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HIV-1 gp120-Induced Axonal Injury Detected by Accumulation of β-Amyloid Precursor Protein in Adult Rat Corpus Callosum

Jingdong Zhang, **Jianuo Liu**, **Bryan Katafiasz**, **Howard Fox**, and **Huangui Xiong**
Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198-5880, USA

Abstract

HIV-1 brain infection induces neurodegeneration. While most studies focus on HIV-1-mediated neuronal injury, relatively few have investigated HIV-1-associated white matter damage. Corpus callosum (CC) is one of frequently involved white matter structures in HIV-1-associated white matter damage. Utilizing a model of ex vivo treatment of brain slice containing CC with HIV-1 glycoprotein 120 (gp120), we examined axonal injury by analyzing β-amyloid precursor protein (β-APP) accumulation in the axon. Incubation of CC slice with gp120 produced a significant higher density of β-APP in the CC tissue compared with non-gp120-treated controls, suggesting the presence of axonal damage in the CC. The gp120-induced CC axonal damage was blocked by a chemokine CXCR4 receptor antagonist T140 but not by an NMDA receptor blocker MK801 as demonstrated by Western blot analysis of β-APP, indicating that gp120 evokes the CC axonal injury through CXCR4 receptor. Immunocytochemical studies revealed a surprisingly high density of CXCR4-positive immunoreactivity in the CC. The CXCR4-positive labeling was distributed along the nerve fibers. Moreover, double labeling of anti-CXCR4 with either anti-neuronal nuclei or anti-myelin/oligodendrocyte-specific protein antibody revealed co-localization of CXCR4 and myelin/oligodendrocytes in some fiber-like structures, inferring that some neurons and oligodendrocytes in the CC express CXCR4. Taken together, these results indicate that gp120 induced axonal damage via CXCR4 in the CC.

Keywords

HIV-1 gp120; Axonal injury; Corpus callosum; CXCR4; β-Amyloid precursor protein; White matter damage

Introduction

Neurological disorders remain prevalent in HIV-infected individuals even in the era of combination antiretroviral therapy. Autopsy and clinical brain image studies have shown that white matter pallor is a key feature of neuropathological change and can be detected by magnetic resonance or diffusion tensor imaging in HIV-infected patients with AIDS or even early during asymptomatic infection (Navia et al. 1986; Gray et al. 1996; Bell 1998; Filippi et al. 2001; Chen et al. 2009; Gongvatana et al. 2009). Such white matter pallor has been considered as a sign of white matter damage in HIV infection as it corresponds to changes in axon injury revealed by a sensitive marker—β-amyloid precursor protein (β-APP) (An et al. 1997; Giometto et al. 1997; Raja et al. 1997). The most frequently involved structures in HIV-1-associated white matter damage are the corpus callosum (CC), internal capsule, and

hxiong@unmc.edu .

U fibers in *centrum semiovale* (Navia et al. 1986; Gosztonyi et al. 1994; Gongvatana et al. 2009; Wohlschlaeger et al. 2009). Nevertheless, the mechanism underlying HIV-associated white matter damage is largely unknown. Autopsy studies have revealed a correlation between white matter pallor and the distribution of microglial nodules/multinucleated giant cells (Navia et al. 1986; Gray and Lescs 1993; Bell 1998), as well as between pallor and the presence of HIV-infected macrophages in subcortical white matter (Gosztonyi et al. 1994). Thus, the soluble factors released from infected and/or activated microglia and macrophage including, but are not limited to, cytokines, chemokines, and viral proteins are believed to be toxic to bypassing axons (Kaul et al. 2001). Amongst such viral proteins is HIV-1 glycoprotein 120 (gp120), an envelope protein which is toxic to neural cells at very low concentration.

Gp120 has been shown to induce neuronal apoptosis (Brenneman et al. 1988; Muller et al. 1992; Lannuzel et al. 1997). Transgenic mice expressing HIV-1 gp120 manifest some neuropathological features that are also found in human AIDS brain (Toggas et al. 1994). These features include apparent loss of dendrites and presynaptic terminals. However, it is unclear whether gp120 could promote white matter damage and/or axonal function change. To address this issue, we examined the effect of gp120 on the CC fiber bundles in adult rat brain slices containing the CC. The gp120-induced axonal impairment in the CC was evaluated by immunocytochemical and Western blot analyses of β-APP accumulation in the CC axonal fiber bundles as β-APP is a sensitive marker for axon injury. β-APP is synthesized in neuronal soma and transported anterogradely to axon terminals by fast axon transport (Buxbaum et al. 1998; Kaether et al. 2000). If axonal function is impaired, β-APP will accumulate in axons, and this accumulation can be detected by either immunocytochemistry or Western Blot (Gentleman et al. 1993; Blumbergs et al. 1995; McKenzie et al. 1996). In this study, we observed an enhancement of β-APP immunoreactivity in the CC following treatment by HIV gp120. The gp120-induced enhancement of β-APP immunoreactivity was blocked by a CXCR4 receptor antagonist T140, indicating that gp120 induces CC axonal damage via chemokine receptor CXCR4.

Materials and methods

Animals

A total of 37 healthy adult Sprague–Dawley rats (150–250 g) were used in this study. All experimental protocols and animal care were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care of Laboratory Animals in Research and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. All efforts were made to minimize animal suffering and the number of animals used in this study.

Preparation and incubation of the brain slices—Animals were anesthetized with isoflurane and decapitated, and brains were quickly removed from the cranial cavity. The brains were placed into an ice-cold (4°C) oxygenated artificial cerebrospinal fluid (ACSF) contained (in millimolar): NaCl, 124.0; KCl, 3.0; $CaCl_2$, 2.0; $MgCl_2$, 2.0; NaH_2PO_4, 1.25; $NaHCO_3$, 26.0; and glucose, 10.0. The ACSF was equilibrated with 95% O_2 and 5% CO_2 and had a pH of 7.4–7.5. Coronal brain slices containing the CC (500 μM in thickness) were cut using a NVSLM1 Vibroslicer (WPI, Sarasota, FL). The slices for Western Blot were divided into four groups: control (Ctrl); treated with 1 nM gp120 (recombinant HIV-1 IIIB gp120, ImmunoDiagnostic Inc., Woburm MA.); treated with gp120 (1 nM)+10 μM MK801 (Sigma-Aldrich, St. Louis, MO); and treated with gp120 (1 nM)+50 nM T140 (kindly provided by Dr. Nobutaka Fujii from the Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan). Incubation was conducted for 8–13 h at room temperature.

After incubation, the slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) overnight. Then, the slices were incubated in 10%, 20%, and 30% sucrose in 0.1 M PB, followed by mixture of 30% sucrose and OCT half by half. Finally, the slices were embedded in OCT and stored in −20°C until cutting with cryostat.

Western blot—The slices were oxygenated at 4°C until the CC tissue was dissected out under a dissecting microscope. Approximately 0.2 g of the CC tissue was transferred in 2 ml Tissue Extraction Reagent 1 (FNN0071, Invitrogen, Camarillo, CA) with 1/1000 protease inhibitor (P-2714, Sigma-Aldrich, St. Louis, MO) and homogenized. Protein concentrations were determined using BCA method with bovine serum albumin as a standard. The homogenate was subjected to 10% sodium dodecyl sulfate-polyamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were then blocked with 5% non-fat milk in Tris-buffered saline–Tween 20 and incubated with monoclonal mouse anti-β-APP antibody (1:100; Millipore, CA), followed by horseradish peroxidase (HRP)-conjugated anti-mouse (1:5,000; Jackson ImmunoResearch Inc. West Grove, PA). For gel loading control, mouse anti- β-actin (1:10,000; Sigma-Aldrich, St. Louis, MO) was used to incubate PVDF membrane and followed by HRP-conjugated anti-mouse (1:5,000–10,000; Jackson, West Grove, PA). Immunoreactive bands were detected using enhanced chemiluminescence and developed with autoradiograph film.

Immunocytochemistry—Ten-micrometer sections were cut with a cryostat and mounted on slides immediately. Slides were incubated with 10% normal goat serum in 0.01 M PB saline (PBS, pH 7.2–7.4) containing 0.5% Triton-X-100 at room temperature for 1 h. Rabbit or mouse anti-β-APP (1:200; Millipore, Billerica, MA), mouse anti-NMDAR1 (1:100; Invitrogen, Camarillo, CA), and rabbit anti-CXCR4 (1:100; Abcam, Cambridge, MA) were used for incubation of slides at room temperature overnight. Antibodies for double labeling were mouse anti-myelin/oligodendrocyte-specific protein (M/OSP, 1:200; Millipore, Temecula, CA) and mouse anti-neuronal nuclei (NeuN, 1:200; Millipore, Temecula, CA). Alexa Fluor 488 or 594-conjugated anti-mouse or anti-rabbit (Molecular Probes, CA) were used for immunofluorescent staining. Slides were blocked by 10% normal goat serum and stained with either Alexa Fluor 488 or 594 anti-mouse or anti-rabbit for control. All slides were mounted using Vectashield with DAPI (Vector Labs, Burlingame, CA) and observed with Nikon-800 microscope and Zeiss confocal (Zeiss LSM 510).

Statistical analysis—Protein density ratio of β-APP/β-actin in each running sample was measured and collected for statistical analysis. ImageJ (NIH, Bethesda, MD) was used with function of “Gel plot”. The plot figure and *p* value were generated with SigmaPlot 10.0 (Systat Software, Chicago, IL) and SPSS 11.5 (SPSS Inc. Chicago, IL). Statistical analyses were performed by one-way ANOVA and LSD multiple comparisons or by Student’s *t* tests. A minimum *p* value of 0.05 was estimated as the significance level for all tests.

Results

HIV-1 gp120-induced accumulation of β-APP in the CC—To explore potential role of gp120 in causing white matter damage, we examined β-APP accumulation in the CC axonal fibers after gp120 treatment. Rat brain slices containing the CC were incubated with 500 pM, 1 nM, and 2 nM gp120 for either 8 or 13 h. Western blotting detected a higher density of β-APP in the CC treated with gp120 (1–2 nM) than that observed in the control CC (Fig. 1a). Paired *t* test showed a significant difference between control and gp120-treated (1–2 nM, 8–13 h) groups (n=8, $p<0.05$; Fig. 1b). In parallel with the Western blotting results, confocal microscopy revealed a higher β-APP-like immunoreactivity in the gp120-treated CC (Fig. 1c, d).

It has been shown that gp120 induced neuronal injury via either NMDA receptors or chemokine receptor CXCR4. To examine whether gp120-induced β-APP accumulation in the CC is mediated via NMDA receptor or CXCR4, we tested the effects of NMDA receptor antagonist MK801 and CXCR4 antagonist T140 on its blockade of gp120-induced accumulation of β-APP in the CC as detected by Western blot. Addition of MK801 to the incubation solution did not decrease the β-APP accumulation in the CC in comparison with the results obtained from the CC treated with gp120 alone (data not show). In contrast, the gp120-induced β-APP accumulation was significantly reduced by addition of T140 in the incubation (Fig. 2, one-way ANOVA, $n=5$, $p<0.05$). One-way ANOVA and LSD multiple comparisons showed significant difference between control (Ctrl) and gp120 groups and between gp120 and gp120+T140 groups (Fig. 2). These results suggest that gp120 produces β-APP accumulation in the CC via chemokine receptor CXCR4.

CXCR4, CXCR4+M/OSP, and CXCR4+NeuN immunocytochemistry—As gp120-induced accumulation of β-APP in the CC was blocked by T140, we conducted immunocytochemical studies to examine the expression of CXCR4 in the normal CC. Abundant CXCR4-positive immunocytochemistry staining was observed in the CC areas (Fig. 3a), including many soma-like structures (Fig. 3b, arrows). The CXCR4-positive structures appeared to be distributing along the nerve fibers in the CC (Fig. 3b–d). When examined by confocal microscopy, both soma-like and fiber stainings were evident (Fig. 3c–d). To determine the identity of the CXCR4-positive soma-like labeling, we performed double labeling using anti-CXCR4 and anti-M/OSP or NeuN to distinguish oligodendrocytes and neurons. The control staining for CXCR4 (Fig. 3e), M/OSP, and NeuN (Fig. 3f) was conducted without the primary antibodies.

Representative examples of anti-M/OSP and anti-CXCR4 double-labeled oligodendrocytes are shown in Fig. 4. Since anti-M/OSP antibody labels both oligodendrocyte cell bodies and myelin sheath, some fiber-like structures were also double labeled with anti-M/OSP and anti-CXCR4 (Fig. 4a–c). Arrowheads in Fig. 4a–c indicate a representative of fiber-like dual labeling. We randomly selected 20 visual fields viewed under the 40× objective from eight sections with six from frontal part of the CC above basal ganglion and two from the parietal CC above hippocampus to count the labeling. The anti-CXCR4-labeled cells were eight to 25 in each visual field with a total of 319 from 20 visual fields, while the anti-M/OSP-positive cells were nine to seven per visual field with a total of 95. The total numbers of anti-CXCR4 and anti-M/OSP double-labeled cells were nine in the 20 visual fields. Therefore, a total of 9.5% (nine of 95) anti-M/OSP-positive cells express CXCR4, and ~3% of the anti-CXCR4-labeled cells (nine of 319) express the oligodendrocyte marker based on our observations.

The number of neurons labeled with anti-NeuN was low, as expected in the CC. We counted ten visual fields viewed under the 40× objective from eight sections, which resulted in 30 NeuN-positive cells and six double-labeled cells (Fig. 5). Anti-NeuN was designed mainly for staining of nuclei, but light stain could be extended into proximal dendrite (Wolf et al. 1996). In this study, we observed only three typical nuclear stains as shown in Fig. 5a–c, with most of the stain in soma. Consistent with the previous report (Wolf et al. 1996), dendrites were also stained (Fig. 5d–f). Based on our results, 20% of neurons (6 of 30) were co-labeled with anti-CXCR4.

Discussion

The major findings of the current work are that HIV-1 gp120 per se can induce axon injury ex vivo which can be protected by a CXCR4 antagonist and that the CC harbors a high

density of CXCR4 and some of them are observed on oligodendrocytes, a type of brain cells producing the myelin sheath to insulate axons.

The mechanism of white matter damage in HIV-1-associated neurological disorder is still largely unknown. The studies on this topic have been limited because of lacking applicable animal model due to refuse of murine immune system to HIV infection and low efficiency with high cost of primate model (Mosier 1996; van Maanen and Sutton 2003; Stremlau et al. 2004). Although infection with simian immunodeficiency virus (SIV) in macaques provides an excellent model for studying HIV-1-induced CNS pathology, few studies have investigated axonal damage and its underlying mechanism. To these ends, we, using β-APP as a specific marker, investigated how gp120 induces axonal injury in ex vivo brain slices prepared from rat CC, a brain region that is well known to be frequently involved in HIV-1-associated neurological disorders (Navia et al. 1986; Gongvatana et al. 2009; Wohlschlaeger et al. 2009). We chose gp120 as an etiological agent as it has widely been demonstrated to induce neuronal apoptosis in rodent neuronal culture (Dreyer et al. 1990; Lipton et al. 1991; Muller et al. 1992; Dawson et al. 1993) and in transgenic animals (Toggas et al. 1994).

β-APP has been widely used in axonal injury studies in head and spinal trauma, multiple sclerosis, infectious disease, and ischemia (Medana and Esiri 2003). It is a sensitive marker for functional impairment of the axons because any functional change such as downregulation of microtubule ATPase (Brady 1985) or calcium-related activities (Rivera et al. 1995) will slacken the axoplasmic movement and accumulation of the β-APP in axoplasma during its fast transportation from soma to terminal (Buxbaum et al. 1998; Kaether et al. 2000). Indeed, β-APP accumulation in the white matter of SIV-infected macaques develops during SIV infection in close correction with the levels of viral replication in the CNS (Mankowski et al. 2002). Hence, combination of brain slice treatment with β-APP immunoblotting and immunocytochemistry is an applicable approach to study HIV-1-associated axonal injury. However, the gp120 concentration used in this work is relatively higher, comparing with most of the previous studies performed with cultured cells, in which the picomole level of gp120 could evoke obvious change (Dreyer et al. 1990; Lipton et al. 1991; Dawson et al. 1993). The possible reason for such a discrepancy may result from the thickness of the brain slice which could hamper effective penetration of large protein molecules like gp120 although higher concentration of proteins may also increase the chance of interaction between proteins and cells in the slice. Other reasons such as indirect effect, dominative, or less effective interaction of HIV-1 proteins upon rodent tissue receptors (van Maanen and Sutton 2003; Blanco et al. 2009) cannot be excluded.

In addition to examination of the effects of gp120 on rodent CC axonal injury, the mechanism underlying gp120-induced axonal injury was also investigated in the present study. In the light of previous studies on gp120-associated neurotoxicity, two major pathways seem to be involved in the mechanism, one is via NMDA receptors (Lipton et al. 1991; Savio and Levi 1993; Toggas et al. 1994; Barks et al. 1997; Wang et al. 1999) and the other is through chemokine receptor CXCR4 (Hesselgesser et al. 1998; Meucci et al. 1998; Pandey and Bolsover 2000; Bachis and Mocchetti 2004; Dong and Xiong 2006). To examine whether these two pathways are involved in gp120-induced axonal injury in the CC, we tested NMDA receptor antagonist MK801 and CXCR4 antagonist T140 on their protection of the axonal injury induced by gp120. Interestingly, we found that T140, not MK801, can alleviate the gp120-induced axonal injuries in the CC, which implicates that HIV-1 gp120 could give rise to axonal injury through CXCR4 pathway. This implication is supported by our immunocytochemical results that the CXCR4-like immunoreactivity distributed along the nerve fibers in the CC, inferring a close relationship of these receptors to the nerve fibers. Further, we found that some neurons and oligodendrocytes in the CC were CXCR4 positive, though the number of double-labeled cells was fairly low. We note

that other researchers did not find CXCR4-expressing neurons and oligodendrocytes in rat CC, perhaps due to different antibodies or staining conditions (Banisadr et al. 2002).

It is not clear at present whether gp120 produces axonal injury directly through the CXCR4 on neurons and/or oligodendrocytes because majority of CXCR4 labelings were on neither neuronal nor oligodendrocyte soma. As anti-M/OSP stains oligodendrocyte soma and processes as well as myelin sheath, we observed that some anti-M/OSP-positive fiber-like structures were also labeled by anti-CXCR4 and that some anti-CXCR4 labeling extended into neuronal processes morphologically more resembled dendrites rather than axons, though there has been no report yet describing G protein-coupled receptor on axon or myelin sheath. Early studies showed that astrocyte and microglia express large amount of CXCR4 (Cartier et al. 2005). Immunocytochemistry work revealed certain amount CXCR4-positive astrocytes and microglia in the CC (van der Meer et al. 2000; Banisadr et al. 2002). Astrocytes participate in formation of myelinated fibers by enveloping Ranvier's nodes with their processes (Butt et al. 1994). Hence, indirect effect of gp120 through CXCR4 on astrocyte and microglia may also play an important role in gp120-induced axonal injury. Since oligodendrocytes participate in formation of myelinated fibers by enveloping Ranvier's nodes with their processes (Butt et al. 1994), gp120 may act directly on the CXCR4 expressed on oligodendrocytes leading to axonal injury as several early studies demonstrated that gp120 could induce functional alteration and apoptosis in cultured mature oligodendrocytes (Bernardo et al. 1997) and inhibited myelin formation in rat cerebral cortex culture (Kimura-Kuroda et al. 1994). However, in a very recent study using an experimental murine model of demyelination mediated by the copper chelator cuprizone, Klein and her colleagues (Patel et al. 2010) showed that the remyelination in the CC after cuprizone-induced demyelination was inhibited by in vivo CXCR4 RNA silencing or prevented by CXCR4 antagonism. The discrepancy may stem from different etiological factors, experimental approaches, and animal species.

In summary, the present study demonstrated that gp120 induces axonal injury via CXCR4 in the CC as detected by accumulation of β-APP in axoplasma which resulted from impairment of axonal transport function. Although the detailed mechanism for gp120-induced axonal injury remains to be determined, our results indicate that detection of axonal injury by β-APP Western blot and immunocytochemistry in rat slice preparations is an applicable approach in studying HIV-1-associated white matter damage.

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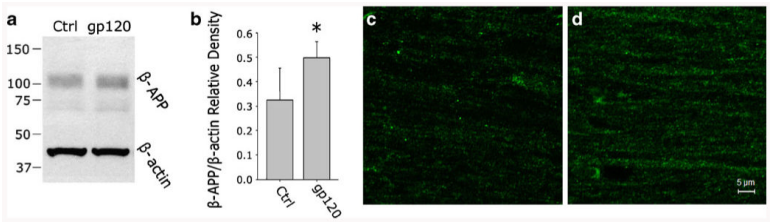

Fig. 1.

Gp120-induced accumulation of β-APP in the CC. **a** Representative Western blot of control (*Ctrl*) and gp120-treated (*gp120*) CC slice protein extracts. The CC-containing slices were incubated in ACSF alone (*Ctrl*) or in the ACSF contained 1–2 nM gp120, for 13 h prior to protein extraction. The protein extracts were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were probed with β-APP antibody, and β-actin was shown for loading control. **b** Bar graph illustrating β-APP expression normalized to β-actin in *Ctrl* and *gp120* groups (n=8, p<0.05). **c**, **d** The immunostaining for β-APP in control and gp120-treated CC sections, respectively. Note that the immunostain for β-APP was increased in the CC treated with gp120 in comparison with that observed in non-gp120-treated CC sections

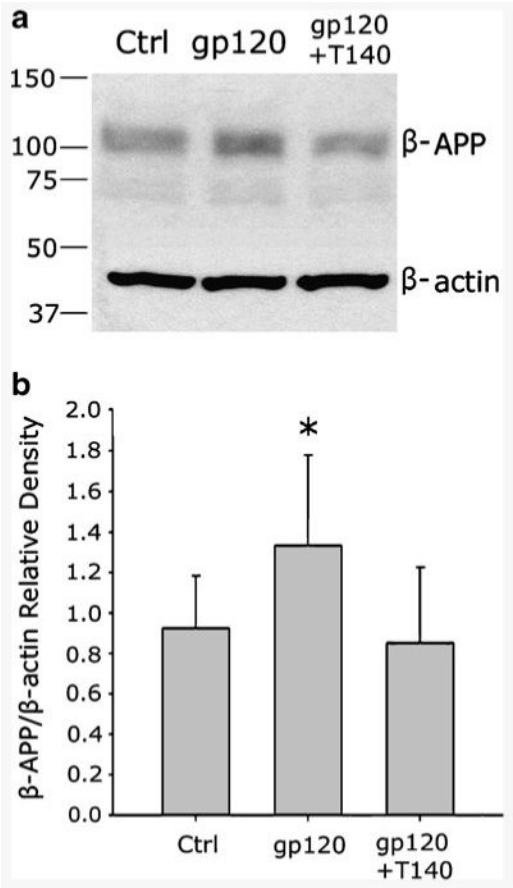

Fig. 2.

Attenuation of gp120-induced accumulation of β-APP in the CC by a CXCR4 antagonist T140. **a** Representative Western blot results from control (*Ctrl*), gp120-treated (*gp120*), and gp120-treated+T140 (*gp120+T140*) CC slice protein extracts. Proteins were resolved by SDS-PAGE, transferred to PDVF, and blotted for β-APP and β-actin. **b** A bar graph displaying β-APP expression in different experimental groups. The β-APP expression was normalized to β-actin loading control groups (n=5; one-way ANOVA and LSD, p<0.05)

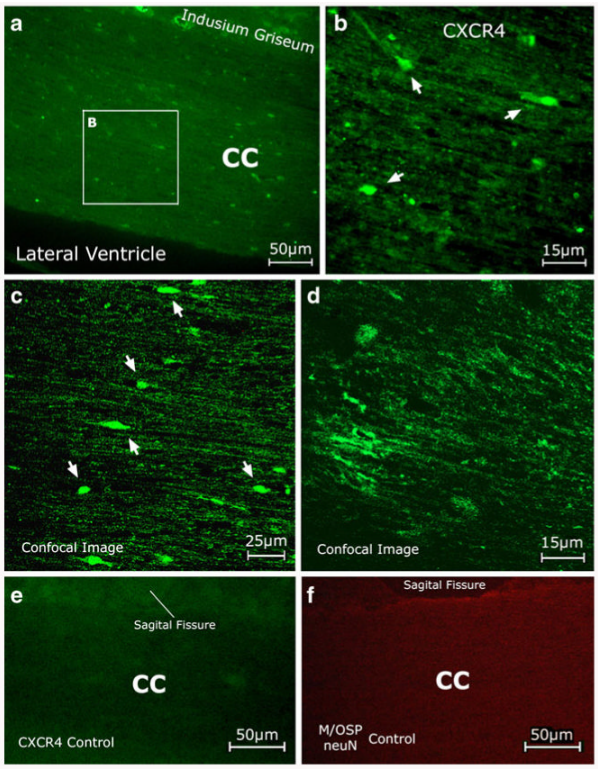

Fig. 3.

Expression of CXCR4 along nerve fibers and in scattered somata of cells in the CC. **a** Lower magnification conventional microscopic image of a CC section stained for CXCR4. *b* An *inset* of box in **a**, showing the area of the section with a higher magnification. Staining for CXCR4 was observed along nerve fibers and in scattered somata (*arrows*) in the CC. **c** Lower magnification confocal microscopic imaging, *arrows* point to somata. **d** Higher magnification observation. **e**, **f** Taken from control sections without primary antibodies

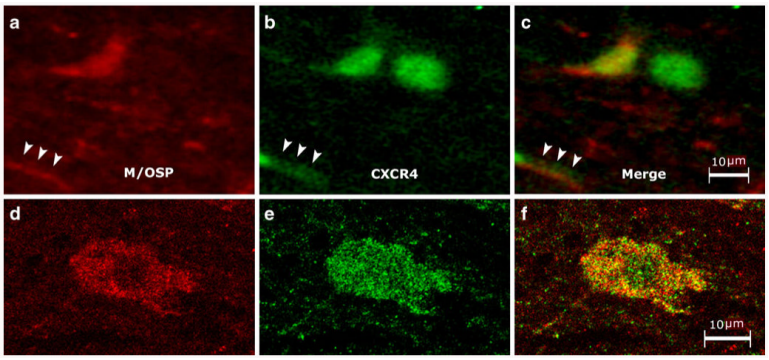

Fig. 4.

Expression of CXCR4 in oligodendrocytes in the CC. The sections were double stained with anti-M/OSP (**a**, **d**) and anti-CXCR4 (**b**, **e**). The merged images (**c**, **f**) illustrate the presence of CXCR4 in CC oligodendrocytes. Some fiber-like structures were also double stained with anti-M/OSP and anti-CXCR4 (**a**–**c**) and *arrowheads* point to a representative fiber-like dual labeling

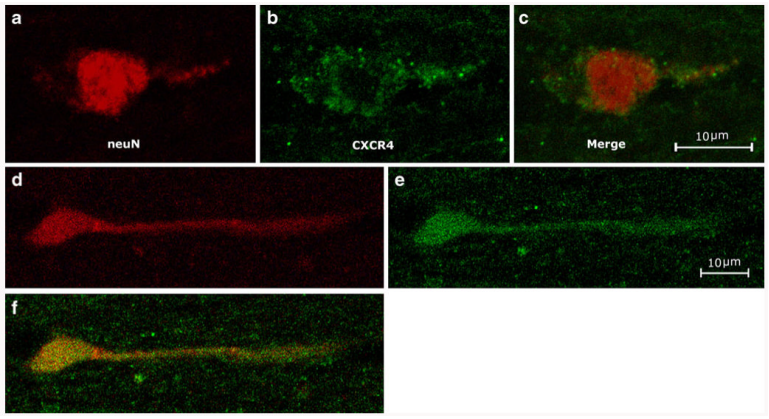

Fig. 5.

Neuronal expression of CXCR4 in the CC. The sections were double stained with NeuN (**a**, **d**) and CXCR4 (**b**, **e**). Merged images (**c**, **f**) indicate the presence of CXCR4 in CC neurons. The CXCR4 expression extends long distance along the dendrite of a ganglion cell-like neuron (**d**–**f**)