrogliosis (Tani et al., 1996; Tanabe et al., 1997). Overexpression of CXCL1 in astrocytes resulted in a milder course of EAE disease pathology (Omari et al., 2009) and upregulation of CXCL1 has been reported at the periphery of MS lesions coincident with the accumulation of OPCs (Omari et al., 2005). Furthermore, proliferation of OPCs has been determined in organotypic human fetal slice cultures to be dependent on CXCL1/CXCR2 signaling (Filipovic

and Zecevic, 2008). By contrast, CXCR2 null animals have been reported to have increased NG2+ cells that may facilitate recovery of demyelination (Padovani-Claudio et al., 2006). Here we demonstrate that in a chemical demyelination model (LPC), and an immune mediated demyelination model (MOG_{35-55} peptide induced EAE), blocking CXCR2 enhances recovery and remyelination in spinal cords of affected animals suggesting inhibition of CXCR2 may represent a potential target for myelin repair.

MATERIALS AND METHODS

Animals and Induction of EAE

All animal procedures were conducted according to approved guidelines established by the National Institutes of Health Animal Protection Guidelines and were approved by Institutional Animal Care and Utilization Committee of Case Western Reserve University School of Medicine

The chronic EAE model was induced in C57BL/6 mice using synthetic myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}, 200ul at 200μg/animal). Mice were injected subcutaneously with an emulsion of MOG_{35-55} peptide mixed with complete Freud's adjuvant with 500μg *Mycobacterium tuberculosis* followed by two intraperitoneal (IP) injections of 500μg pertussis toxin, one immediately after immunization and a second 24hrs later (Bai et al., 2009). Clinical scores were obtained on a 5 point scoring system in which a score of 0 equates to no clinical symptoms ; 1, limp tail; 2, paralysis of one limb; 3, paralysis of two hind limbs; 4, paralysis of front limbs; 5, death as previously described (Bai et al., 2009). Treatment of animals with either CXCR2 antagonist (Tocris; 20ng/kg) or vehicle was begun when the animals showed the initial signs of disease. In general, this occured 10-14 days post immunization. Animals received IP injections daily for the remainder of the study or two weeks post disease induction.

Lysolecithin induced demyelination and delivery of antagonists

Twelve week old female Sprague-Dawley rats (220-240 grams) were anesthetized with ketamine hydrochloride, xylazine hydrochloride, and acepromazine. Following a laminectomy at thoracic vertebrae level 10, three microliters of 1 percent LPC (L-α-lysophosphatidyl-choline, lysolecithin) (Sigma, St. Louis, MO) in 0.9 % sodium chloride solution were microinjected using a pulled glass pipette into the dorsal column of the spinal cord at a rate of 0.25 μl/min . Post-operatively, animals received a subcutaneous injection of 5ml of saline to promote hydration. For double injection of either CXCR2 neutralizing antibody (R&D systems, 100μg/ml) or CXCR2 antagonist (Tocris, 100μg/ml) or appropriate vehicle controls, animals were anesthetized 2 days post lesion and injected with either 3μl CXCR2 antibody or 3μl CXCR2 antagonist, using the same paradigm as above. Systemic delivery of CXCR2 antagonist (Tocris, 20ng/kg) was performed IP on the day of surgery and everyday thereafter. Animals were then allowed to recover for 10 days prior to sacrifice. Control animals received an equivalent injection of either isotype control antibody or vehicle.

Primary spinal cord cultures

Mixed cell cultures were prepared from postnatal day 3 rat spinal cords and plated on poly-L-lysine (PLL) coated coverslips. The media was changed the following day and cells allowed to grow for 3 days. Cells were grown in media consisting of DMEM, 10ng/ml platelet derived growth factor AA (PDGFAA), 10ng/ml fibroblast growth factor (bFGF), 1% FBS, and N2 supplement. Cells were treated with small molecule inhibitor against CXCR2 at various concentrations (40ng/ml, 80ng/ml, 160ng/ml) and/or the ligand CXCL1 (0.5 ng/ml) overnight and the effect on OPC development assessed.

Immunocytochemistry of primary cell cultures

Cells were fixed in 4% paraformaldehyde and incubated in primary antibody for 30 minutes in PBST (0.03% triton) (MBP: SMI99, Sternberger Monoclonals, 1:500) followed by corresponding secondary antibodies and mounted using Vectashield with DAPI (Vector Laboratories Burlingame, CA). Labeling with O4, A2B5 and O1 was performed on live cells. Cells were post-fixed using 5% acetic acid in methanol. To analyze proliferation of cells in S phase, bromodeoxyuridine (BrdU) (10μM) was added to the media at least 18 hours prior to fixation. Images were collected using a Leica DM5000B microscope and Leica Applications Suite Software. The proportion of each different cell type relative to the total number of DAPI positive cells were counted by an observer blinded to the treatment from 6 randomly selected fields taken from at least 2 different coverslips from 4 separate preparations. The data were pooled and presented at mean +/− standard deviation.

Immunohistochemistry and immunofluorescence

Animals were perfused with 4% PFA in saline. Transverse frozen sections of spinal cord were dried on slides and stored at −80°C. Sections were rinsed blocked in 0.03% PBST and 5% NGS and incubated in primary antibody overnight at 4°C (GFAP: Dako 1:500; Iba1: Wako 1:250; MBP: SMI99, Sternberger Monoclonals 1:500, Iba1: Dako, 1:100, ED1: Santa Cruz, 1:100). Sections were rinsed and incubated in anti-rabbit IgG or anti-mouse IgG fluorescently conjugated secondary antibodies (Sigma, 1:500) for 1 hour at room temperature followed by 10 minutes in DAPI (1:1000; Invitrogen) to label nuclei. The sections were briefly rinsed and mounted using Citifluor mounting media (Ted Pella, Redding PA). Images were collected using a Leica DM5000B microscope and Leica Applications Suite software. The number of Iba1 positive cells were counted in defined regions of spinal cord grey and white matter to determine relative amount of microglia and macrophages in response to CXCR2 treatment in EAE animals. The mean cell number was determined from at least 4 different sections taken from 2 different animals. Data was pooled and presented as mean+/− standard deviation.

Electron Microscopy

Animals were anesthetized and perfused initially with PBS followed by 3% formaldehyde/3% gluteraldehyde in 0.1M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA). Tissue was dissected and 300μm thick vibratome sections post fixed in 1% OsO4. Samples from EAE animals were taken from lumbar segments of the spinal cord. Sections from lysolecithin (LPC) animals were selected from midlesion as indicated by luxol fast blue staining as previously described (Mi et al., 2005). Sections were dehydrated through a series of ethanol dilutions, stained using uranyl acetate and embedded in a Poly/Bed812 resin (Polysciences Inc., Warrington, PA). Thin (1μm) sections were stained with toluidine blue and appropriate areas for ultrathin sectioning were selected. Ultrathin (0.1μm) from matching areas of experimental and control tissue blocks were cut and visualized using an electron microscope (JEOL1200CX) at 80kV. G ratios were calculated by dividing the axonal area and myelin thickness for randomly selected myelinated axons. Three separate measurements of radial thickness of myelin were taken for each axon and averaged to provide mean myelin thickness.

Statistical analyses

Data was analyzed using t-test assuming equal variances in Microsoft Excel software. Statistical significance was set to $p<0.05$. Values are described in the text as mean +/− standard deviation followed by the p-value, and plotted either as mean +/− standard deviation or mean +/− two standard errors of the mean.

RESULTS

Local delivery of CXCR2 blocking reagents reduce the size of LPC-induced lesions

To determine whether CXCR2 antagonists alter the rate of recovery in models of demyelination and remyelination, the effect of local injection of neutralizing anti-CXCR2 antibody on lesion load was assayed in a lysolecithin model (Bambakidis et al., 2003; Mi et al., 2005). Lysolecithin injection induces rapid local demyelination that is mostly developed by 2 days post injection. Direct injection of a CXCR2 neutralizing antibody into a region adjacent to the lesion 2 days after LPC injection had a profound effect on lesion volume (Fig 1). Animals that received control antibodies had a lesion volume of 5 +/− 2.8mm^3 after a survival of 10 days and the lesions appeared to contain relatively little myelin (Fig 1 A,C, E, and G). By contrast, in animals that received neutralizing CXCR2 antibodies, the lesion size was reduced to 1+/− 1.3mm^3 after 10 days and only the central core was devoid of myelin (Fig 1 B, D, F, and G). Three dimensional reconstruction of the area of the lesion occupying the spinal cord revealed a reduction in rostral caudal and lateral extent of the lesion in anti-CXCR2 treated animals although the lesions still spanned the depth of the dorsal column (Fig 1 F and G) (n=5). To confirm the specificity of the anti-CXCR2 antibody effects, the studies were repeated using a single injection of a small molecule CXCR2 inhibitor. Local injection of the Tocris CXCR2 antagonist (Busch-Petersen, 2006) resulted in smaller LPC lesions and increased number of myelinated axons at 10 days post insult (Fig 2). For example, the proportion of myelinated fibers was less than 6 +/− 4% in the middle lesion of animals that received vehicle only (Fig 2 A, C and E) and there were substantial cellular infiltrates throughout the lesion while similar areas of CXCR2 antagonist treated animals (Fig 2 B, D and E) contained a significant proportion (54 +/− 7%) of remyelinated axons scattered throughout the lesion and fewer infiltrated cells. Ultrastructural studies confirmed the presence of relatively thinly myelinated axons (arrows) scattered throughout the lesion (Fig 2D). The lesions that develop in response to LPC injections are primarily a result of direct chemical demyelination rather than immune cell infiltration (Ousman and David, 2000) and these studies suggest that treatment with CXCR2 antagonists significantly accelerates myelin repair in a non-inflammatory setting through a mechanism that likely includes a direct effect on oligodendrocytes and their precursors (Robinson et al., 1998; Tsai et al., 2002).

Systemic delivery of CXCR2 antagonist does not significantly enhance recovery of LPC induced demyelination

To determine whether CXCR2 inhibition in the LPC lesions was primarily local or systemic, the CXCR2 antagonist was delivered IP directly after lysolecithin lesions (Fig 3). Injection of CXCR2 antagonist daily for 10 days after induction of a dorsal spinal cord LPC lesion had only a small effect on lesion volume (2.6mm in experimental versus 3mm in controls). Consistent with this limited effect of systemic blockade of CXCR2 on lesion size, morphological studies demonstrated only a marginal although significant effect on repair in the LPC model. In middle areas of lesions in control animals (Fig 3C) 94 +/− 2% of the axons were demyelinated (asterisks) and 6+/−3% were myelinated, similarly in animals that received CXCR2 antagonists (Fig 3D) 76+/−4% axons were demyelinated while 24 +/− 6% were remyelinated (arrows) (p=0.004)(Fig 2E, Fig 3). In all cases the total number of axons and the proportion that were affected by LPC injection were not different suggesting there is not a protective effect on lesion formation following systemic inhibition of CXCR2. Further analyses suggested no significant difference in MBP expression in the lesion or cellularity of the lesion with systemic CXCR2 inhibition (data not shown) suggesting that the primary benefit derived from direct injections reflected local inhibition of CXCR2 signaling on CNS cells.

CXCR2 antagonists decrease CXCL1 induced OPC proliferation and increases OPC differentiation in vitro

Previous studies suggest that OPCs express the CXCR2 receptor and respond directly to CXCL1 stimulation (Robinson et al., 1998; Tsai et al., 2002). To assess whether CXCR2 antagonists had direct effects on neural cells, the antagonist was added to spinal cord cultures to determine the effect on oligodendrocyte differentiation (Fig 4). Spinal cord cultures from P3 animals were grown for 3 days and treated for one day with CXCL1 (Fig 4 B,E, and H), the CXCR2 antagonist (Fig 4 C, F, and I) or control medium (Fig 4 A, D, and G). Oligodendrocyte precursors were identified through binding of mAb A2B5. In the presence of CXCL1 (0.5ng/ml), the proportion of A2B5+ cells increased dramatically due to proliferation (46% p=0.002) (Fig 4B). This increase was abolished by treatment with CXCR2 antagonist (26%) and the residual cells were weakly A2B5+ and appeared to have a larger cell body and more differentiated phenotype suggesting treatment with CXCR2 antagonist promoted OPC differentiation (Fig 4C). Cultures exposed to CXCR2 antagonist contained increased O1+cells (20 +/− 4% versus 34 +/−3%) and 50% more MBP positive cells than those in control conditions (18 +/− 1% versus 52 +/− 5% p=0.007) (Fig 4 D-I). Not only were there increased number of differentiated oligodendrocytes but their morphology was also substantially different. More of the MBP+ cells contained sheet like processes in the presence of CXCR2 antagonist than in control conditions (Fig 4I). Consistent with previous studies (Tsai et al., 2002) the presence of CXCR2 antagonists reduced the proliferation of A2B5+ cells (42 +/− 4% BrdU+/A2B5+ cells) in the presence or absence of CXCL1 (data not shown). These data indicate that the CXCR2 antagonist blocks the mitogenic effects of CXCL1 and facilitates OPC differentiation.

Functional improvement in EAE mice treated with CXCR2 antagonist

While analyses of recovery from lysolecithin (LPC) lesions has many advantages for the study of myelin repair, the immunological aspects of demyelinating disease such as MS are effectively modeled by experimental allergic encephalitis (EAE). Previous studies have implicated CXCR2 in the progression of EAE (Carlson et al., 2008) and to investigate whether systemic treatment with CXCR2 antagonists reduced disease burden, animals immunized with the MOG_{35-55} peptide (Bai et al., 2009) were treated daily with 20ng/kg CXCR2 antagonist (Tocris) while control animals received vehicle only (Fig 5). Treatments were initiated when immunized animals began to show functional deficits. On average this occurred during the second week following immunization. Animals that received vehicle rapidly developed increasing functional deficits (Fig 5A). EAE animals initially presented with flaccid tail and hindlimb weakness that progressed over a three-week period to forelimb paralysis. Some animals were severely compromised by day 24 and were subsequently sacrificed. By contrast, animals that received CXCR2 antagonists initially demonstrated functional deficits including hindlimb weakness but, with continued treatment, all animals (n=12) exhibited some functional recovery and no animals progressed to forelimb paralysis. Indeed, a subset (9/12) of animals had total functional recovery after two weeks of treatment (Fig 5A). These data suggest that even after the onset of disease antagonizing CXCR2 signaling promotes functional recovery in a chronic model of EAE.

Enhanced myelination and reduced lesion load in EAE mice treated with CXCR2 inhibitor

Histological analyses of CXCR2 antagonist and vehicle treated EAE animals supported the functional assessment. Animals treated with the CXCR2 antagonist had a reduced lesion load in the spinal cord. Luxol fast blue staining showed smaller demyelinated lesions after 14 days of treatment with CXCR2 antagonist (Fig 5C) compared to vehicle controls (Fig 5B). Although CXCR2 treated animals were not entirely free of demyelinated lesions, quantitation of relative lesion load by 3D reconstruction of the area occupied by lesions demonstrated a reduction of 39 +/− 2 % ($3.3mm^3$ vehicle versus $1.3mm^3$ CXCR2 antagonist treated animals) in the extent

of spinal cord demyelination between the EAE control and CXCR2 antagonist treated animals (Fig 5F; p=0.001). Ultrastructural analyses confirmed that following 14 days of treatment with CXCR2 antagonist, lesion areas from treated animals contained larger numbers of remyelinated axons (Fig 6 D and E) than non-treated EAE controls (Fig 6 C and E). For example, while greater than 90% of the axons were demyelinated (asterisks) in lesion areas from non-treated EAE animals less than 25% of axons were demyelinated in antagonist treated animals (Fig 6; p<0.0001). Many of the myelinated axons in treated animals had altered G ratios consistent with remyelination (Fig. 6F). Vehicle only treated EAE animals had a ratio close to 1 suggesting the myelinated axons were not remyelinated. By contrast, CXCR2 antagonist treated animals had a mean ratio closer to 3 indicative of significantly thinner myelin sheaths which indicates remyelination (Figure 6F; p=0.02). To confirm the effects of blocking CXCR2 on demyelination/remyelination, sections from control and treated EAE animals were labeled with antibodies to the major myelin protein MBP (Fig 7). Inhibition of CXCR2 increased the intensity of MBP labeling by 63% over control EAE animals (Fig 7 A-D and reduced the size of the demyelinated areas) suggesting that oligodendrocytes are being recruited and/or protected in lesion areas by reducing CXCR2 signaling.

Infiltration and activation of macrophage/microglial cells are reduced in MOG35-55 induced EAE animals treated with CXCR2 inhibitors

The reduction in demyelinated areas in CXCR2 antagonist treated EAE animals correlated with a reduction in inflammatory cell infiltration. For example, H&E stained sections from vehicle EAE animals revealed substantial cell infiltrates associated with lesion areas (Fig 5D) characteristic of lesion pathology and the recruitment of immune cells. By contrast, in animals treated with the CXCR2 inhibitor only rare mononuclear cells were seen in presumptive lesion areas (Fig 5E).

The preservation of cytoarchitecture and reduction in vascular cuffing was most clearly seen in toluidine blue stained 1 μm section**s** (Fig 6 A and B) and confirmed at the ultrastuctural level. Vehicle treated EAE animals (Fig 6A) had significant cuffing and infiltration while CXCR2 antagonist treated EAE animals (Fig 6B) exhibited fewer cells around blood vessels and infiltration into the parenchyma of the spinal cord. Microglia were present in all spinal cords but their reactivity, defined by a more stellate phenotype and shorter process, was reduced in animals treated with CXCR2 antagonists (Fig 7 E-H). Vehicle-treated EAE animals contained large numbers of Iba1+ cells within lesions (Fig 7E) as well as in adjacent grey and white matter areas of the spinal cord. Animals treated with CXCR2 antagonists, however, demonstrated microglial activation associated with smaller lesions and a 50+/− 5% decrease of Iba1 cells in adjacent grey and white matter regions (Fig 7 F and H). EAE animals that were removed from treatment after 1 week exhibited a return of demyelination in the absence of inflammation (Fig 8B). These animals also exhibited a return in functional impairment associated with demyelination (data not shown). In contrast, EAE animals that were treated with CXCR2 antagonists for a longer period of time (2 weeks), demonstrated an enhancement in remyelination in toluidine blue sections (Fig 8A) although they retained some inflammatory cell infiltration into the parenchyma of the spinal cord (Fig 8A). Taken together, our studies suggest that antagonism of CXCR2 signaling promotes functional recovery and facilitates the establishment of normal cytoarchitecture in the spinal cord in animal models of CNS demyelination.

DISCUSSION

Current therapies for demyelinating diseases such as MS are largely focused on regulating the immunological attack on neural tissue. Successful long term functional recovery in MS is likely to require substantial myelin repair. Here we demonstrate that blocking signaling through the

chemokine receptor CXCR2 promotes functional recovery in two demyelinating models and OPC differentiation is enhanced. Focal injections of CXCR2 antibody, or a CXCR2 antagonist, enhanced myelin repair in LPC lesions and promoted the differentiation of OPCs in culture. Systemic injection of CXCR2 antagonist resulted in limited enhancement of repair most likely reflective of the limited involvement of immune cells in this model. Consistent with this hypothesis, systemic treatment with CXCR2 antagonist resulted in significant functional recovery in a model of chronic EAE. This functional recovery was correlated with a reduced disease burden. For example, compared to vehicle treated EAE animals, those that received daily IP injections of CXCR2 antagonist had smaller demyelinated lesions and higher levels of myelin basic protein expression in the spinal cord that was correlated with higher numbers of remyelinated axons. Treatment with CXCR2 antagonist also reduced the extent of microglial activation and the degree of inflammatory cell infiltration.

The enhanced recovery from CNS demyelinating lesions mediated by antagonists of CXCR2 likely reflects influences on both neural and immune cells. Within the CNS, CXCR2 has been reported to be expressed on a range of cells including OPCs and other glia (Luo et al., 2000; Nguyen and Stangel, 2001). Within the spinal cord oligodendrocyte lineage, stimulation of CXCR2 on A2B5+ OPCs results in enhancement of PDGF driven proliferation and inhibition of cell migration (Tsai et al., 2002; Kadi et al., 2006). The accelerated repair in LPC lesions following local inhibition of CXCR2 signaling may reflect the ability of OPCs to ignore the migrational blockade from locally expressed CXCL1 and effectively repopulate the lesion. Alternatively, in order to progress to myelination, OPCs must complete their differentiation and mature. The control of differentiation has been shown to be inversely correlated with proliferation. If OPCs are refractory to the mitogenic stimulation of CXCL1 through CXCR2 blockade, they are more likely to differentiate and myelinate. Consistent with this hypothesis, inhibition of CXCR2 in spinal cord cultures resulted in enhanced numbers of mature MBP+ oligodendrocytes. Other neural cells have also been reported to express CXCR2 receptors including astrocytes. Astrocytes are thought to be a potential source of the ligand, raising the possibility of an autocrine loop that also contributes to the regulation of remyelination either through release of CXCL1 or other cytokines.

The robust functional recovery seen in EAE models after systemic delivery of CXCR2 antagonists may reflect CXCR2 signaling responses in immune tissues. Recent studies demonstrated that transfer of encephalitogenic TH17 cells into naïve host animals induces both CXCL1 and CXCR2 expression (Carlson et al., 2008). Furthermore, in relapsing remitting EAE, blockade of CXCR2 resulted in functional improvement and a reduction in leukocyte infiltration. Likewise, CXCR2−/− animals were relatively refractory to disease induction. In this model, the functional deficits are largely a result of immune signaling since transplantation of CXCR2+ leukocytes into CXCR2−/−animals resulted in disease induction. Taken together with the current data from a chronic model of EAE these data strongly suggest that CXCR2 signaling is a major contributor to immune mediated demyelination.

The roles of the chemokine CXCL1 and its receptor CXCR2 in demyelination and remyelination are complex. In a model of demyelination induced by cuprizone intoxication no apparent differences were seen in CXCR2+/+ and CXCR2 null animals (Lindner et al., 2008). The basis for this discrepancy is unclear and may represent either differential mechanisms of demyelination, repair, or the quantitation of recovery. In a more complex transgenic animal model, recent studies suggest that overexpression of CXCL1 driven by the GFAP promoter in astrocytes reduces lesion load and enhances repair in models of relapsing and remitting EAE (Omari et al., 2009). By contrast, in the current studies, blocking CXCR2 signaling enhances recovery in chronic models of EAE and in LPC induced demyelination. Several factors may account for the apparent contradiction in these results. First, in vitro studies demonstrate that the levels of chemokine stimulation are critical in promoting OPC

proliferation and inhibiting migration. Dose response analyses demonstrate that at high concentrations of CXCL1, OPC responses are negated, presumably, through receptor desensitization, a common characteristic of chemokine receptors (Mueller et al., 1997; Richmond et al., 1997). Thus, in the setting of EAE, astrocytes are highly responsive and, presumably, the levels of CXCL1 are highly elevated. This might be expected to lead to a transient stimulation of OPC proliferation while more persistent high level ligand expression leads to desensitization of the receptor facilitating functional recovery in relapsing EAE (Richmond et al., 1997). Elevated levels of CXCL1 may stimulate receptors other than CXCR2. For example, while CXCR2 is the major receptor for CXCL1, there is substantial promiscuity in ligand receptor interactions and other receptors such as CXCR1 are known to bind the ligand. The functional role of these alternate receptors is currently unknown. Finally, although overexpression of CXCL1 by oligodendrocytes leads to leukocyte accumulation in the CNS (Smith et al., 1983; Omari et al., 2009), overexpression in astrocytes did not appear to do so. Thus, the effect of the chemokine in this model may be restricted to specific regions of the CNS parenchyma that harbor differentially responsive cells.

We propose several characteristics that position CXCR2 as an attractive target for therapeutic development in demyelinating diseases such as multiple sclerosis. The receptor influences response in OPCs, the primary target cells mediating repair, as well as in peripheral leukocytes, the primary effector cells in immune mediated demyelination. In addition, CXCR2 antagonists have been utilized as potential therapeutics in other inflammatory based diseases (Keane et al., 2004; Gorio et al., 2007). For example, clinical trials are currently ongoing to evaluate CXCR2 antagonists for the treatment of chronic obstructive pulmonary disease, asthma, and cystic fibrosis based on the modulation of neutrophil function (Barnes, 2001; Traves et al., 2004; Govindaraju et al., 2006). Taken together, current data suggests that CXCR2 antagonists may enhance myelin repair in demyelinating diseases such as multiple sclerosis.

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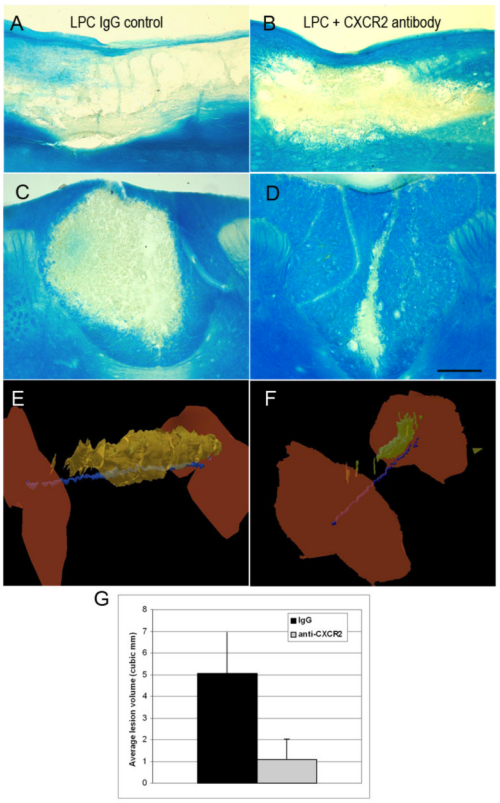

Figure 1. Local delivery of anti-CXCR2 antibodies reduces the size of LPC induced demyelinated lesions

Vibratome sections labeled with Luxol fast blue (A-D) of LPC lesions 10 days after induction treated with isotype control IgG (A and C) or neutralizing antibodies against CXCR2 (R&D Systems; 100μg/ml) (B and D) 2 days after lesion induction. Longitudinal sections (A and B) and cross sections (C and D) from mid-lesion areas demonstrate a reduction in lesion volume in anti-CXCR2 treated animals (B and D) compared to isotype controls (A and C). Three dimension reconstruction of LPC lesions (yellow) from isotype controls (E) and anti- CXCR2 treated animals (F). Comparison of the average LPC lesion volume in IgG control and anti-CXCR2 treated animals. n = 8 p= 0.005. Bar = 100μm in A-D (G). In E and F, blue = central canal, yellow = lesion, and red = outline of the spinal cord.

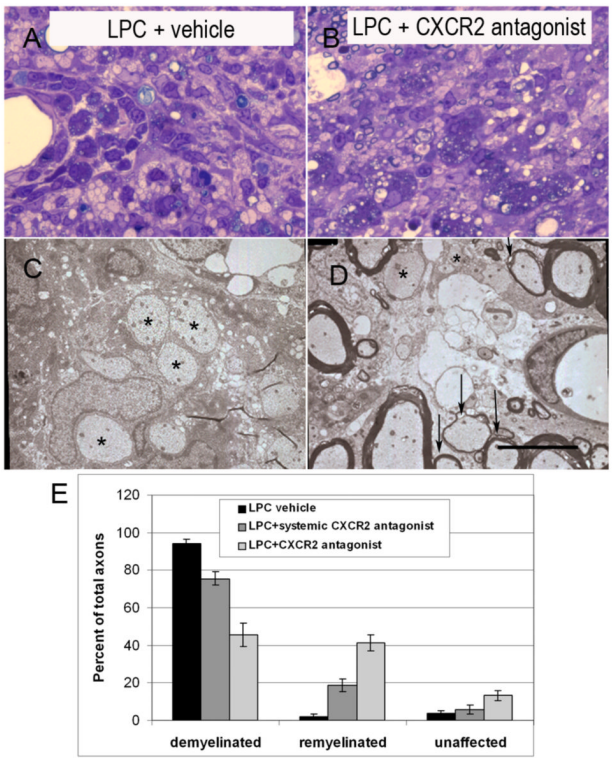

Figure 2. Local delivery of CXCR2 antagonists enhances remyelination in LPC lesions

Labeling of 1 μm sections through the middle LPC lesion area with Toluidine blue (A and B) demonstrates an increase in the number of myelinated axonal profiles in animals that received a single injection of a CXCR2 antagonist 2 days after LPC injection. Ultrastructural analyses confirmed the presence of unmyelinated (asterisks) and thinly myelinated (arrows) axons in experimental animals (D). Lesions from control animals contain largely unmyelinated (asterisks) axons and a higher number of cell bodies (C). Quantification of the proportion of remyelinated versus demyelinated axons in lesions from vehicle and small molecule antagonist treated animals demonstrated a 20-fold increase ($p=0.002$) in remyelinated axons versus control with direct delivery into LPC lesions and a 9-fold increase in remyelinated axons ($p=0.004$) with systemic delivery (E). Bar = 20μm in A and B and 2μm in C and D.

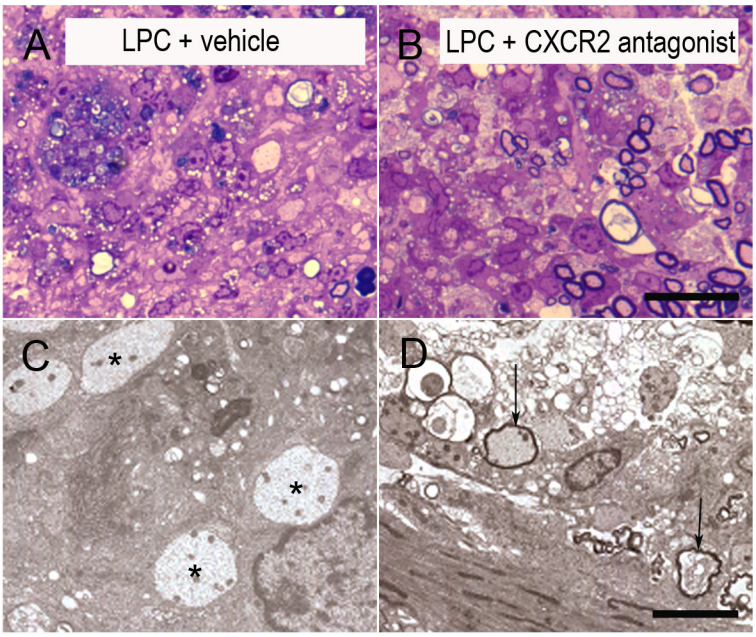

Figure 3. Systemic delivery of CXCR2 antagonists has limited effect on repair of LPC lesions

Labeling of 1 μm sections with toluidine blue (A and B) demonstrated no significant change in overall lesion size in animals treated daily with IP injections of CXCR2 antagonist, although experimental animals demonstrated an apparent reduction in the cellularity of lesions (B and D). Electron microscopy confirmed the limited effect of systemic inhibition of CXCR2 in promoting LPC lesion repair (C and D). Occasional thinly myelinated axons (arrows) were present scattered throughout the lesion in CXCR2 antagonist treated animals (D) that were absent in controls (C) which had a higher number of unmyelinated axons (asterisks). The myelin surrounding remyelinating axons was frequently uneven, possibly reflecting active remyelination. Bar in A and B = 20 μm and C and D = 2μm.

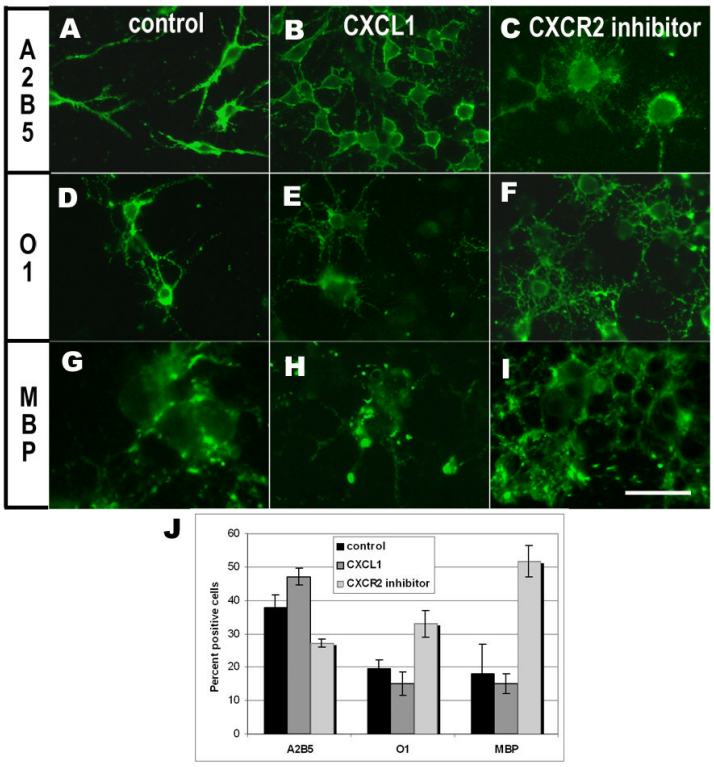

Figure 4. Inhibition of CXCR2 promotes the differentiation of spinal cord OPCs in vitro

Spinal cord mixed cell cultures (P3) grown for 5 days in vitro contain 35% A2B5+ cells (A) and approx 19% O1+ (D) and 17% MBP+ cells (G). Exposure to 0.05ng/ml CXCL1 for 24hrs results in an increase in A2B5+ cells (46%) (B) but a decrease in O1+ (12%) (E) and MBP+ (12%) cells (H). By contrast, exposure to CXCR2 antagonist (40ng/ml) for 24hrs results in a decrease in A2B5+ cells (26%) (C) but an increase in O1+ (31%) (p=0.05) (F) and MBP+ cells (50%) (p=0.007) (I) to the proportion of immunopostive cells compared to DAPI+ cell numbers (J). Data represents mean +/− standard deviation taken from 2 coverslips from at least 3 separate experiments. Bar = 20 μm in A-F and Bar = 40μm in G-I.

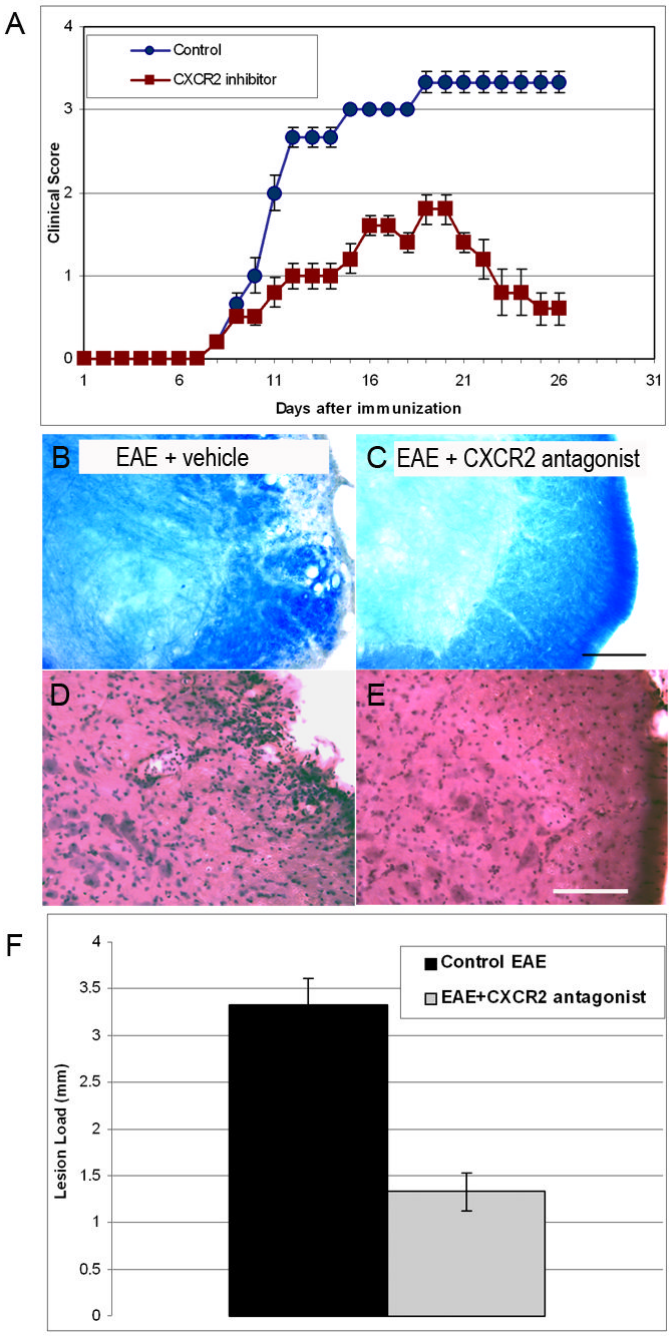

Figure 5. Systemic inhibition of CXCR2 results in functional improvement in MOG_{35-55} peptide induced EAE animals

(A) Injection of MOG_{35-55} peptide induces a robust functional deficit after 7-10 days that plateaus in the 2nd week (circles). Systemic daily delivery of CXCR2 inhibitor (20ng/kg) results in a slowing of disease progression and functional recovery that is maintained with continuous treatment (squares). Data represent mean clinical scores for 12 animals from 3 separate studies. Luxol fast blue staining on transverse spinal cord sections showed areas of demyelination in control EAE animals (B) that were significantly reduced in animals treated with CXCR2 antagonists (C). Control EAE animals showed significant cellular infiltrates in areas of demyelination (D) that were reduced in animals treated with CXCR2 antagonists (E).

Quantitation of the relative lesion load in the spinal cord of control EAE animals and CXCR2 antagonist treated animals at day 28 after immunization (F). Data represents the mean +/− standard deviation taken from 20 Luxol stained sections from each of 4 animals in each group. p= 0.0001. Bar = 100μm in B and C and 50μm in D and E.

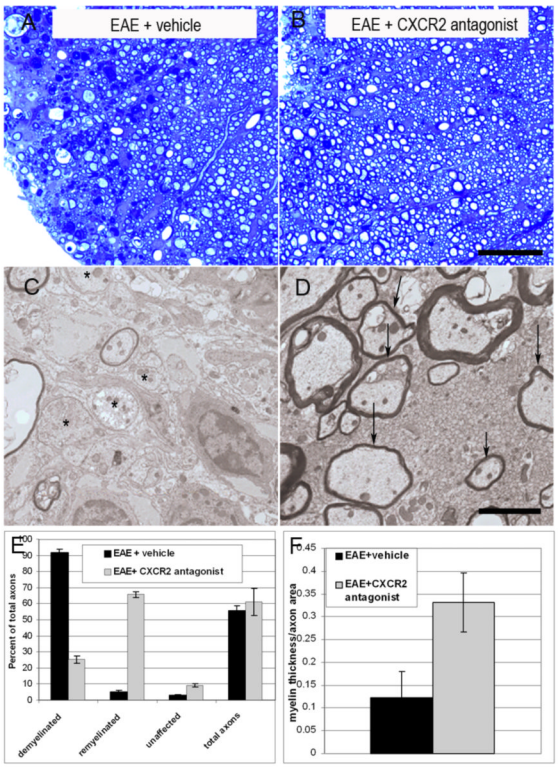

Figure 6. Systemic inhibition of CXCR2 results in decreased cell infiltration and increased remyelination in MOG_{35-55} induced EAE

In control EAE animals, 28 days after disease induction, lesion areas in the spinal cord were characterized by demyelination and cellular infiltrates (A). By contrast, in EAE animals treated daily with CXCR2 antagonists the extent of demyelination and cellular infiltration is substantially reduced (B). Ultrastructural studies confirmed the presence of extensive demyelinated axons (asterisks) in lesion areas associated with substantial cell infiltration (C). In animals treated with CXCR2 antagonist (D) the number of demyelinated axons was reduced and the number of thinly myelinated (arrows) axons increased consistent with widespread remyelination. Quantification of the relative numbers of demyelinated (asterisks), remyelinated (arrows), and unaffected axons in lesion areas of control CXCR2 antagonist treated EAE animals (E). The number and proportion of demyelinated axons is significantly decreased in CXCR2 antagonist treated animals compared to controls (90% vs 25%) while the number and proportion of remyelinated axons was significantly increased (10% vs 65%) ($p= 0.000002$) (E). The total number of axons was not significantly different in treated and untreated animals and the proportion of unaffected axons was below 10% in both groups (E). Remyelinated axons were characterized as having thinner myelin sheaths and quantification of 20 axons per lesion from 5 lesion areas in each of 3 animals demonstrated a significant reduction ($p= 0.02$) in myelin thickness/axon diameter ratio in treated animals (F). Control EAE animals had a ratio close to 1 suggesting little remyelination while CXCR2 antagonist treated animals had a mean ratio around 3, indicative of substantially thinner myelin sheaths (F). Bar = 75μm in A and B and 2 μm in C and D.

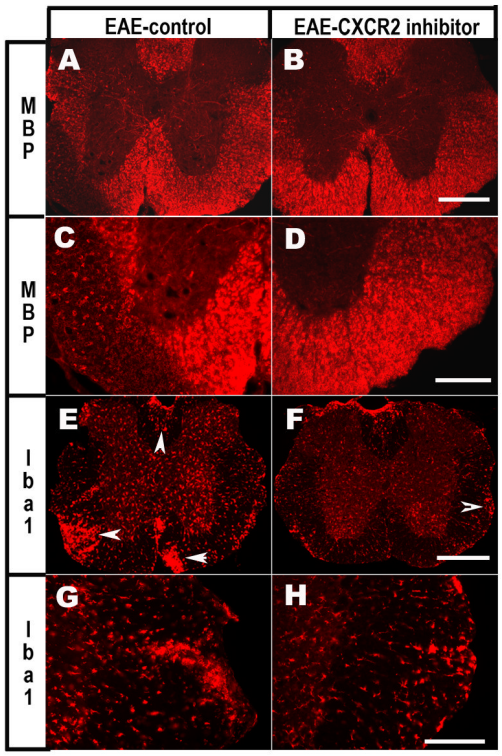

Figure 7. Systemic treatment with CXCR2 antagonists result in increased MBP and decreased Iba1 expression in EAE animals

Control EAE animals demonstrated reduced MBP labeling (A and C) and high levels of Iba1 + cells in lesion areas (arrowheads) (E and G. By contrast, animals treated with CXCR2 antagonist demonstrated higher levels of MBP expression (B and D) and fewer Iba1+ cells (F and H)in lesion areas (arrowheads). The number of Iba1+ cells in CXCR2 antagonist treated EAE animals was decreased by 50 +/− 5% in grey and white matter regions compared to vehicle treated animals (G and H). . Bar = 500μm in A, B, E and F and 200μm in C, D, G and H. .

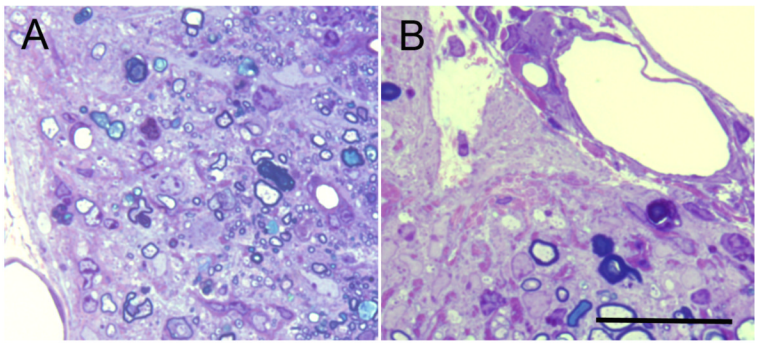

Figure 8. Long-term but not short-term treatment with CXCR2 antagonist results in sustained remyelination

EAE animals treated with CXCR2 antagonist systemically for a period of 2 weeks and then removed from treatment demonstrated persistent remyelination 1 week later (A). By contrast, ., EAE animals removed from CXCR2 antagonist treatment after 1 week of treatment demonstrated a lack of remyelination, although there was an apparent reduction in inflammatory cell infiltration (B). . Bar = 50μm.