

RESEARCH ARTICLE

A CXCR4 receptor agonist strongly stimulates axonal regeneration after damage

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Introduction

The peripheral nervous system (PNS) is exposed to a variety of damages, from mechanical traumas to autoimmune or neurotoxic attacks. In humans, peripheral nerve injuries may also derive from surgical resections like those occurring during excision of tumors. For its essential roles in physiology and survival, the PNS has retained throughout evolution the capability to regenerate.^{1–5} Nevertheless, several factors, including injury type, its anatomical location, and the extent of associated

Abstract

Objective: To test whether the signaling axis CXCL12 α -CXCR4 is activated upon crush/cut of the sciatic nerve and to test the activity of NUCC-390, a new CXCR4 agonist, in promoting nerve recovery from damage. **Methods:** The sciatic nerve was either crushed or cut. Expression and localization of CXCL12 α and CXCR4 were evaluated by imaging with specific antibodies. Their functional involvement in nerve regeneration was determined by antibody-neutralization of CXCL12 α , and by the CXCR4 specific antagonist AMD3100, using as quantitative read-out the compound muscle action potential (CMAP). NUCC-390 activity on nerve regeneration was determined by imaging and CMAP recordings. **Results:** CXCR4 is expressed at the injury site within the axonal compartment, whilst its ligand CXCL12 α is expressed in Schwann cells. The CXCL12 α -CXCR4 axis is involved in the recovery of neurotransmission of the injured nerve. More importantly, the small molecule NUCC-390 is a strong promoter of the functional and anatomical recovery of the nerve, by acting very similarly to CXCL12 α . This pharmacological action is due to the capability of NUCC-390 to foster elongation of motor neuron axons both *in vitro* and *in vivo*. **Interpretation:** Imaging and electrophysiological data provide novel and compelling evidence that the CXCL12 α -CXCR4 axis is involved in sciatic nerve repair after crush/cut. This makes NUCC-390 a strong candidate molecule to stimulate nerve repair by promoting axonal elongation. We propose this molecule to be tested in other models of neuronal damage, to lay the basis for clinical trials on the efficacy of NUCC-390 in peripheral nerve repair in humans.

inflammation, can limit the extent of the repair process. The mechanisms and players of peripheral nerve regeneration are intensively investigated, as the molecular and cellular understanding of the regeneration process will lead to the discovery of chemical and biological molecules to improve rehabilitation of PNS neurons after accidents. In addition, there is the possibility that the same actors may be beneficial for the central nervous system, where regeneration is poor.

Recently, we found that the chemokine CXCL12 α potently promotes the recovery of the neuromuscular

junction (NMJ) activity after degeneration of the motor axon terminal.⁶ In this process, CXCL12 α is synthesized and released by perisynaptic Schwann Cells (SC), and acts *via* the CXCR4 receptor re-expressed on the tip of the regenerating motor axon terminal.⁶

CXCL12 α (also known as SDF-1) is a secreted chemokine discovered as a growth factor for the bone marrow pre-B cells,^{7,8} and later shown to be involved in the development of various regions of the central nervous system.^{9–11} As other chemokines, it acts *via* a G protein coupled receptor, dubbed CXCR4, forming a signaling axis involved in a variety of responses in the immune and nervous systems.^{12–18} Given its nature as modulator of the immune system, CXCL12 α is susceptible to post-translational modifications and hydrolysis in body fluids that block/attenuate its biological activity, bioavailability and half-life, resulting in poor pharmacokinetics properties.¹⁹ In addition, CXCL12 α structure consists of 93 amino acids, 5 cysteines, and 2 disulfide bridges which makes its production *via* recombinant methods complicated and very costly.

On the other hand, as CXCR4 is the co-receptor driving the entry of HIV into human immune cells, in addition to its involvement in a variety of regulatory and modulatory activities of the immune and nervous systems,^{15,20} an intensive search for CXCR4 antagonists and agonists with favorable pharmacokinetics is ongoing. A novel group of CXCR4 ligands was recently identified and, among them, a new molecule, dubbed NUCC-390, displayed the best capability to activate the receptor in a cell line.²¹

Here, we describe the finding that the expression of CXCL12 α and CXCR4 increases at the site of sciatic nerve crush, and we report compelling evidence that the stimulation of CXCR4 with the agonist NUCC-390 accelerates neurotransmission rescue mainly by promoting axonal elongation.

Materials and Methods

Reagents

NUCC-390 synthesis was performed as previously described.²² AMD3100, a strong CXCR4 antagonist, was from Abcam (120718). Cytosine β -D-arabinofuranoside (C6645), DNase I (DN25), poly-L-lysine (P1274), laminin (L2020), and trypsin (T4799) were from Sigma Aldrich. μ -conotoxin GIIIB was from Alomone. Primary antibodies: β ₃-tubulin (302302, Synaptic System), NF (ab4680, Abcam), CXCR4 (ab1670, Abcam), GAP43 (ab75810, Abcam), CXCL12 α (Cell Signaling, BK3740S). Secondary antibodies were from Thermo Scientific.

Animals and Ethical statement

C57BL/6 mice expressing cytosolic GFP under the *plp* promoter²³ were kindly provided by Dr. W. Macklin (Aurora, Colorado) and Dr. T. Misgeld (Munich, Germany). They were used in imaging experiments. CD1 mice were from the local animal facility, and were employed for electrophysiological recordings. Animal care and experimental procedures were performed in accordance with National laws and policies (D.L. n. 26, March 14, 2014), with the guidelines established by the European Community Council Directive (2010/63/EU), and were approved by the local authority veterinary services.

Sciatic nerve injury, electrophysiology, and immunostaining

Six-eight week-old CD1 or C57BL6-J *plp-GFP* mice were anesthetized with xylazine (48 mg/kg) and zoletil (16 mg/kg) *via* i.p. injection. The sciatic nerve was exposed and crushed or cut as described.^{24,25} Briefly, an incision was made in the skin that is gently dissected from the underlying musculature. Then the fascial plane between the gluteus maximus and the anterior head of the biceps femoris was open revealing the sciatic nerve. The nerve was then gently freed from the surrounding connective tissue, and cut using small surgical scissor or, alternatively, placed on the bottom jaw of a super-fine haemostatic forceps for the crush. The nerve was crushed once for 40 sec with three clicks of the haemostatic forceps pre-dipped in powdered carbon, used to mark the crush site. Finally, the gluteal musculature was re-opposed and the skin sutured using 6-0 braided silk, nonabsorbable sutures (ETHLCON2 biological instruments, 8697). After surgery, mice were i.p. injected with the indicated treatments: NUCC-390 26 mg/kg (daily), or AMD3100 4 mg/kg (twice daily), or AMD3100 plus NUCC-390, or 100 μ g neutralizing anti-CXCL12 α antibody (weekly, R&D MAB310). At indicated time points compound muscle action potentials (CMAP) were recorded in gastrocnemius muscles of anesthetized mice upon supramaximal stimulation of the sciatic nerve (15 V, 4 ms and 0.5 Hz), approximately 0.5 mm below the sciatic notch. Electromyographic signals were amplified with a GrassP6 Amplifier (Grass, USA), fed to a PC using an A/D interface (BNC-2110, NI, USA). Traces were recorded with WinEDR, and analyzed using pClamp10. CMAP values from one hind limb muscle were normalized to those from the contralateral one of the same mouse, which received the same treatment (i.p. injection of either the agonist, or the antagonist, or of the neutralizing antibodies), thus making the result independent of muscle fiber dimensions.

For immunostaining, sciatic nerves from C57BL6-J *plp-GFP* mice were fixed in 4% paraformaldehyde for 10 min at RT, quenched in 50 mmol/L NH_4Cl , and saturated in blocking solution containing 0.5% Triton X-100. Incubation with antibodies was performed for >48 h in blocking solution. After washings and incubation with secondary antibodies, images were collected with a SP5 confocal microscope (Leica, Germany). Where indicated, whole mount staining was performed as in.²⁶

Neuronal cultures and axon length quantification

Cultures of spinal cord motor neurons (SCMNs) were prepared as previously described.²⁷ After plating, neurons were exposed for 24 h to the indicated concentrations of either NUCC-390, or AMD3100, or their combination in culture medium. Cells were fixed for 10 min (4% paraformaldehyde), stained for β_3 -tubulin, and examined by epifluorescence microscopy (Leica DMIRE2). Axon length was quantified with NeuronJ (ImageJ).

Statistical analysis

A group size of seven mice allowed to detect a difference in the mean values between groups with a power of 80% and a statistical significance of 5% in *in vivo* experiments. At least three independent replicates for cell culture experiments were conducted in blind. Data are expressed as mean \pm SEM. GraphPad Prism software was used for all statistical analyses. Statistical significance was calculated by ANOVA with Tukey post-test, or two-tailed unpaired Student's t-test depending on the number of groups analysed. Data were considered statistically different when * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Results

The CXCR4 receptor and its ligand CXCL12 α are strongly expressed in the sciatic nerve after crush

We tested the expression of CXCL12 α and CXCR4 after axonal damage in a well-established model of traumatic neuronal injury consisting in the compression (crush) of the sciatic nerve.^{5,24} The crush causes interruption of many axons, mostly of myelinated ones, with preservation of Schwann cells (SC) and the basal lamina (BL), which are essential for nerve regeneration to take place. Figure 1 shows the expression of CXCR4 in longitudinal (A–B) and cross-sections (C) collected consecutively (slices of 20 μm thickness each) in control sciatic nerves or over

the lesion site in after crush. CXCR4 is barely detectable in noninjured nerves, but its signal strongly increases in the axonal compartment after damage where it co-localizes with neurofilament (NF). Neurons negative for CXCR4 are likely to be those neurons whose axons were spared by the crush, or that were already beyond the regeneration front. Also CXCL12 α expression increases in the crushed area 3 and 7 days after injury (D), but in this case the signal mainly colocalizes with GFP, suggesting expression mainly by SC. However, given the known biological activities of CXCL12 α and the inflammatory reaction triggered by nerve compression, it is possible that inflammatory cells recruited in the damaged area, as well as resident cells such as endoneural, endothelial cells and fibroblasts, do express CXCL12 α , thus explaining the staining pattern at 3 days post crush (Fig. 1 panel D). We did not pursue further this aspect as we mainly focused on the signaling axis formed by CXCL12 α and CXCR4 expressed by SC and neurons, respectively, following axonal damage. The comparison of the crushed nerves at 3 and 7 days indicates that the increase in CXCL12 α expression induced by the injury is transient, with the chemokine almost disappearing after 7 days. At variance, the expression of CXCR4 is more persistent, a finding that speaks in favor of a therapeutic use of a CXCR4 agonist.

The CXCL12 α -CXCR4 axis is involved in the functional recovery upon crush

To assess the functional role of the CXCL12 α -CXCR4 axis in the recovery from nerve injury we measured the compound muscle action potential (CMAP) in gastrocnemius muscles of crushed CD1 mice treated either with a specific antibody neutralizing CXCL12 α , or with AMD3100, a specific CXCR4 antagonist, as schematically indicated in Figure 2A. Both treatments cause a strong delay in neuro-transmission recovery. This is quantified in panel B of Figure 2, which reports the CMAP areas of injured nerves (2 weeks after crush), showing that the inhibition of either components of the axis delays regeneration.

The CXCR4 agonist NUCC-390 promotes neuromuscular recovery after sciatic nerve crush

The finding that CXCL12 α is expressed for a limited time period after nerve injury (Fig. 1D) suggests that it may function as initiator of the repair process. At the same time, as CXCR4 expression persists longer (Fig. 1C), even when its ligand is no more detectable, we reasoned that a prolonged stimulation of the receptor might foster regeneration. As CXCL12 α displays a very poor pharmacokinetics

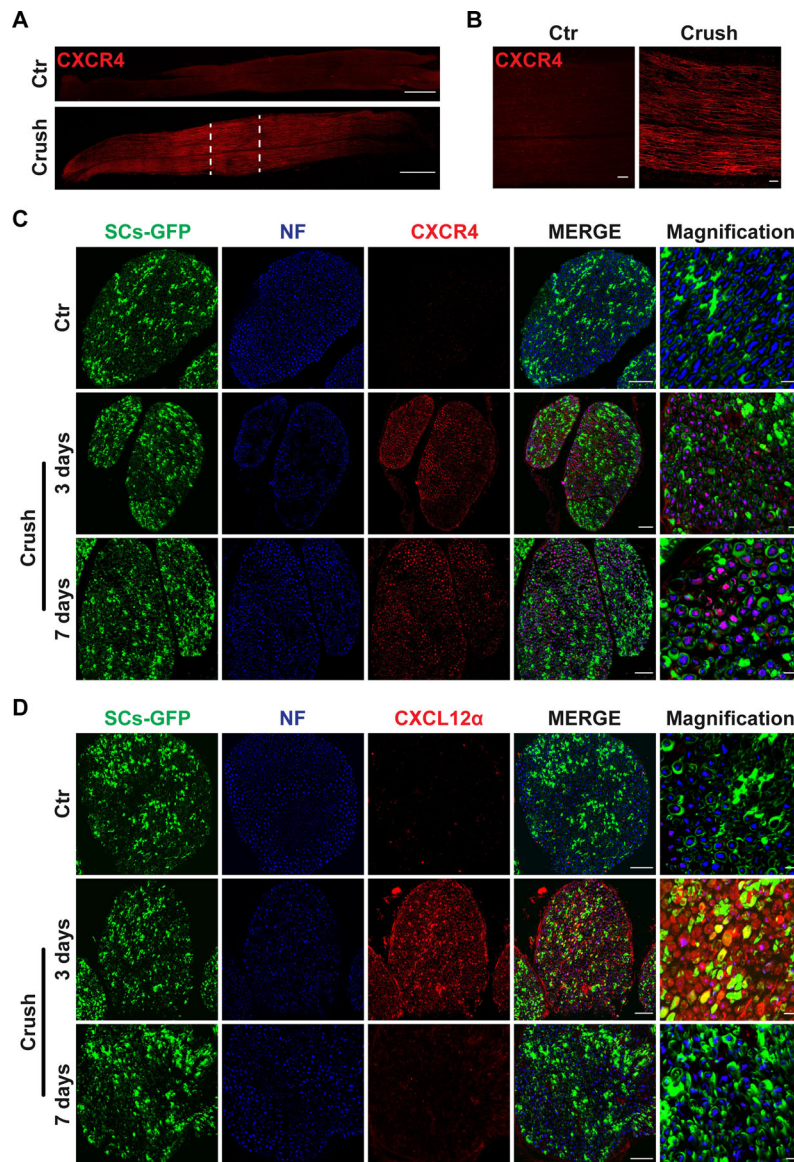


Figure 1. CXCR4 and CXCL12 α are expressed at the crushed site of the sciatic nerve. (A) Longitudinal cryo-sections of control (top) and crushed (bottom) sciatic nerves show CXCR4 expression (red). Dotted lines define the crushed area (the proximal site of the injury is on the left). (B) Higher magnification of the crush site. Scale bars: 500 μ m (A), 50 μ m (B). (C) Cross-sections of sciatic nerves from mice expressing cytosolic GFP (green) specifically in SC, of controls (top panels), and after 3 (middle panels) or 7 days (bottom panels) from nerve crush. Neurofilament (NF) staining (blue) identifies the axonal compartment; CXCR4 is in red. CXCR4 co-localizes with NF, restricting its expression to the axonal compartment. Scale bars: 50 μ m. Right panels show higher magnifications (scale bar: 10 μ m). (D) Cross-sections of sciatic nerves show CXCL12 α expression (red) at the crushed site 3 and 7 days after crush. Right panels show higher magnifications. Scale bars: 50 μ m (right panels: 10 μ m). Images are representative of at least three independent sets of experiments.

in vivo,¹⁹ we treated crushed mice with the chemical compound NUCC-390, which was recently shown to act similarly to CXCL12 α in a cell culture system.²¹ CMAP areas measured in gastrocnemius muscles 7, 14, and 28 days after injury clearly indicate that NUCC-390 is a good promoter of the functional recovery of the sciatic nerve after crush (Fig. 2D–E). The accelerated recovery of

neurotransmission by NUCC-390 is also indicated by the faster disappearance of the multiple peaks beside the main biphasic shape in the CMAP trace, indicative of a synchronous stimulation of the muscle fibers upon electrical stimulation of the entire nerve. Such a pharmacological effect arises from the specific interaction of NUCC-390 with CXCR4, as it is prevented by AMD3100, a selective

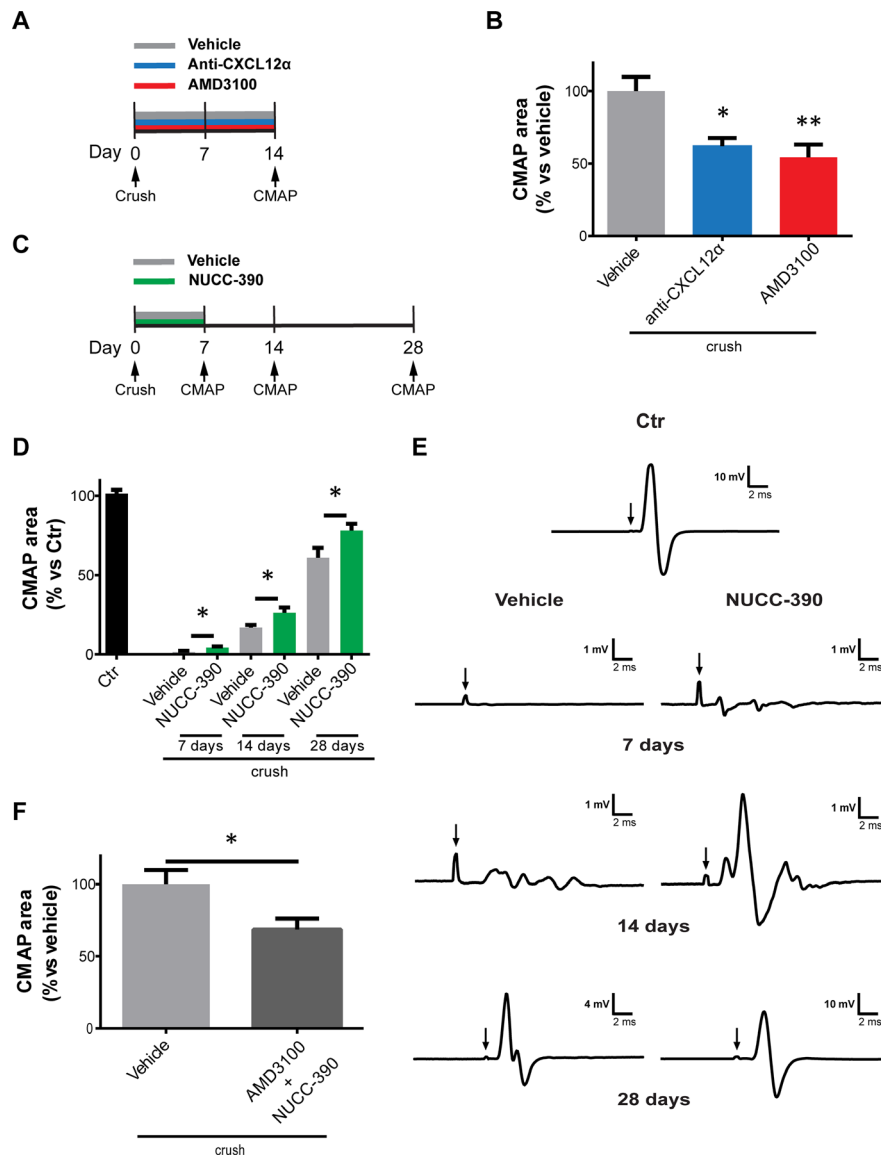


Figure 2. CXCR4 engagement promotes the recovery of neuromuscular activity after sciatic nerve injury. (A) Scheme of the experimental workflow. After sciatic nerve crush mice were treated either with an antibody neutralizing CXCL12 α , or with AMD3100, or with vehicle. CMAP was recorded at day 14. (B) Histogram reports the area of CMAP traces 14 days after sciatic nerve crush. (C) Scheme of NUC-390 administration after sciatic nerve crush, and time-course of CMAP recordings. (D) Histogram reporting the area of CMAP traces at the indicated time points after sciatic nerve crush (\pm NUC-390). (E) Representative CMAP traces at different time points after sciatic nerve crush (\pm NUC-390). Black arrows indicate the stimulation artifact. For each trace, the scale of CMAP amplitude is reported on the right. (F) Histogram showing the area of CMAP traces 14 days after sciatic nerve crush in mice treated either with vehicle, or with NUC-390 in combination with AMD3100. [Correction added on 06 December 2019 after first online publication: Figure 2 has been updated.]

antagonist of the CXCR4 receptor (Fig. 2F). These results suggest that NUC-390 holds a great potential as therapeutics for damaged peripheral nerves.

NUCC-390 stimulates axonal elongation

By using an *in vitro* system consisting of primary cultures of spinal cord motor neurons (SCMNs) we found that

NUCC-390 induces elongation of motor axons (Fig. 3A,B), and that this activity is mediated by CXCR4, being it blocked by AMD3100 (Fig. 3C,D).

The axonal elongation activity of NUC-390 is displayed also *in vivo* after nerve transection (an injury that cuts both axons and BL, making regeneration incomplete) as indicated by the evident staining of GAP43, a biochemical marker of active axonal re-growth (Fig. 3E).²⁸

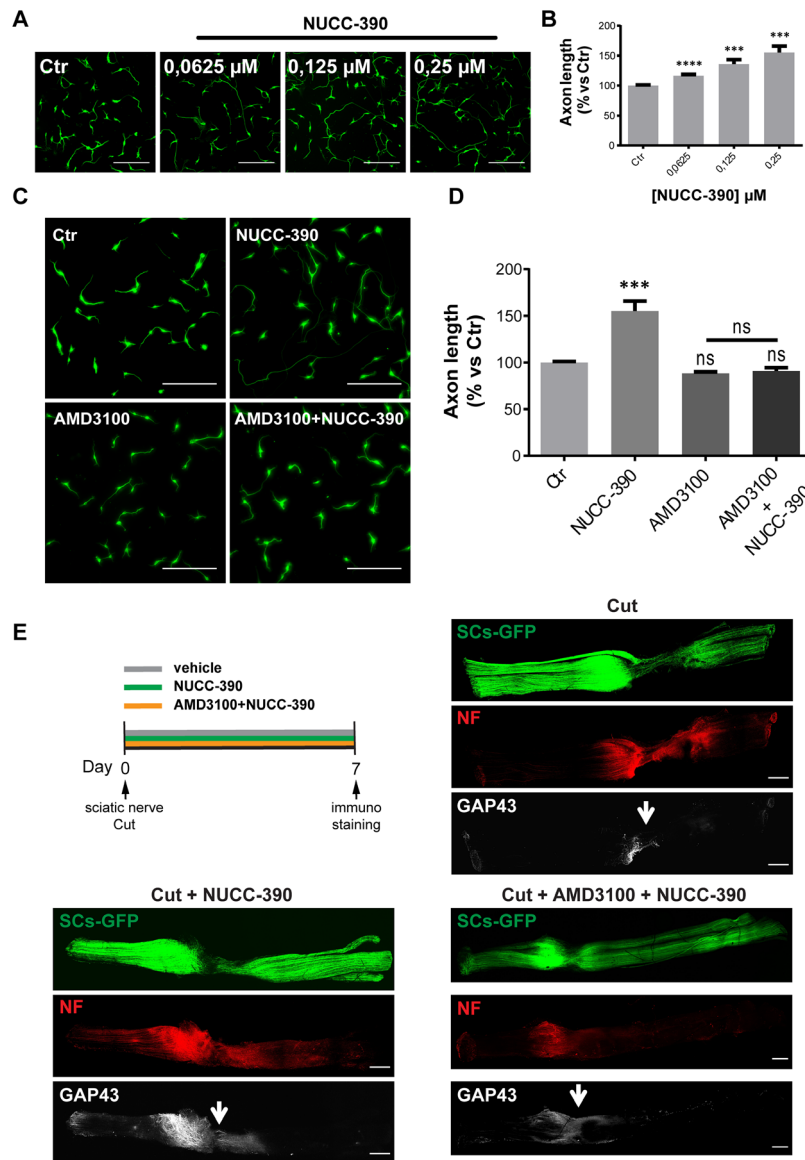


Figure 3. NUCC-390 stimulates elongation of motor axons *via* CXCR4. (A) Rat spinal cord motor neurons (SCMNs) were treated for 24 h with the indicated concentrations of NUCC-390 added to the culture medium, then fixed and stained for β_3 -tubulin (green) to identify axons. Scale bars: 200 μ m. (B) Axons were traced and their length measured as a function of NUCC-390 concentration. Histogram represents mean values reported as a percentage of untreated controls (Ctr). Bars represents the SEM calculated from at least three independent experiments. (C) After SCMNs adhesion, the culture medium was conditioned with either 0.25 μ mol/L NUCC-390, or 10 μ mol/L AMD3100, or their combination. Twenty-four hours later neurons were stained as above, and axon length measured as in A. Scale bars: 200 μ m. (D) Quantification of C. (E) Scheme of the experimental workflow. Sciatic nerve transection was performed in mice expressing the GFP protein in SC cytosol (green), followed by treatment either with vehicle, or with NUCC-390, or with AMD3100, or with their combination. Seven days later the nerve was stained for NF (red) and GAP43 (white) to monitor active axonal re-growth. Scale bars: 500 μ m. [Correction added on 06 December 2019 after first online publication: Figure 3 has been updated.]

NUCC-390 treatment increases the density of axons in active regrowth in the proximal side, and the number of axons entering the bridge area. On the contrary, AMD3100 prevents the pharmacological effect of NUC-390, by reducing both the density and the number of GAP43-positive axons entering the bridge segment connecting the distal and the proximal stumps of the nerve.

This result confirms that NUCC-390 action on axonal regeneration takes place *via* CXCR4.

Discussion

The major findings of the present work are the following: (i) an injury to the sciatic nerve triggers the expression of

the signaling axis CXCL12 α -CXCR4 at the damaged site; (ii) this axis is functionally involved in nerve regeneration because specific inhibition of both components delays the recovery of neuromuscular function; (iii) the CXCR4 agonist NUCC-390 promotes the functional rescue of peripheral nerves by stimulating motor axon elongation. The latter activity, shared by CXCL12 α (the natural CXCR4 ligand) and NUCC-390 (CXCR4 agonist), is suggested by the *in vitro* experiments with cultured motor neurons, by the *in vivo* effects on CMAP rescue, and by the imaging with specific markers. However, one cannot exclude that the *in vivo* effects of CXCL12 α and NUCC-390 may also result from still unknown activities or signaling mediated by CXCR4 in the neuronal cell body within ganglia and in the ventral horn of the spinal cord.

The *in vivo* activity of NUCC-390 described here holds a great potential translational value. In fact, given the ongoing intense research for novel therapies for injured peripheral neurons,^{29,30} these results have relevant clinical implications, as they indicate NUCC-390 as a potential pro-regenerative drug to be tested in different animal models of PNS disorders. We recently found that NUCC-390 strongly promotes the functional recovery of motor nerve terminals after the acute degeneration induced by α -latrotoxin at the neuromuscular junction,²² a model that recapitulates several aspects of autoimmune neuropathies caused by auto-antibodies against peripheral axon antigens.³¹ In addition, NUCC-390 activity could be tested in neurodegenerative conditions, like amyotrophic lateral sclerosis, where fostering regenerative programs may help in slowing down disease progression. If positive results will arise from these studies, NUCC-390 would become a strong candidate therapeutics to be evaluated in human peripheral neurotraumas, neuropathies, and neuromuscular disorders.

Author Contributions

M.R., M.P., and C.M. conceived the project and supervised the study; G.Z., S.N., A.Ma, and A.M. performed the experiments; F.L. and S.F. contributed to data analysis; M.R., M.P., and C.M. wrote the manuscript, with contributions of all authors.

Conflicts of Interest

Nothing to report.

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