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ORIGINAL PAPER

CXCL1 and CXCL2 Inhibit the Axon Outgrowth in a Time- and Cell-Type-Dependent Manner in Adult Rat Dorsal Root Ganglia Neurons

Antonia Teona Deftu[1] · Ruxandra Cioroescu[1] · Roxana-Olimpia Gheorghe[1] · Dan Mihăilescu[1] · Violeta Ristoiu[1]

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Abstract

The ability to regrow their axons after an injury is a hallmark of neurons in peripheral nervous system which distinguish them from central nervous system neurons. This ability is influenced by their intrinsic capacity to regrow and by the extracellular environment which needs to be supportive of regrowth. CXCL1 [Chemokine (C-X-C motif) Ligand 1] and CXCL2 [Chemokine (C-X-C motif) Ligand 2] are two low-molecular-weight chemokines which can influence neuronal proliferation, differentiation and neurogenesis, but which are also upregulated by injury or inflammation. In this study we investigated the effects of long-term incubation (24, 48 and 72 h) with different concentrations of CXCL1 (0.4, 4 or 40 nM) or CXCL2 (0.36, 3.6 or 36 nM) on the axon outgrowth of adult rat dorsal root ganglia neurons in culture. The results showed that both chemokines significantly inhibited the axon outgrowth, with large and medium NF200 (NeuroFilament 200) (+) dorsal root ganglia neurons affected quicker, compared to small IB4 (Isolectin B4) (+) dorsal root ganglia neurons which were affected after longer exposure. Blocking CXCR2 (C-X-C motif chemokine receptor 2) which mediates the effects of CXCL1 and CXCL2 prevented these effects, suggesting that CXCR2 may represent a new therapeutic target for promoting the axon outgrowth after a peripheral nerve injury.

Keywords Chemokine (C-X-C motif) ligand 1 · Chemokine (C-X-C motif) ligand 2 · Axon growth · Dorsal root ganglia neurons

Introduction

After axotomy, neurons in the peripheral nervous system (PNS) are capable of regeneration [1], whereas neurons in the central nervous system (CNS) are generally not [2]. In addition in PNS, even though the axons regeneration can be quite precise [3], sometimes a loss of specificity can occur [4] or the number of axons that reach their targets can be limited [5]. Therefore, understanding which factors can enhance or limit PNS regeneration is very important for developing therapies to treat nerve injury.

Regeneration of axons in vivo is not cell autonomous, rather it is highly influenced by non-neuronal cells, in particular Schwann cells and macrophages [1]. Macrophages act not only locally at the lesion site where they are involved in Wallerian degeneration, but also distantly, at the DRG (dorsal root ganglia) level, where, through a number of secreted molecules they promote the expression of certain regeneration-associated genes in DRG neurons [6, 7].

CXCL1 [Chemokine (C-X-C motif) Ligand 1] and CXCL2 [Chemokine (C-X-C motif) Ligand 2] are two low-molecular-weight members of the ELR (Glu-Leu-Arg: the ELR motif) (+) CXC chemokine family with 78% homology of their sequence [8]. They are secreted by macrophages, but also by neutrophils, microglia, astrocytes or endothelial cells as response to injury or inflammatory signals [8, 9]. Interestingly, CXCL2 was also localized inside cortical neurons after a moderate lateral fluid percussion injury [10], which suggest a neuronal source for this chemokine, as well. Both of them are known to have pleiotropic effects on neurons or immune cells, ranging from stimulating proliferation to increasing pain [11]. In particular, CXCL2 has been shown to significantly increase

✉ Violeta Ristoiu
v_ristoiu@yahoo.com

[1] Department of Anatomy, Animal Physiology and Biophysics, Faculty of Biology, University of Bucharest, Splaiul Independentei 91-95, Sector 5, 050095 Bucharest, Romania

axon outgrowth in embryonic hippocampal neurons downstream of HGF (hepatocyte growth factor) signaling [12], with the same signaling pathway being also involved in the neurite outgrowth in adult rat cultured DRG neurons [13]. There are no data about a direct influence of CXCL1 on neurites outgrowth in any type of neurons, although a significant neurite sprouting was noticed in PC12 cell after treatment with injured intervertebral disc media containing CXCL1 among other pro-inflammatory factors [14]. In addition, CXCL1 can promote axon myelination in multiple sclerosis [15, 16] which may suggest a possible indirect effect on DRG axons as well.

Recently, we have shown that CXCL1 and CXCL2 may interfere with the functioning of TRPV1 (Transient Receptor Potential 1) receptors in TRPV1 (+)/IB4 (Isolectin B4) (+) DRG neurons, either directly or by altering their desensitization rate [11, 17, 18]. These effects of CXCL1 and CXCL2 on TRPV1 receptors are important because studies have shown that TRPV1 receptors are physically and functionally present at dynamic neuronal extensions, including growth cones of embryonic and adult DRG neurons. Activation of TRPV1 at this level inhibits sensory neuronal extension and reduces motility by facilitating the disassembly of microtubules [19, 20].

Given all these data, it is conceivable that CXCL1 and CXCL2 may also influence the neurite/axon outgrowth in the adult rat DRG neurons. Possibly secreted by the macrophages infiltrated in a DRG after a peripheral lesion, CXCL1 and CXCL2 might represent some of the components of the extracellular medium which could influence the regenerative capacity of DRG neurons. In this study we investigated the effects of long-term incubation (24, 48 and 72 h) with different concentrations of CXCL1 and CXCL2 on the axon outgrowth of adult rat DRG neurons in culture. The results showed that both chemokines significantly inhibited the axon outgrowth, with large and medium diameter neurons affected quicker, compared to small diameter neurons which were affected after longer exposure.

Materials and Methods

Animals

For this study, 36 adult male Wistar rats (100–150 g) from the animal facility of the "Ion Cantacuzino" National Institute, Bucharest, Romania were used. All procedures were carried out in accordance with the Directive 2010/63/EU and the Romanian Law 43/2014 on the use of animals for scientific purposes, and were approved by the Ethics Committee of the Faculty of Biology, University of Bucharest (Approval No. 34/10.07.2019).

Cell Culture

Rats were sacrificed by inhalation of 100% CO_2 followed by decapitation. DRG from all spinal segments were removed and prepared for the cell culture as previously described [21], since this culture model is well established and suitable for neurite outgrowth studies [22, 23]. Briefly, neurons were dissociated in 1 mg/ml collagenase IA and 2 mg/ml dispase (Gibco, 17105041) for 1 h at 37 °C, then plated on pretreated 13-mm glass coverslips and cultured in a NGF-free 1:1 mixture of 7.4 mM glucose DMEM (D5523) and Hams's F10 medium (N6635) with 10% horse serum, 0.5% Penicillin/Streptomycin and 1% L-glutamine (Thermo Fisher Scientific, A1286001) for 24 h, at 37 °C, in 5% CO_2 in air. In order to increase the adherence to the substrate and facilitate the neurite growth, the glass coverslips were first treated with H_2SO_4 for 24 h, washed with distilled water and ethanol 100%, heat-dried in a flame and kept in ethanol 100% until use. In the day of the culture, the coverslips were coated first with poly-D-lysine (0.1 mg/ml, P0899) for 30 min at room temperature, then with laminin (10 µg/ml, L2020) for 2 h at room temperature [24] and maintained in phosphate buffer (PBS) until plating. If not otherwise specified, all reagents were from Sigma. After 24 h the neurons were treated with 0.4, 4 or 40 nM CXCL1 (Promokine, E-65420) or 0.36, 3.6 or 36 nM CXCL2 (Promokine, E-65430) for 24, 48 or 72 h. The concentrations were established based on a previous study in which we identified an EC_{50} of 4 nM for CXCL1 and 3.6 nM for CXCL2 as effective on primary DRG neurons after 4 h of incubation [18]. Consequently, in this study we tested the effect of long term-incubation with CXCL1 and CXCL2 at concentrations below, at or above the EC_{50} concentration. The contribution of CXCR2 (C-X-C motif chemokine receptor 2) in mediating the effects of CXCL1 and CXCL2 on the axon outgrowth was confirmed using SB265610 (Tocris, 2724), a CXCR2 antagonist.

Cell Viability Assay

Primary DRG culture cells were plated into 96-well plates (3×10^3 cells/well) in 100 µl of medium and were allowed to adhere overnight. Then the cells were subjected to drug treatments for the indicated times. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the cleavage of the yellow tetrazolium salt to purple formazan crystals by mitochondrial and cytosolic dehydrogenases of living cells. Briefly, cells were incubated with MTT solution (1 mg/ml, Sigma, M2128,) in

serum- and antibiotics-free culture medium for 3 h. The culture medium was then removed, and the formazan product was solubilized by DMSO. Absorbance at wavelengths of 540 and 690 nm (reference) was measured with a microplate reader (FlexStation 3, Molecular Devices, USA). The values were expressed as percentage of cell survival relative to the untreated cells.

Immunocytochemistry (ICC)

Cells cultured on coverslips were fixed in 4% paraformaldehyde solution (Roth) in 0.01 M PBS for 20 min, permeabilized with a 0.3% Triton X-100 solution in PBS for 1 h and incubated for 1 h in blocking solution (0.3% Triton X-100 and 4% normal goat serum in PBS). After overnight incubation at 4 °C with the primary antibodies, cells were washed, incubated for 1 h with the secondary antibodies and cover-slipped with Prolong Gold antifade reagent (Life Technologies, P36931). The slides were visualized under an Olympus IX73 (Carl Zeiss, Germany) fluorescence microscope and processed with Image-J software (Wayne Rasband, National Institute of Health, SUA). The primary antibodies were anti-βIII-tubulin (1:1000, mouse monoclonal, Abcam, ab78078), anti-NF200 (1:400, mouse monoclonal, Sigma, N0142) and biotinylated isolectin B4 (1:200, Vector Laboratories, B-1205). The secondary antibodies were (from Life Technologies): goat anti-mouse Alexa Fluor 568 (1:1500, A11004), goat anti-mouse Alexa Fluor 488 (1:1500, A11001) and Streptavidin-Alexa Fluor 488 Conjugate (1:1500, S32354).

Axon length was measured using Image J's plugin *Simple Neurite Tracer* (https://imagej.net/Simple_Neurite_Tracer, ver 3.1.3), with axon length defined as the distance from the soma to the end of the longest neurite (if there was more than one neurite) or to the end of the longest branch at each branch point for branched neurites. Only the cells with at least a neurite longer than the diameter of the cell body and with a constant fluorescence along its length, which were not in contact with other cells or at the very edge of the coverslip, were considered. Typically, 15–20 neurons were measured/condition in ≥ 3 independent experiments. Images were inverted using Photoshop CC 2015 software and are shown on a white background for a clearer visualization of their morphology. The average axon length for each group was calculated to evaluate the extent of neuronal differentiation and compared to the corresponding untreated control.

Statistical Analysis

All data were given as means ± SEM, statistical significance was tested using a two tailed Student's t-test and 1 way ANOVA with Bonferonni post-test (GraphPad Prism 5.0 software). A value of $P < 0.05$ was considered to be statistically significant, with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Results

CXCL2, but not CXCL1, had a Cytotoxic Effect at Higher Concentrations

Before testing the effect of different concentrations of chemokines on axon outgrowth, we investigated first if any of them had a cytotoxic effect on cell viability. The MTT test indicated that CXCL1, at all concentrations (0.4, 4 and 40 nM) and at all time points (24, 48 and 72 h), had no cytotoxic effect on cell viability (Fig. 1A). Even more, after 72 h of incubation, smaller concentrations of CXCL1 seemed to have a beneficial effect, with cell viability significantly increased after 0.4 and 4 nM treatment (Fig. 1A). In contrast, treatment with CXCL2 showed no cytotoxic effects only for 0.36 and 3.6 nM concentrations at all time points, while 36 nM seemed to be cytotoxic as early as one day of treatment (Fig. 1B). Therefore, for CXCL2 we decided to go further only with the non-toxic concentrations, 0.36 and 3.6 nM.

CXCL1 and CXCL2 Inhibits Axon Outgrowth in Adult Rat Dorsal Root Ganglia Neurons

In order to investigate if extended presence of CXCL1 and CXCL2 chemokines around dorsal root ganglia neurons might have morphological effects on them, axon length was measured at 3 different time points (24, 48 and 72 h) after treatment with different concentrations of CXCL1 (0.4, 4 and 40 nM) or CXCL2 (0.36 and 3.6 nM). The neurons were stained with anti-βIII-tubulin antibody, a pan-neuronal marker of cell cytoskeleton which can't distinguish between the small, medium or large sub-populations of DRG neurons. The results showed that both CXCL1 (Figs. 2 and 4A) and CXCL2 (Figs. 3 and 4B) significantly decreased axons outgrowth. In control conditions, all DRG neurons showed a progressive increase in axons length with the time in culture. Upon CXCL1 treatment, starting from the first day of treatment, all concentrations significantly inhibited the axon outgrowth which was not compensated by the time spent in culture: after 72 h the inhibitory effect was even stronger than after one day of treatment (Fig. 4A). Similarly, all concentrations of CXCL2 significantly inhibited the axon outgrowth from the first day of treatment, but in contrast to CXCL1 the effect was not as persistent and it was compensated by the time in culture: after 72 h the inhibitory effect although present was less strong than after one day of treatment (Fig. 4B). Table 1 (first row, β3T (+) neurons) summarizes the mean axons' length values after CXCL1 treatment,

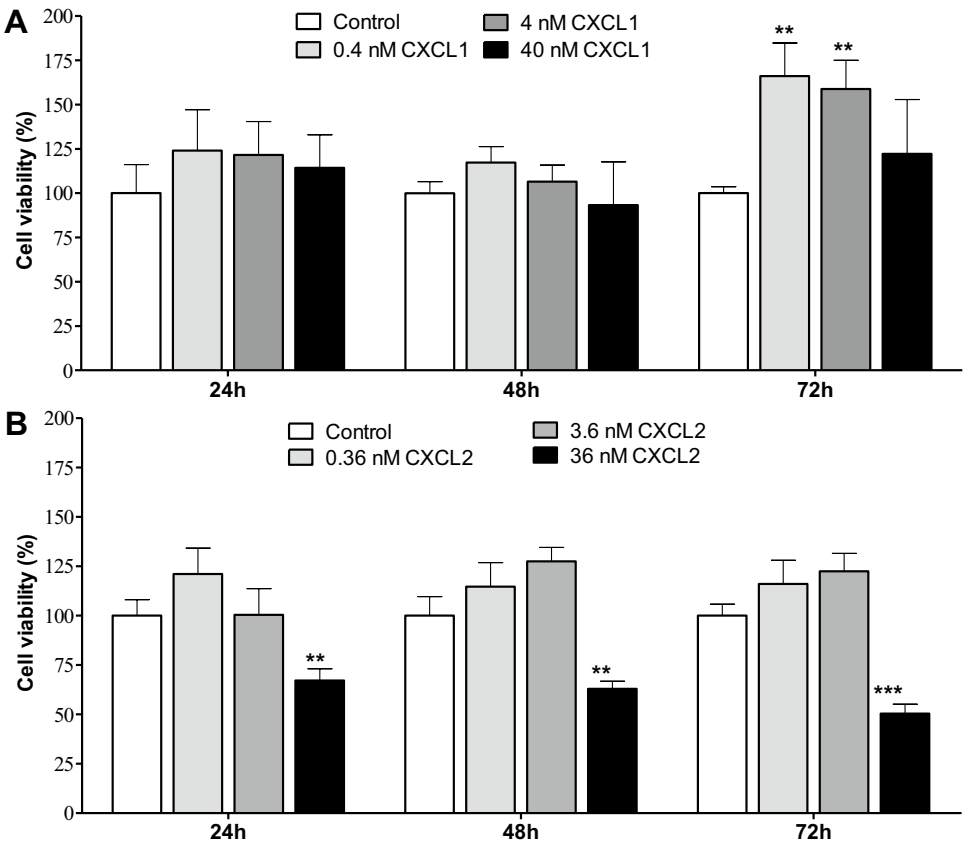

Fig. 1 CXCL2 is more cytotoxic at higher concentrations than CXCL1. Primary culture of DRG neurons were treated with different concentrations of CXCL1 and CXCL2 for 24, 48 and 72 h, and cell viability was measured by the MTT colorimetric assay. **A** CXCL1 had no cytotoxic effect at any concentration and at all time points. **B** CXCL2 had beneficial effects on cell viability at smaller concentrations (0.36 and 3.6 nM) and at all time points, and significant cytotoxic effect at higher concentrations (36 nM) and all time points (**$P<0.01$, ***$P<0.001$)

and Table 2 (first row, β3T (+) neurons) summarizes the mean axons' length values after CXCL2 treatment.

Axons of NF200 (+) DRG Neurons were Affected Faster by CXCL1 and CXCL2

To distinguish which of the subpopulations of DRG neurons were more sensitive, the axon outgrowth in large NF200 (+) DRG neurons was evaluated for the same time points and concentrations of CXCL1 or CXCL2. The neurons were stained with anti-NF200 antibody to identify the cell type and measure the axon outgrowth. NF200 is a neurofilament with high molecular weight (200 kD), particularly abundant in the axons of large and medium DRG neurons [25].

The results showed that NF200 (+) DRG neurons were significantly more sensitive to both CXCL1 (Fig. 5) and CXCL2 (Fig. 6) after 24 and 48 h of treatment, with axon outgrowth significantly inhibited (Fig. 7A and B). After 48 h mainly the lower concentrations, i.e. 0.4 and 4 nM for CXCL1 (Fig. 7A) and 0.36 nM for CXCL2 (Fig. 7B) continued to be inhibitory, while larger concentrations, i.e. 40 nM for CXCL1 and 3.6 nM for CXCL2 lost their inhibitory properties. After 72 h of exposure to CXCL1 and CXCL2, large DRG neurons seemed to become insensitive to all concentrations of the two chemokines (Fig. 7A and B). Interestingly, the axon outgrowth in control conditions did not show the progressive increase in length with the time in culture as noticed after staining with anti-βIII-tubulin antibody (Fig. 4), as if the large NF200 (+) neurons grow their axon to their maximum from the very first day in culture, and after that just maintain them, not extend anymore. This is most likely the consequence of laminin coating which was shown to facilitate neurite outgrowth in NF200 (+) cells [26]. Table 1 (2nd row, NF200 (+) neurons) summarizes the mean axons' length values after CXCL1 treatment, and Table 2 (2nd row, NF200 (+) neurons) summarizes the mean axons' length values after CXCL2 treatment.

Axons of Small IB4 (+) DRG Neurons are Affected Later by CXCL1 and CXCL2

To understand who is responsible for the inhibitory effect of CXCL1 and CXCL2 on axon outgrowth after 72 h of incubation, we investigated the axon outgrowth in small IB4 (+) DRG neurons for the same time points and concentrations of CXCL1 (Fig. 8) or CXCL2 (Fig. 9). The neurons were stained with isolectin B4 and anti-βIII-tubulin antibodies to identify the cell type and also measure the axon outgrowth. Isolectin B4 is a lectin from *Griffonia simplicifolia*, used as a specific marker for a subpopulation of non-peptidergic small DRG neurons [27].

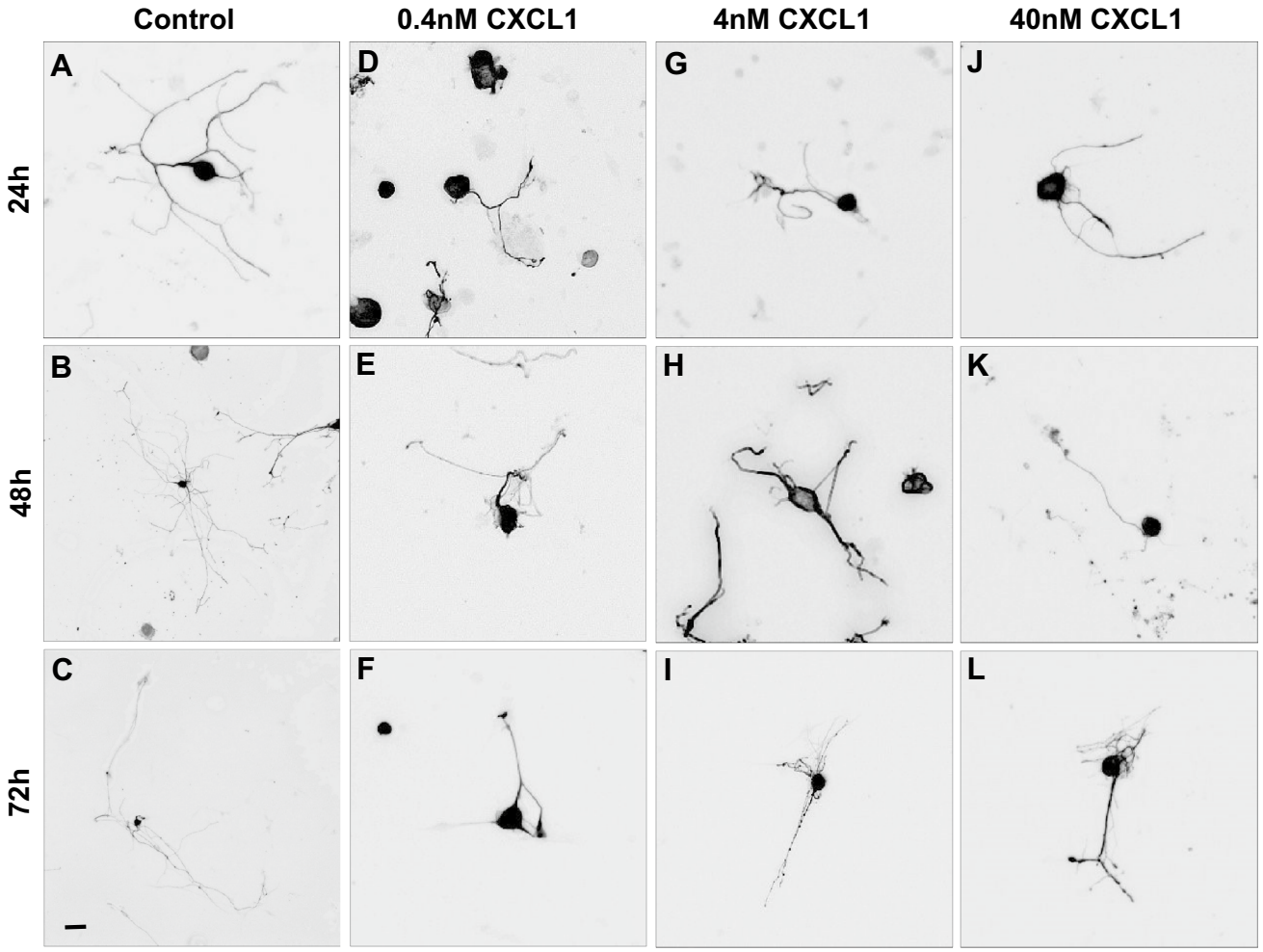

Fig. 2 Representative images of βIII-tubulin (+) adult DRG neurons in control conditions or after treatment with different concentrations of CXCL1 for 24, 48 or 72 h. Scale bar for images **B** and **C** = 100 µm; for the rest = 20 µm

The results showed that the axon outgrowth in small IB4 (+) DRG neurons was not affected after 24 h of treatment, but it started to be inhibited after 48 h and it was maintained after 72 h of continuous treatment (Fig. 10A and B). In contrast to NF200 (+) neurons, small IB4 (+) DRG neurons were significantly inhibited by all concentrations of CXCL1 and CXCL2 after 48 h of treatment and maintained the same response after 72 h of incubation. The strong inhibitory effect of CXCL2 at 72 h on IB4 (+) DRG neurons, combined with a lack of effect on NF200 (+) neurons, could explain the results in Fig. 4B in which the inhibitory effect although present, was less strong at 72 h than after one day of treatment. Interestingly, the axon outgrowth in control conditions showed the same progressive increase in length with the time in culture, although at a much smaller scale, as noticed after staining with anti-βIII-tubulin antibody (Fig. 4). This was somehow in contrast with previous studies in which laminin coating, without any neurotrophic factors, was unable to support neurite outgrowth in IB4 (+) DRG neurons [26]. Most probably this response was a consequence of the pre-coating treatment of the coverslips with H_2SO_4 which better prepared the surface for cell adherence. Table 1 (3rd row, IB4 (+) neurons) summarizes the mean axons' length values after CXCL1 treatment, and Table 2 (3rd row, IB4 (+) neurons) summarizes the mean axons' length values after CXCL2 treatment.

Blocking CXCR2 Prevented CXCL1 and CXCl2 Inhibitory Effects of Axon Outgrowth

To confirm that the inhibitory effects of CXCL1 and CXCL2 on axon outgrowth were mediated by CXCR2, additional experiments using SB265610 (a specific CXCR2 antagonist) have been performed. The effect on large NF200 (+) DRG neurons was tested after 24 h co-treatment with 1 µM SB265610 and 4 nM CXCL1 or 3.6 nM CXCL2, while the effect on small IB4 (+) DRG neurons was tested after 48 h co-treatment with 1 µM SB265610 and 4 nM CXCL1 or

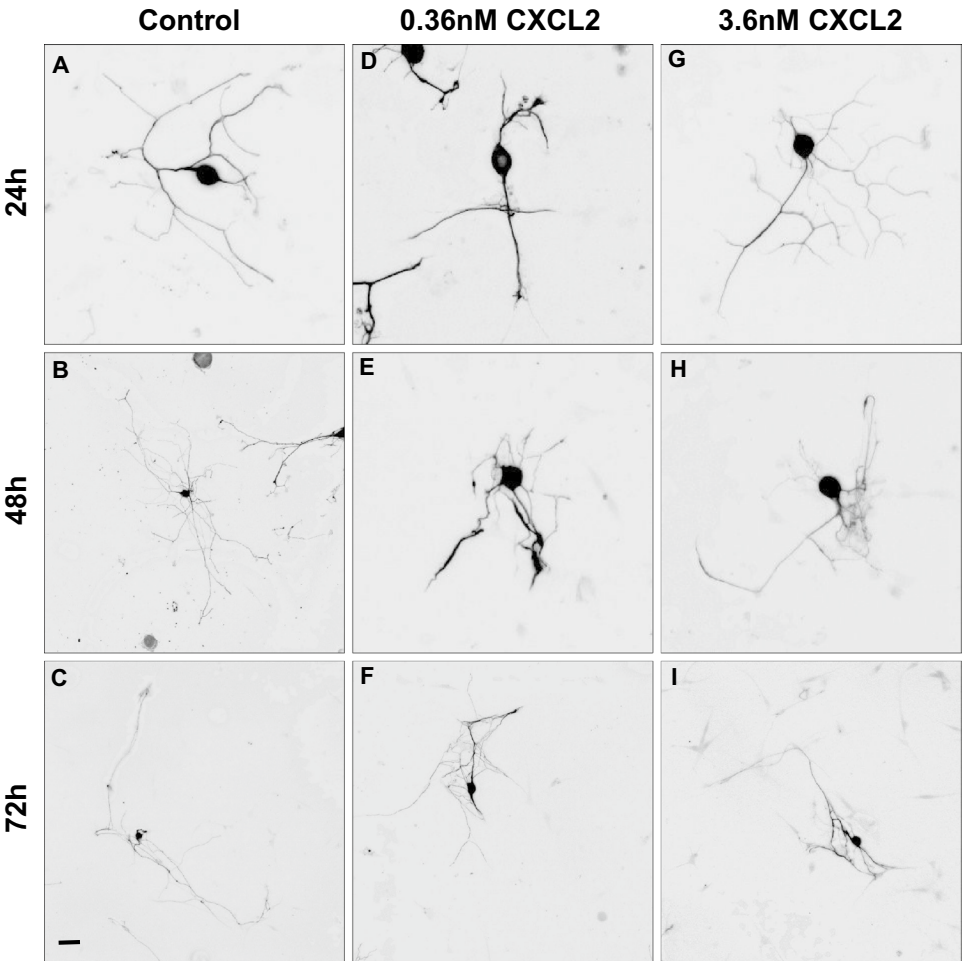

Fig. 3 Representative images of βIII-tubulin (+) adult DRG neurons in control conditions or after treatment with different concentrations of CXCL2 for 24, 48 or 72 h. Scale bar for images **B** and **C** = 100 µm; for **F** and **I** = 50 µm; for the rest = 20 µm

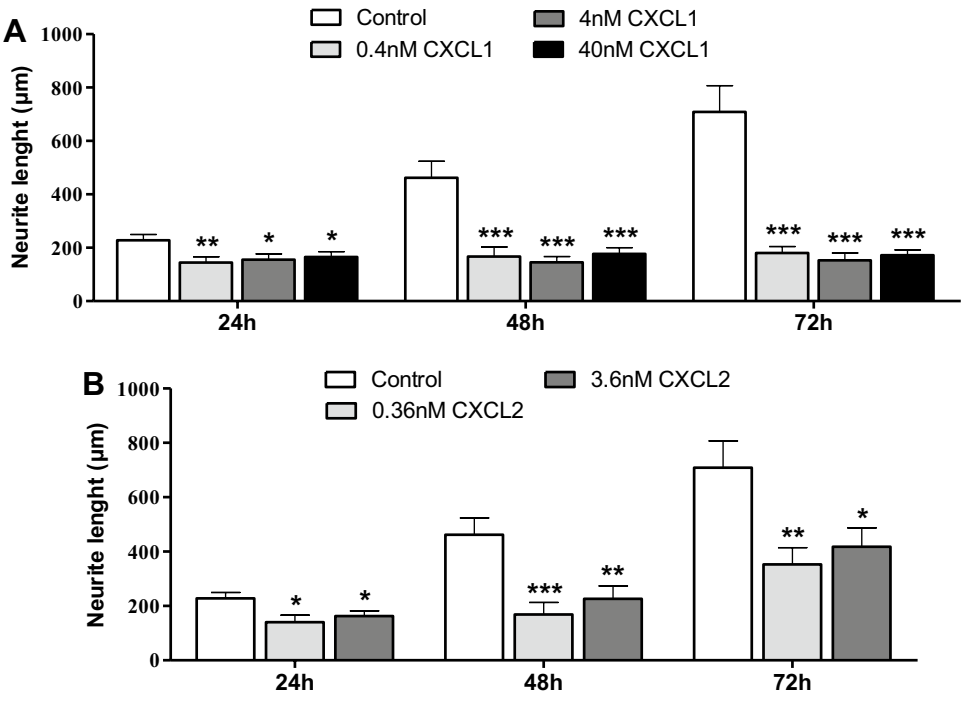

Fig. 4 Both CXCL1 and CXCL2 inhibited axon outgrowth starting from the first day of treatment. **A** Graph bar representing mean βIII-tubulin (+) axon lenght in control conditions or after treatment with different concentrations of CXCL1 for different time intervals. **B** Graph bar representing mean βIII-tubulin (+) axon lenght in control conditions or after treatment with different concentrations of CXCL2 for different time intervals. In both cases, the inhibition was significant compared to the control conditions for all time points (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$)

Table 1 Axon outgrowth after treatment with CXCL1 chemokine

Population type	Control			0.4 nM CXCL1			4 nM CXCL1			40 nM CXCL1		
	Mean (μm)	SEM	N	Mean (μm)	SEM	N	Mean (μm)	SEM	N	Mean (μm)	SEM	N
β3T (+) neurons	228	21.66	20	144.9	21.25	20	155.3	22.07	20	212.8	26.64	20
	462.3	61.3	20	166.8	36.04	20	145.2	21.77	20	177.1	23.32	20
	709.1	98.3	17	180.6	24.26	20	153	27.45	20	172.3	19.5	20
NF200 (+) neurons	366.5	46.95	20	184.1	25.19	20	144	15.28	20	221.9	40.07	20
	381.5	59.34	20	209.5	35.61	20	220.6	52.65	20	292.6	44.01	20
	399.6	68.79	17	267.1	44.76	20	273.6	40.26	20	382.2	63.72	20
IB4 (+) neurons	87.89	10.79	20	75.49	9.181	20	85.52	7.99	20	81.28	9.17	20
	168.1	19.81	17	91.36	14.02	20	102.4	15.65	20	98.97	11.18	20
	286.7	62.96	15	140.2	26.95	17	81.42	8.03	20	96.8	16.54	16

Table 2 Axon outgrowth after treatment with CXCL2 chemokine

Population type	Control			0.36 nM CXCL2			3.6 nM CXCL2		
	Mean (μm)	SEM	N	Mean (μm)	SEM	N	Mean (μm)	SEM	N
β3T (+) neurons	228.00	21.66	20	140.10	25.74	20	162.70	19.57	19
	462.30	61.30	20	168.50	44.32	20	226.40	47.05	20
	709.10	98.30	17	353.10	61.73	20	417.90	68.81	20
NF200 (+) neurons	366.50	46.95	20	150.70	31.54	18	180.70	20.66	20
	381.50	59.34	20	231.10	33.19	20	303.10	36.15	20
	399.60	68.79	17	284.50	45.36	20	313.40	39.45	20
IB4 (+) neurons	87.89	10.79	20	93.91	12.42	20	79.87	12.61	20
	168.10	19.81	17	100.40	11.64	20	106.40	16.99	15
	286.70	62.96	15	122.70	16.73	20	89.29	13.56	17

3.6 nM CXCL2. Only one time point was chosen for these experiments because for NF200 (+) DRG neurons the effect was the strongest after 24 h exposure to the two chemokines, while for IB4 (+) DRG neurons the inhibitory effects were as strong at 48 h as after 72 h of chemokine treatment. Only one concentration was selected for the two chemokines (i.e.: 4 nM for CXCL1 and 3.6 nM for CXCL2) because it represents the EC_{50} for CXCL1 or CXCL2 established as effective on primary DRG neurons in a previous study [18], and we considered them as the most representative.

The results showed that 1 μM SB265610 prevented the inhibitory effects of both CXCL1 and CXCL2 on NF200 (+) DRG neurons, with the axon length not statistically different compared to control conditions: for CXCL1 treatment, axon length in control conditions = 366.50 ± 46.95 μm (n = 20), and after 4 nM CXCL1 + 1 μM SB265610 = 308.20 ± 23.99 μm (n = 20), $P > 0.05$ (Fig. 11A and B); for CXCL2 treatment, axon length in control conditions = 366.50 ± 46.95 μm (n = 20), and after 3.6 nM CXCL2 + 1 μM SB265610 = 250.70 ± 34.10 μm (n = 20), $P > 0.05$ (Fig. 11C and D).

Similarly, on IB4 (+) DRG neurons 1 μM SB265610 prevented the inhibitory effects of both CXCL1 and CXCL2, with the axon length not statistically different compared to control conditions: for CXCL1 treatment, axon length in control conditions = 168.10 ± 19.81 μm (n = 17), and after 4 nM CXCL1 + 1 μM SB265610 = 196.70 ± 20.22 μm (n = 20), $P > 0.05$ (Fig. 12A and B); for CXCL2 treatment, axon length in control conditions = 168.10 ± 19.81 μm (n = 17), and after 3.6 nM CXCL2 + 1 μM SB265610 = 247.2 ± 40.45 μm (n = 15), $P > 0.05$ (Fig. 12C and D).

Discussion

In this study we showed that long-term treatment with CXCL1 or CXCL2 chemokines had an inhibitory effect on axon outgrowth of the adult rat DRG neurons, with large and medium neurons responding faster than small DRG neurons.

The ability to regrow their axons after an injury is a hallmark of neurons in PNS which distinguish them from CNS neurons [1, 2]. This ability is influenced by their intrinsic capacity to regrow, which is better for PNS neurons than for CNS neurons [28], and by the extracellular environment which needs to be supportive of regrowth. Previous studies on rats have showed that adult CNS neurons can regrow their severed axon if the non-permissive CNS environment is

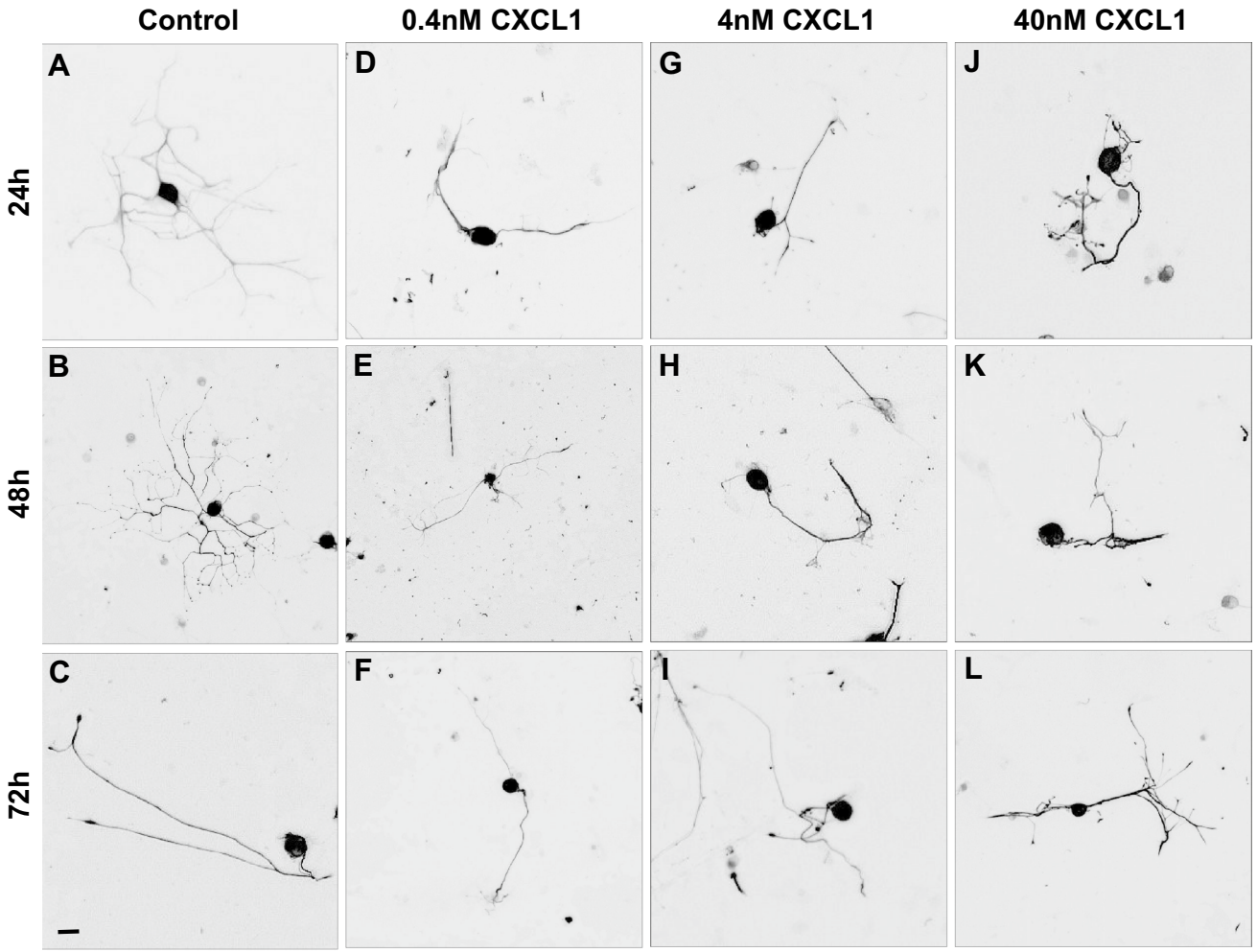

Fig. 5 Representative images of NF200 (+) adult DRG neurons in control conditions or after treatment with different concentrations of CXCL1 for 24, 48 or 72 h. Scale bar for images **B**, **E**, **F** and **I** = 50 µm; for the rest = 20 µm

manipulated to induce regeneration [29, 30], suggesting that the environment is a key player in the regeneration process.

Chemokines are small proteins classified into four subgroups referred to as CXC/α, CC/β, CX3C/δ or C/δ families [31]. They are mostly known for their role in immunesurveillance and inflammatory responses, but they have been also implicated in proliferation, neurogenesis and neuronal differentiation of neural precursors [32]. In particular, **CXCL1** promotes neurogenesis in the developing rat ventral midbrain [33] and in the adult hippocampal dentate gyrus of mouse [34], it facilitates the adoption of a neuronal fate in human embryonic stem cells [35] and regulates differentiation of adult murine neural precursor towards oligodendrocyte [36]. There are no data about a direct influence on neurites outgrowth in any type of neurons, but it may promote neurites sprouting in PC12 cell upon treatment with an injury-conditioned medium containing CXCL1 among other pro-inflammatory factors [14]. On the other hand, **CXCL2** was not directly involved in neurogenesis, but it was shown to promote olfactory ensheathing cells proliferation which remove by phagocytosis degenerated and apoptotic neural tissue debris [37] and to be neuroprotective against amyloid beta-induced neurotoxicity in mice [38]. In contrast to CXCL1, CXCL2 has been shown to significantly increase axon outgrowth in embryonic hippocampal neurons downstream of HGF signaling [12], with the same signaling pathway being also involved in the neurite outgrowth in adult rat cultured DRG neurons [13].

Usually, CXCL1 and CXCL2 are not part of a normal environment inside a dorsal root ganglia, but their expression can be upregulated after an inflammatory reaction or a peripheral nerve lesion. Thus, CXCL1 shows strong, rapid upregulation in dorsal root ganglion in both nerve injury and inflammatory models [39–41], but the data for CXCL2 are not very clear and may strongly depend of the type of lesion [42, 43]. The source of CXCL1 and CXCL2 in the DRG neurons could be the macrophages, which after an inflammatory reaction or a peripheral nerve lesion strongly

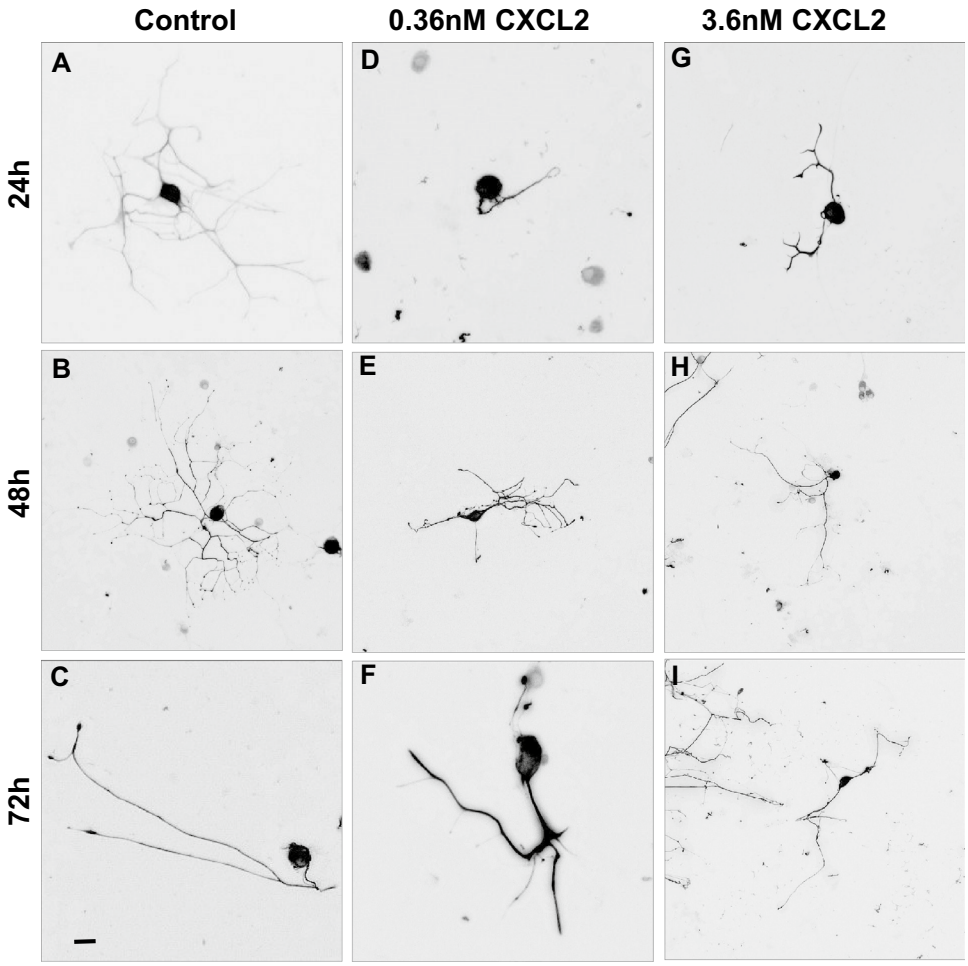

Fig. 6 Representative images of NF200 (+) adult DRG neurons in control conditions or after treatment with different concentrations of CXCL2 for 24, 48 or 72 h. Scale bar for images **B**, **E**, **G**, **H** and **I** = 50 µm; for the rest = 20 µm

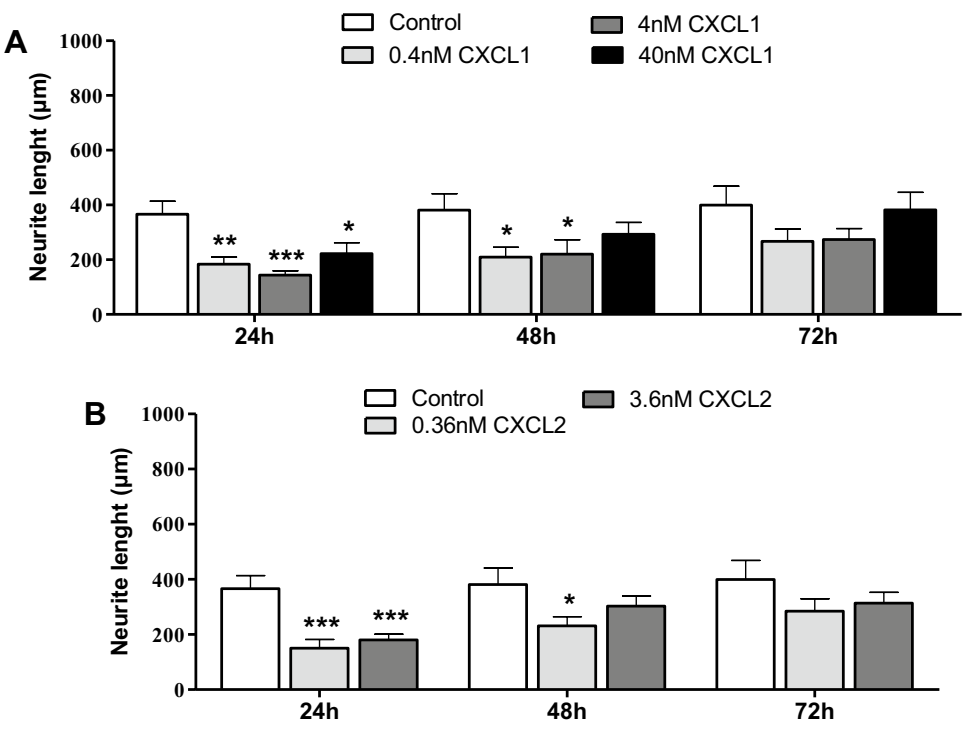

Fig. 7 Axons of NF200 (+) DRG neurons are affected first by CXCL1 and CXCL2. **A** Graph bar representing mean axons' lenght in control conditions or after treatment with different concentrations of CXCL1 for different time intervals. **B** Graph bar representing mean axons' lenght in control conditions or after treatment with different concentrations of CXCL2 for different time intervals. In both cases, the inhibition was significant compared to the control conditions only for 24 h and 48 h (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$)

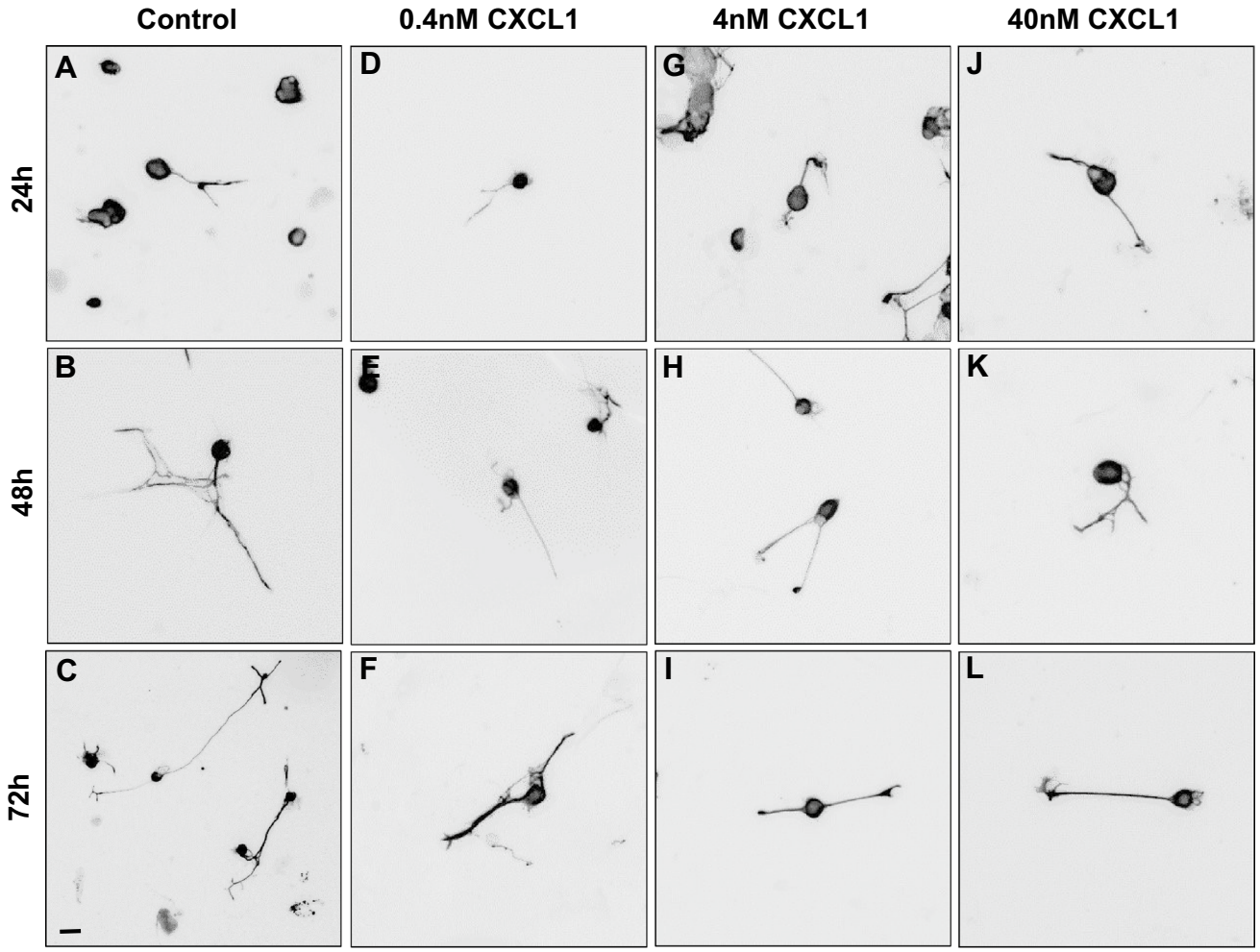

Fig. 8 Representative images of IB4 (+)/βIII-tubulin (+) adult DRG neurons in control conditions or after treatment with different concentrations of CXCL1 for 24, 48 or 72 h. Scale bar for image **C** = 50 µm; for the rest = 20 µm

infiltrate inside DRG and through a number of secreted molecules influence DRG neurons functioning [7].

In our study, both CXCL1 and CXCL2 proved to significantly inhibit the axon outgrowth in adult DRG neurons starting with 24 h and maintained up to 72 h, strongly reducing the progressive increase of axon length directly proportional with the time in culture noticed in control conditions (Fig. 4). This was not a cytotoxic effect, since the MTT assay showed no effect on cell viability (Fig. 1), and no dose–effect since different concentrations in nM range showed the same effect. These results came as a surprise, because according to the data in the literature both chemokines have beneficial effect on neuronal survival, differentiation and even on axon outgrowth as in the case of CXCL2 [12]. However, the data on CXCL2 and axon growth were obtained on embryonic hippocampal neurons, which have a different genetic profile and intracellular signaling pathways compared to an adult DRG neuron.

In order to understand where this inhibitory effect came from, we separately investigated the axon ourgrowth in large and medium NF200 (+) DRG neurons and small IB4 (+) DRG neurons with the same concentrations of CXCL1 and CXCL2 and for the same time points. The results showed that all DRG sub-populations were affected, but in a secquential manner: for the first 24 h only NF200 (+) DRG neurons were affected, with the inhibitory effect fading away after 48 of exposure to both chemokines (Fig. 7), while IB4 (+) DRG neurons started to be strongly inhibited by both chemokines after 48 h and maintained the response up to 72 h (Fig. 10).

DRG neurons are highly heterogenous. Large and medium DRG neurons transmit mechanoreceptive and proprioceptive signals via thickly myelinated afferents (Aβ-fibers) to spinal lamina III-V and express neurofilament 200 (NF200) [25], while small diameter DRG neurons give rise to unmyelinated axons (C-fibers) and thinly myelinated axons (Aδ-fibers)

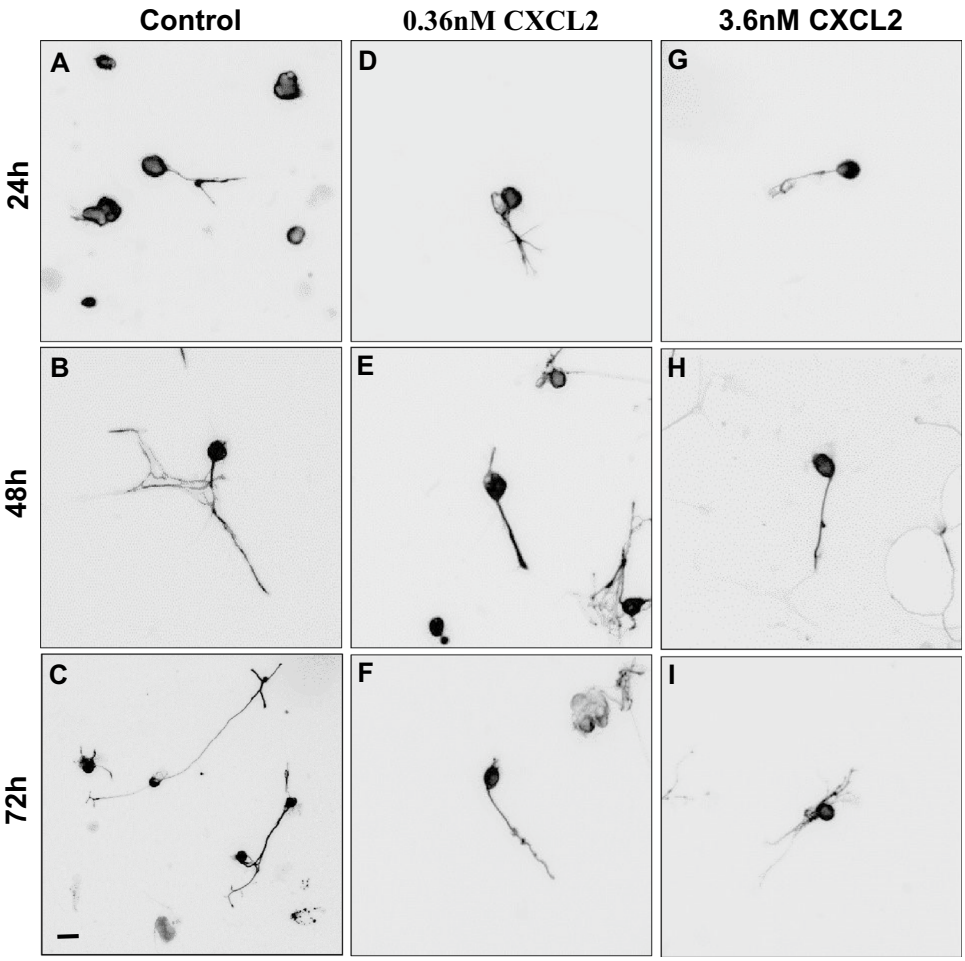

Fig. 9 Representative images of IB4 (+)/βIII-tubulin (+) adult DRG neurons in control conditions or after treatment with different concentrations of CXCL2 for 24, 48 or 72 h. Scale bar for image **C** = 50 µm; for the rest = 20 µm

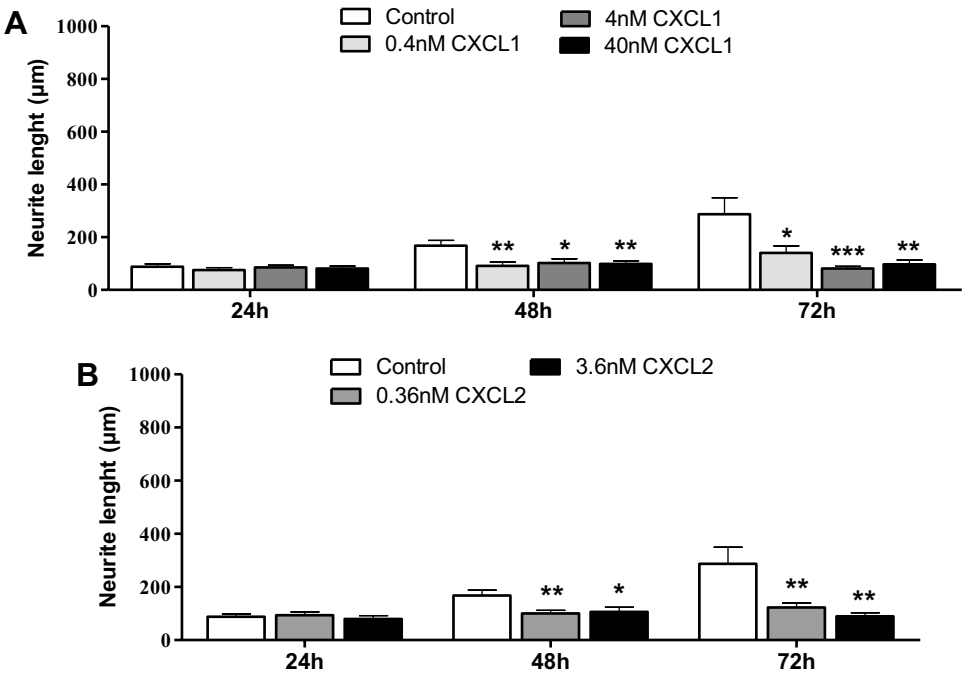

Fig. 10 Axons of small IB4 (+) DRG neurons respond slower to CXCL1 and CXCL2. **A** Graph bar representing mean axons' lenght in control conditions or after treatment with different concentrations of CXCL1 for different time intervals. **B** Graph bar representing mean axons' lenght in control conditions or after treatment with different concentrations of CXCL2 for different time intervals. In both cases, the inhibition was significant compared to the control conditions only for 48 h and 72 h (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$)

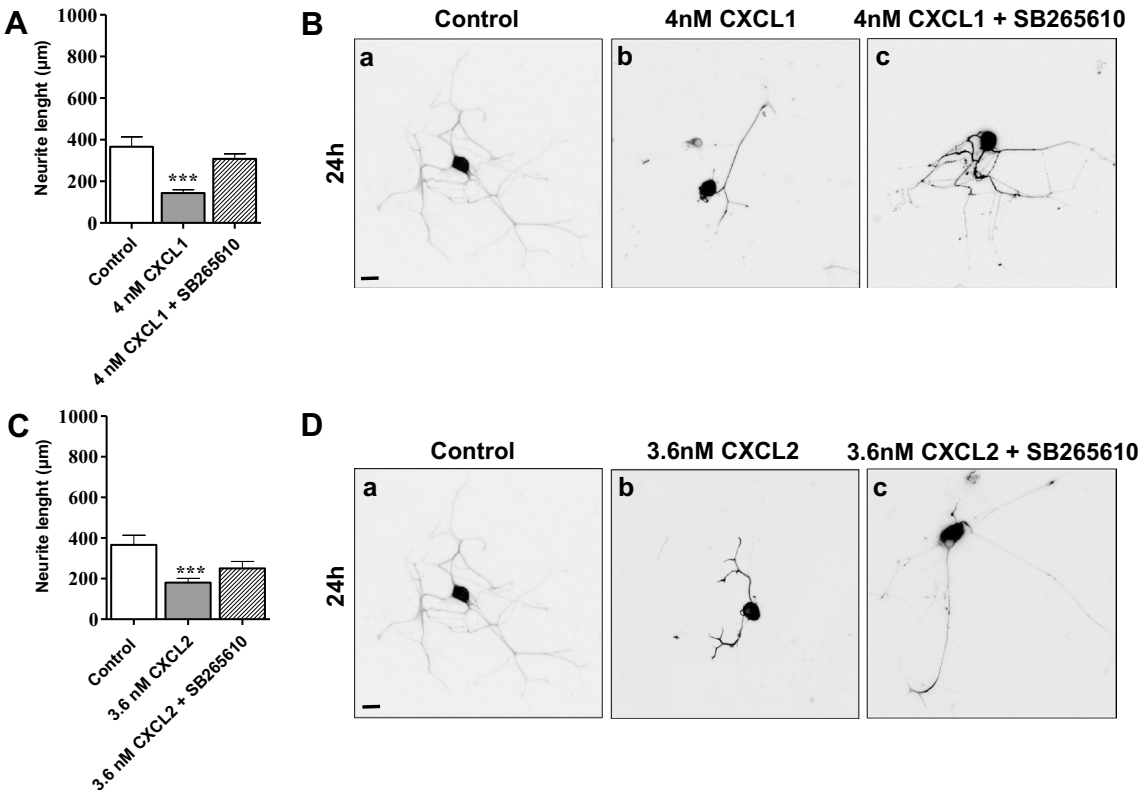

Fig. 11 Blocking CXCR2 prevented inhibitory effects of CXCL1 and CXCL2 on NF200 (+) DRG neurons' axon outgrowth. **A** Graph bar representing mean axons' lenght in control conditions, after treatment with 4 nM CXCL1 or with 4 nM CXCL1 + 1 µM SB265610 for 24 h. Blocking of CXCR2 allowed axon outgowth. **B** Representative images of NF200 (+) adult DRG neurons in control conditions, after treatment with 4 nM CXCL1 or with 4 nM CXCL1 + 1 µM SB265610 for 24 h. Scale bar = 20 µm. **C** Graph bar representing mean axons' lenght in control conditions, after treatment with 3.6 nM CXCL2 or with 3.6 nM CXCL2 + 1 µM SB265610 for 24 h. Blocking CXCR2 allowed axon outgowth. **D** Representative images of NF200 (+) adult DRG neurons in control conditions, after treatment with 3.6 nM CXCL2 or with 3.6 nM CXCL2 + 1 µM SB265610 for 24 h. Scale bar for image Db = 50 µm; for the rest = 20 µm

that convey the nociceptive, thermal and mechanoreceptive signals to neurons in lamina I-II of the spinal cord [44]. Traditionally, small neurons have been classified into non-peptidergic/IB4 (+) and peptidergic/IB4 (−) expressing neuropeptide substance P and calcitonin gene-related peptide (CGRP) [27]. Recenty, by integrating single-cell techniques such as high-coverage RNA-seq, in vivo patch clamp recording and single-cell PCR, DRG neurons were further classified into 10 types and 14 subordinate subtypes with distinct transcriptional patterns, molecular markers and functional properties [25, 45]. Such a high heterogeneity of DRG neurons may explain why the responses to CXCL1 and CXCL2 were cell type-dependent. In addition, CXCR2 which mediate the effects of both CXCL1 and CXCL2 and are expressed by all DRG neurons [18], may also contribute. CXCR2 have a very complex functioning: usually they form dynamic and temporal assembly with different adaptor signaling proteins called "chemosynapses" and subsequently activate various intracellular pathways, making its responses to specific ligands to be highly variable [46]. In our experiments, blocking CXCR2 prevented the inhibitory effects of CXCL1 and CXCL2 on the axon growth, confirming thus their implications. Therefore, a combination of cell-type and CXCR2 specificities could be responsible for the cell type- and time-dependent effects we have noticed in our experiments.

A possible connection between the CXCL1 and CXCL2 and neuronal cytoskeleton are TRPV1 receptors. Recently we have shown that both CXCL1 and CXCL2 may alter the functioning of TRPV1 receptors in TRPV1 (+)/IB4 (+) DRG neurons. The results showed that in an itch-sensitive TRPV1 (+)/IB4 (+) sub-population of DRG neurons CXCL1 either directly activated TRPV1 receptors (upon acute application) or it reduced their desensitization rate (upon short-term incubation) and thus it mediated itch, while in the rest of the TRPV1 (+)/IB4 (+) population CXCL1 only reduced TRPV1 desensitization (upon short-term incubation) with other possible consequences [17, 18]. Reduced desensitization is associated with increased neuronal excitability, because under this condition TRPV1 receptors are still

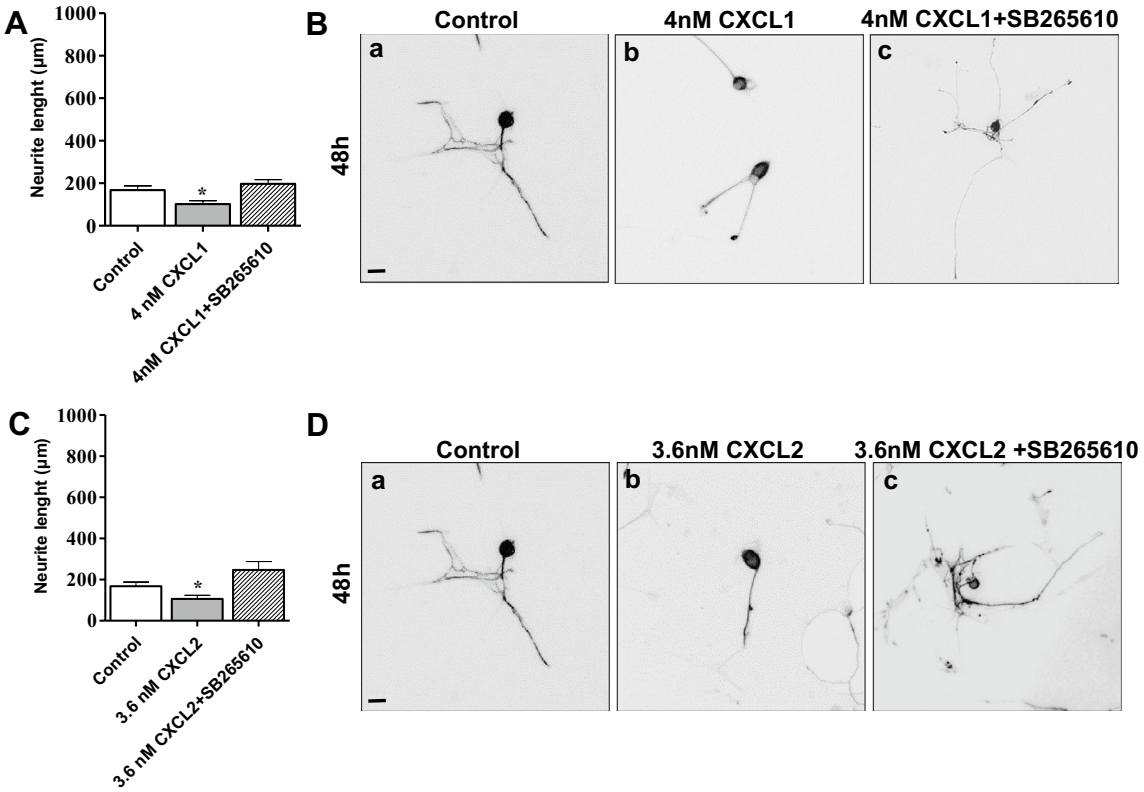

Fig. 12 Blocking CXCR2 prevents inhibitory effects of CXCL1 and CXCL2 on IB4 (+) DRG neurons' axon outgrowth. **A** Graph bar representing mean axons' lenght in control conditions, after treatment with 4 nM CXCL1 or with 4 nM CXCL1 + 1 µM SB265610 for 48 h. Blocking of CXCR2 allowed axon outgowth. **B** Representative images of IB4 (+) adult DRG neurons in control conditions, after treatment with 4 nM CXCL1 or with 4 nM CXCL1 + 1 µM SB265610 for 48 h. Scale bar for image Bc = 50 µm, for the rest = 20 µm. **C** Graph bar representing mean axons' lenght in control conditions, after treatment with 3.6 nM CXCL2 or with 3.6 nM CXCL2 + 1 µM SB265610 for 48 h. Blocking of CXCR2 allowed axon outgowth. **D** Representative images of IB4 (+) adult DRG neurons in control conditions, after treatment with 3.6 nM CXCL2 or with 3.6 nM CXCL2 + 1 µM SB265610 for 48 h. Scale bar for image Dc = 50 µm, for the rest = 20 µm

able to open to new/repetitive stimuli. In contrast to CXCL1, CXCL2 activated the non-itch sensitive TRPV1 (+)/IB4 (+) DRG neurons, more by reducing TRPV1 desensitization and a lot less by directly activating TRPV1 receptors [18]. If maintained for long-term (24 h), CXCL1 had no longer any effect on TRPV1 functioning, while CXCL2 became inhibitory by reducing TRPV1 current density and increasing its desensitization rate [11].

Studies have shown that TRPV1 receptors are physically and functionally located at the dynamic neuronal extensions, including growth cones of embryonic and adult DRG neurons, and that their prolonged activation results in shortening of neurites in the majority of IB4 (+) DRG neurons due to a Ca^{2+}-independent disassembly of microtubules [19, 20, 47]. These consequences of TRPV1 activation were seen after resiniferatoxin (RTX) application (~1 min at 100 nM concentrations), an ultra-potent agonist of TRPV1 which activates the current as strongly as capsaicin and does not deactivate even after prolonged washout [48]. In our previous experiments, CXCL1 induced an ~11% activation of TRPV1 receptors compared to a response to capsaicin, after 12 min of application and only in the itch-sensitive TRPV1 (+)/IB4 (+) DRG neurons, while CXCL2 activated much less the non-itch sensitive TRPV1 (+)/IB4 (+) DRG neurons [17, 18]. It is not clear if this reduced, but extended activation of TRPV1 by CXCL1 or CXCL2 would be the equivalent of RTX application, so that a shortening of the neurites to be initiated from the very first moments of CXCL1 or CXCL2 application, and maintained for 24, 48 or 72 h. To complicate the situation even more, in a previous study we showed that 24 h incubation with CXCL1 had no effect on TRPV1 functioning, while CXCL2 inhibited TRPV1 currents [11].

Considering all these data and the complex network of signaling pathways that may be activated downstream CXCR2 after CXCL1 and CXCL2 binding, we may speculate that in TRPV1 (+)/IB4 (+) DRG neurons, during the first few hours of incubation, the shortening of axon is initiated because of some TRPV1-associated CXCL1- or CXCL2-dependent mechanisms, while later on the shortening is maintained by some CXCL1- or CXCL2-independent

mechanism. The same mechanisms might also apply for the IB4 (−)/CGRP (+)/substance P (+) DRG neurons, which were not investigated in this study, but which equally express TRPV1 receptors [49]. Other CXCL1- or CXCL2-dependent, but TRPV1-independent mechanisms could be responsible for the axon shortening effect we have noticed in the NF200 (+) neurons since they express very little TRPV1 receptors [49].

In conclusion, in our study we have shown that extended presence of CXCL1 and CXCL2 around DRG neurons did not stimulate the regenerative capacities of the neurons, but exerted the oposite effects in a time- and cell-type-dependent manner. Blocking their specific receptor, CXCR2, prevented this effect, suggesting that CXCR2 may represent a new therapeutic target for promoting the axon outgrowth after a peripheral nerve injury associated with an increase in CXCL1 and CXCL2 concentration.

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Compliance with Ethical Standards

Conflict of interest The authors declare no competing or financial interests.

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