Intravenous neural stem cells abolish nociceptive hypersensitivity and trigger nerve regeneration in experimental neuropathy

Silvia Franchi [a], Anna E. Valsecchi [a], Elisa Borsani [b], Patrizia Procacci [c], Daniela Ferrari [d], Cristina Zaffa [d], Patrizia Sartori [c], Luigi F. Rodella [b], Angelo Vescovi [d], Sabatino Maione [e], Francesco Rossi [e], Paola Sacerdote [a], Mariapia Colleoni [a], Alberto E. Panerai [a,*]

[a] Dipartimento di Farmacologia Chemioterapia e Tossicologia Medica, Università degli Studi di Milano, Milano, Italy
[b] Divisione di Anatomia Umana, Dipartimento di Scienze Biomediche e Biotecnologie, Università di Brescia, Brescia, Italy
[c] Dipartimento di Morfologia Umana e Scienze Biomediche, Università degli Studi di Milano, Milano, Italy
[d] Dipartimento di Biotecnologie e Bioscienze, Università Milano-Bicocca, Milano, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Foggia, Italy
[e] Dipartimento di Medicina Sperimentale – Sezione di Farmacologia "L. Donatelli", Seconda Università di Napoli, Napoli, Italy

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

ARTICLE INFO

ABSTRACT

A nonphysiological repair of the lesioned nerve leading to the formation of neurinomas, altered nerve conduction, and spontaneous firing is considered the main cause of the events underlying neuropathic pain. It was investigated whether neural stem cell (NSCs) administration could lead to a physiological nerve repair, thus to a reduction of neuropathic pain symptoms such as hyperalgesia and allodynia in a well-established model of this pain (sciatic nerve chronic constriction injury [CCI]). Moreover, since we and others showed that the peripheral nerve lesion starts a cascade of neuroinflammation-related events that may maintain and worsen the original lesion, the effect of NSCs on sciatic nerve pro- and anti-inflammatory cytokines in CCI mice was investigated. NSCs injected intravenously, when the pathology was already established, induced a significant reduction in allodynia and hyperalgesia already 3 days after administration, demonstrating a therapeutic effect that lasted for at least 28 days. Responses changed with the number of administered NSCs, and the effect on hyperalgesia could be boosted by a new NSC administration. Treatment significantly decreased proinflammatory, activated antiinflammatory cytokines in the sciatic nerve, and reduced spinal cord Fos expression in laminae I-VI. Moreover, in NSC-treated animals, a reparative process and an improvement of nerve morphology is present at a later time. Since NSC effect on pain symptoms preceded nerve repair and was maintained after cells had disappeared from the lesion site, we suggest that regenerative, behavioral, and immune NSC effects are largely due to microenvironmental changes they might induce at the lesion site.

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1. Introduction

Neuropathic pain resulting from damage to or dysfunction of peripheral nerves due to compressing tumors, toxins used as chemotherapy, metabolic (diabetes) or viral (herpes zoster) diseases, severe ischemic insults, trauma and disc herniation that stretches, compresses, or induces a nerve root inflammation [15] is not well understood and is difficult to treat.

Despite over 50 years of research, there are not valid treatments over time, and neuropathic pain can be classified as an incurable disease [34].

The aim of our study was to use neural stem cells (NSCs) to obtain neuropathic pain symptoms (allodynia and hyperalgesia) remission and evaluate their effects on nerve morphology and local immune parameters in a universally accepted experimental model of neuropathic pain (chronic constriction injury [CCI]). This approach was suggested by the observation that mesenchymal stem cells [11,33] transplanted directly into the nervous tissue could ameliorate peripheral induced neuropathic pain. We considered that in a peripheral nerve injury model the use of NSCs, for their neural phenotype [14], could be an advantage in order to prompt a physiological repair of the nerve. We were aware that NSCs or stem cells differentiated into NSCs had been previously used in neuropathic pain derived from spinal cord injuries, yielding both positive [20] and negative [22,27] outcomes. These results, however, were obtained in experimental models more traumatic than the one we propose, for example, after spinal cord crush.

* Corresponding author. Address: Dipartimento di Farmacologia Chemioterapia e Tossicologia Medica, Università degli Studi di Milano, Via Vanvitelli 32, Milano 20129, Italy. Tel.: +39 0250316934; fax: +39 0250316933.

   E-mail address: alberto.panerai@unimi.it (A.E. Panerai).

Moreover, in these experiments, cells were administered directly at the lesion site (intraspinal or perilesional), thus possibly overloading the system [22,27]. We also considered that peripheral systemic NSC administration was a route of administration more easily transferable to a putative clinical use of this therapeutic approach, rather than the perilesional administration used in other studies. Finally, we looked at the NSC effects on local immune activation since this, according to our previous studies [30], could be very important in neuropathic pain pathogenesis and maintenance. Consistently, in addition to integration and differentiation into neurons, astrocytes, and oligodendrocytes, transplanted NSCs were also shown to exert their beneficial effects through an immunomodulatory and neuroprotective action [5,36–39], for example, acting on the neuroimmune cascade that is involved in the genesis and maintenance of the neuropathic lesion and pain [30,42,43]. Interestingly, it has also been shown that intravenous NSCs can migrate from blood vessels into the central nervous system, crossing the blood–brain barrier [5,26,28,45], and might therefore modulate pain, also at the spinal cord dorsal horn level.

Finally, we further elucidated the biochemical mechanisms participating in the NSC effects on the lesioned nerve repair and correlated them with the morphological and functional changes taking place in the lesioned tissues. It has been suggested that NSCs exert a bidirectional interaction with activated immune cells and modulate stimulatory and inhibitory factors induced by the lesions, that could be crucial to obtain a full anatomical and functional repair [9]. The knowledge of the biochemistry, localization, and timing of these interactions will be extremely important for the best use of NSC-based therapy.

2. Materials and methods

2.1. Animals and surgical procedure

All performed experiments were in accordance with Italian State and European regulations governing the care and treatment of laboratory animals (permission No. 94/2000A) and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain [46]. The experimental work was previously reviewed by the Animal Care and Use Committee of the University of Milan.

Painful neuropathy was induced on 20- to 25-g C57BL/6J male mice (Harlan Laboratories, Bresso, Italy). The chronic constriction injury (CCI) model originally described by Bennett and Xie [6] for rats was adapted for mice, as described in our previous studies [30,43]. Sham-operated animals (sciatic exposure without ligation) were used as controls.

2.2. Thermal hyperalgesia and mechanical allodynia evaluation

Measurements were performed on both the ipsilateral and contralateral hind paws of all mice by researchers blind to treatments.

Thermal hyperalgesia was tested according to the Hargreaves procedure [18], slightly modified by us for mouse [30,43], using a Plantar Test Apparatus.

Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer, as previously described in detail [30,43].

2.3. Neural stem cell lines

An adult neural stem cell line was established from the subventricular zone of 2-month-old C57BL/6 mice (Charles River Laboratories, Calco, Italy), as previously described [17].

Our neural stem cell lines are derived and characterized using the neurosphere technique [14]. The cells used for these experiments have been tested for their proliferation ability (growth curves) and tri-lineage differentiation along the neural phenotype, in order to confirm the actual neural stem identity.

For expansion, cells were plated at a density of 8000/cm² in medium containing 20 ng/mL of both epidermal growth factor and fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA).

Transduction of NSCs with a lentiviral vector carrying the Green Fluorescent Protein (GFP) gene was carried as previously described [4,16].

2.4. NIH-3T3 cultures

The mouse fibroblast cell line NIH-3T3 (American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% calf serum and antibiotics at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

2.5. Experimental protocol

Cell administration was always performed starting 7 days after CCI, when the pain hypersensitivity was maximal. Mouse NSCs were mechanically dissociated to a single cell suspension in phosphate-buffered saline solution with 2.5% heparin and injected intravenously into the caudal vein of mice. The amount of cells and their concentration were 1×10^6 cells/200 µL, chosen according to Pluchino et al. [37]. NSCs were administered as follows:

Group 1: 1×10^6 cells injected at day 7 since surgery to CCI and sham mice.
Group 2: 1×10^6 cells injected at day 7 and 1×10^6 cells at day 8 since surgery to CCI and sham mice.
Group 3: 1×10^6 cells injected at day 7, 1×10^6 cells at day 8, and 1×10^6 cells at day 21 since surgery.

For each group, both sham and CCI mice were injected with the same amount of vehicle at the same time points.

Thermal hyperalgesia and mechanical allodynia were evaluated in all mouse groups immediately before surgery and at day 7 after surgery, prior to cell injection (time 0). The subsequent evaluations were performed at days 3, 7, 14, 21, and 28 after the first NSC administration.

In the experiments with non-GFP-transfected NSCs or NIH-3T3 fibroblasts, sham and CCI mice received 1×10^6 cells/200 µL at day 7 and 1×10^6 cells at day 8 from surgery for a total of 2×10^6 cells. Thermal hyperalgesia and mechanical allodynia were evaluated 7 days after either NIH-3T3 or NSC administration.

2.6. Fos and GFP immunohistochemistry

Fos is a nuclear phosphoprotein product of the mammalian c-fos proto-oncogene widely used as morphological marker of neuronal activation induced by various factors including nociceptive stimuli [1,19]. Fos expression was studied 14 days after CCI, that is, 7 days after 2×10^6 NSC administration. The induction of Fos-positive neurons by nonnoxious stimulation was evaluated according to Kosai et al. [25], as previously described in detail [7]. Briefly, the mice received the nonnoxious stimulus 3 times a minute every 30 s and were sacrificed 1.5 h after the stimulation. The lumbar L4–L6 segment was determined in each case with the spinal cord in situ using a dissecting microscope and measuring the distance between the points of entry of the most rostral and the most caudal rootlets of the L4, L5, and L6 dorsal roots. Floating transverse sections (35 µm thick) of all spinal cords (L4–L6 segments) were processed with polyclonal primary antiserum directed against Fos (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The

distribution of the labeled cells in the ipsilateral dorsal horn of all animals was charted using a light microscope equipped with an image analyzer (Immagini e Computer, Milano, Italy). Digitized images of 20 randomly selected sections per animal were captured and the number of the labeled cells in each section of the lumbar spinal laminae, grouped into I-II and III-VI, was evaluated by researchers unaware of the animal group assignment. For each animal, the mean from its 20 sections was obtained and those means were used to further derive group means that were compared by analysis of variance (ANOVA) and by a Bonferroni multiple comparison test.

For GFP, frozen serial longitudinal sections (15 μm thick) of the sciatic nerves or other organs were placed on glass slides, dried, and processed for GFP immunohistochemistry with a polyclonal primary antiserum directed against GFP (1:500, Invitrogen, Eugene, OR, USA). The immunohistochemistry control was performed by omitting the primary antibody and incubating the sections with nonimmune rabbit serum. The immunopositivity was observed using a light microscope (Olympus, Tokyo, Japan). The number of stem cells immunostained for GFP in the sampled section was counted. The total number of transplanted NSCs homing in sciatic nerve was estimated by summing the number of stem cells counted from all histological sections. However, the number of stem cell profiles identified in 2-dimensional plane could not reflect the number of neurons in 3-dimensional space. To address the problem of converting stem cells profiles in 2-dimensional plane to one that represents 3-dimensional space, Abercrombie's correction factor was applied [2,40]. If N is the corrected, true estimate of stem cells in a specimen of serial sections, n is the total number of NSCs from each animal examining all sections obtained from a nerve, T is the thickness (in μm) of the section, and H the average height (9 μm) of the stem cells, then: $N = n [T/(T + H)]$. Data are presented as number of stem cells in sciatic nerve and calculated as the average among animals of each experimental group.

2.7. Substance P and calcitonin gene-related peptide immunofluorescence

For immunofluorescence, frozen floating sections (35 μm thick) of all spinal cords were placed in Tris-buffered saline 1% (TBS) and processed for immunofluorescence. Briefly, the sections were incubated in bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO, USA) blocking solution (5% BSA, 0.25% Triton X-100 in TBS 1%) and then incubated in rabbit polyclonal primary antiserum against substance P (SP) (1:300, Millipore, Temecula, CA, USA) or calcitonin gene-related peptide (CGRP) (1:1000, Millipore) diluted in TBS containing 3% BSA and 0.1% Triton X-100, for 24 h at +4°C. After incubation in the primary antibody, the sections were sequentially incubated with appropriated fluorescent secondary antibodies diluted in TBS (1:200, anti-rabbit Alexa-Fluor 555, red fluorescent dye; Invitrogen, Carlsbad, CA, USA). The immunofluorescence control was performed by omitting the primary antibody and incubating the sections with nonimmune rabbit serum. All floating sections were placed on slides and finally mounted using a special mounting medium (UltraCruz Mounting Medium; Santa Cruz Biotechnology).

The immunofluorescence localization was evaluated on digital images acquired with laser scanning confocal microscopy (LSM 510, Carl Zeiss MicroImaging Gmbh, Göttingen, Germany).

2.8. RNA extraction and real-time reverse transcription polymerase chain reaction for cytokine evaluation

Seven days after NSC double administration, mice were anaesthetized with sodium pentobarbital (60 mg/kg intraperitoneally, 0.1 mL/10 g) and under dissecting microscope the ipsilateral sciatic nerve, proximal to the trifurcation (about 1 cm), before the 3 ligatures in the CCI animals, was removed and immediately frozen in liquid nitrogen and stored at −80°C until the cytokine expression assay. All procedures for real-time reverse transcription polymerase chain reaction interleukin (IL)-1β, IL-6, and IL-10 evaluation have been previously described in detail [30,43]. The results were quantified using the comparative threshold method. The Ct value of the specific gene of interest was normalized to the Ct value of the endogenous control, glyceraldehyde 3-phosphate dehydrogenase, and the comparative Ct method ($2^{-\Delta\Delta Ct}$) was then applied using vehicle-treated sham animals as calibrator.

2.9. Cytokines protein evaluation

The nerve samples were homogenized in 0.40 mL of ice-cold phosphate-buffered saline containing a protease inhibitor cocktail (Sigma–Aldrich, Milano, Italy) and centrifuged at 10,000g for 15 min, as previously described [30].

The supernatant was used to measure IL-1β, IL-6, and IL-10 level and total protein content (Lowry's method). IL-1β, IL-6, and IL-10 protein contents were determined by enzyme-linked immunosorbent assay (ELISA) using ultra-sensitive ELISA kits according to the manufacturer's instruction (IL-1 and IL-6: R&D Systems, Minneapolis, MN, USA; IL-10: BD Pharmingen, San Diego, CA, USA). Cytokine concentrations were determined by interpolation with standard curves assayed on individual plates and normalized to protein content in each sample.

2.10. Nerve morphology

For morphological studies, 16 mice were used. Four were sham operated: 2 animals were NSC (2×10^6 cells) treated and 2 untreated, as controls.

The other animals, in which experimental lesion CCI was performed, were distributed in 2 experimental groups of 6 animals each (6 animals NSC treated and 6 untreated animals, as controls) that were sacrificed at 14 and 21 days, respectively, after lesion.

The animals were deeply anaesthetized with Nembutal (Lundbeck, Deerfield, IL, USA) (48 mg/kg, intraperitoneally), perfused transcardially with a solution containing 2% formaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). The right sciatic nerve of sham-operated mice and the right sciatic nerve segments of 2–3 mm length, immediately distal to the lesion, were removed from each CCI animal and immersed in the same fixative. After about 4 h of fixation, sciatic nerves were washed in cacodylate buffer and postfixed at 0°C for 1.5 h in 2% osmium tetroxide in the same buffer. The specimens were washed in distilled water, stained in 2% aqueous uranyl acetate, dehydrated through an ascending series of ethanol, and embedded in Epon-Araldite resin. Nerves were oriented for cross-sectioning.

Semi-thin sections (0.5 μm), obtained from each nerve with an LKB III ultramicrotome (LKB, Vienna, Austria), were stained with 0.5% toluidine blue in 1% sodium borate and examined in the light microscope (Zeiss Axiophot Photomicroscope) at a final magnification of 1500×. For morphometric analysis, the whole cross-sectional area of each nerve was acquired through AxioVision 4.6 software (Carl Zeiss, Germany).

Ultra-thin sections were examined under an electron microscope (Zeiss EM) [10].

2.11. Morphometric analysis

In mice in which experimental lesion CCI was performed, 9–12 fields, corresponding to at least 50% of the total nerve cross-sectional area, were randomly selected. In each field, counts of Schwann cells, fibroblasts, (i.e., nonneuronal cells) were done manually:
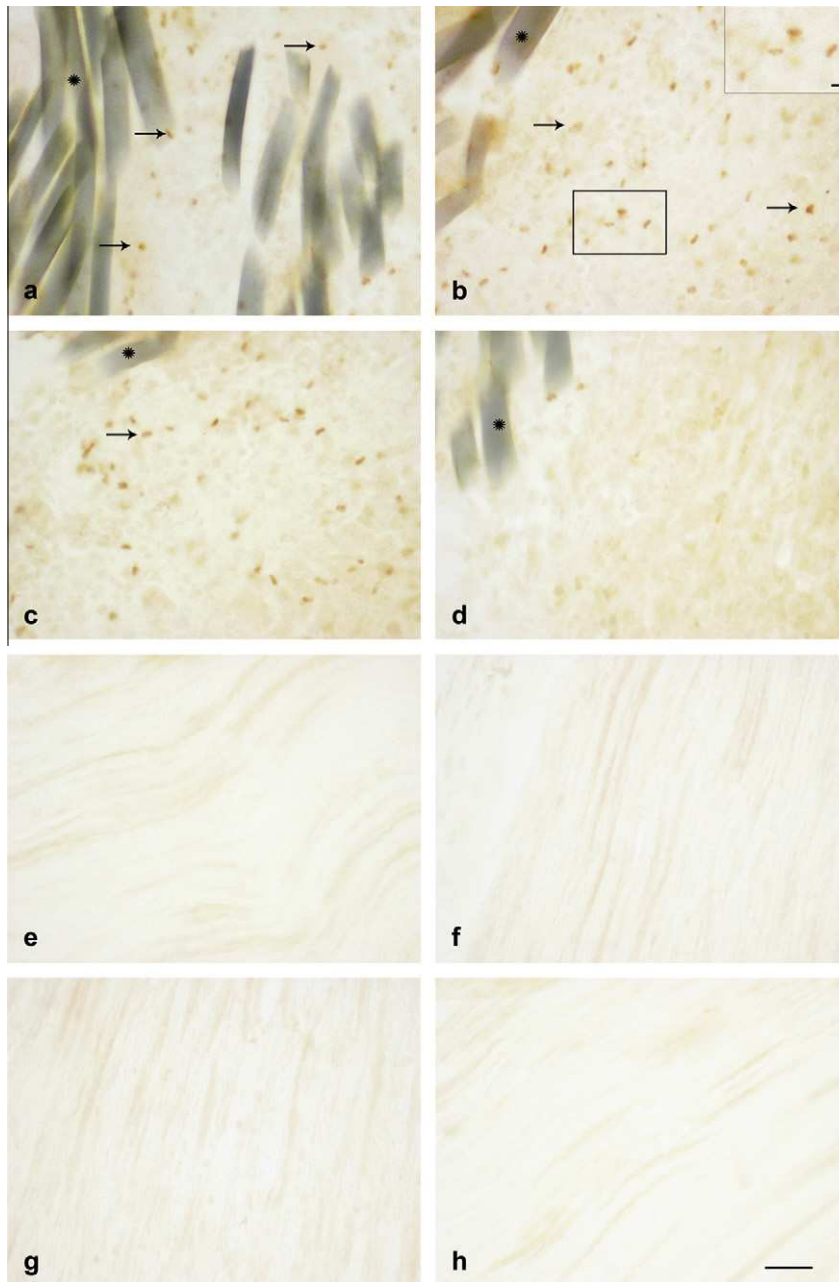
Fig. 1. Neural stem cells (NSCs) in the sciatic nerve: (a) ipsilateral chronic constriction injury (CCI)+NSC 24 h, (b) ipsilateral CCI + NSC 3 days, (c) ipsilateral CCI + NSC 7 days, (d) ipsilateral CCI + NSC 14 days, (e) contralateral CCI + NSC 24 h, (f) contralateral CCI + NSC 3 days, (g) ipsilateral sham + NSC 24 h, (h) ipsilateral sham + NSC 3 days. Arrows indicate NSC; the asterisks (∗) indicate the suture material used for the ligature. Bar 20 μm; bar inset 5 μm. Each group analyzed consisted of 3 animals. Representative sections are shown.

Table 1
Total number NSCs in sciatic nerve of CCI mice.

Time from injection			
24 h	3 d	7 d	14 d
5409 ± 341[a]	4285 ± 256[*]	3658 ± 203[**]	412 ± 35[***]

NSCs, neural stem cells; CCI, chronic constriction injury.
[a] Mean ± SEM, see Section 2.
[*] $P < 0.05$ vs 24 h.
[**] $P < 0.05$ vs 3 d.
[***] $p < 0.05$ vs 7 d.

in particular, only cells that were sectioned at their nucleus level were included in the count. Macrophages in active phagocytosis were separately counted. Total cross-sectional nerve area and diameter of myelinated fibers were also measured (AxioVision 4.6 software); finally, number and axon density were calculated.

2.12. Statistical analysis

The data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Tukey's or Bonferroni test. When indicated, behavioral responses were also evaluated by 2-way ANOVA, considering treatment and time as factors. For morphometric analysis, the 2-tailed Student's *t*-test was used to identify the differences between untreated and treated animal at both 7 and 14 days after treatment. Number of nonneuronal cells nuclei and number of macrophages were expressed as mean ± SEM per 600 μm² of cross-sectional nerve area, whereas

number of myelinated axons was referred as mean ± SEM per 0.1 mm² of cross-sectional nerve area.

Differences were considered significant at $P < 0.05$. All the statistical analyses were performed using GraphPAD 4 software (San Diego, CA, USA).

3. Results

3.1. NSC transplantation and homing to the lesion

Prior to transplant, NSC lines from the subventricular zone of syngeneic C57BL/6J mice [17] were infected with lentiviral vector carrying the GFP gene [4,16], in order to distinguish transplanted NSCs from host cells. Neuropathic mice were treated intravenously with NSCs (1×10^6) after 7 days since sciatic injury and their presence in sciatic nerve and organs evaluated by GFP immunolabeling at 24 h and 3, 7, and 14 days thereafter. As early as 24 h after administration, NSCs were present in the neurinoma of CCI + NSC animals, were still present in a relevant number at days 3 and 7, and were almost absent on day 14 (Fig. 1a–d, Table 1). The NSCs were not present in the contralateral sciatic nerve of CCI mice at any observation time (Fig. 1e and f), nor in dorsal root ganglia, spinal cord, or brain. NSCs were not detected in sham + NSC mice ipsilateral sciatic nerves at any time of observation (Fig. 1g and h). A significant presence of NSCs was not in evidence in extraneural tissues such as lung and liver at any time after treatments. Representative pictures of lung and liver obtained 24 h after NSCs or vehicle in CCI animals are shown in Fig. 2a–d.

3.2. NSCs reduce thermal hyperalgesia and mechanical allodynia

Mice were treated with NSCs after 7 days from sciatic injury, when neuropathic pain was already established, in order to assess their role as therapeutic option in neuropathic pain. Pain behavior was evaluated 3, 7, 14, 21, and 28 days after injection. Fig. 3 shows that the administration of 1×10^6 NSCs in the CCI mice tail vein induced a significant reduction of hyperalgesia (Fig. 3A) and allodynia (Fig. 3B). The effect was already present 3 days after treatment. The effect on thermal hyperalgesia was maintained at least until day 28, while at this time point, the effect on mechanical allodynia was not significantly different from vehicle-treated CCI mice.

When NSCs were administered twice in 2 subsequent days, for a total dose of 2×10^6 cells, the reduction of hyperalgesia (Fig. 3C) was more evident at day 7, but it decreased to the 1×10^6 effect by day 14 and such remained at least until day 28. At difference, a better effect on allodynia was evident at day 28, when the double administration significantly increased thresholds.

When the administration of 1×10^6 cells was repeated 14 days after the first treatment, the effect on hyperalgesia was further increased, while the effect was not as evident on allodynia (Fig. 3E and F).

Two-way ANOVA showed that no differences in CCI + vehicle were present throughout all experiments, both for hyperalgesia and allodynia at any time interval after surgery. Standing this homogeneity in responses, for sake of better evidencing relevant results, Fig. 3G and H illustrate the time course of hyperalgesia and allodynia in CCI + NSC mice, as they result from the average of data obtained in all similar experiments, compared to the respective CCI + vehicle mice.

The analysis of these panels allows some interesting observations. The increase in thermal thresholds obtained by a single 1×10^6 NSC administration is maintained almost identical until day 28. Doubling the number of cells clearly further increases ($P < 0.001$, Fig. 3G) thermal thresholds in the short term, but these promptly decrease to those observed after a single administration and are maintained unchanged until day 28. Moreover, the NSC re-administration 14 days after the first administration again increases thresholds measured 1 week later. Fig. 3H shows that, different from what was observed for hyperalgesia, the increase in tactile thresholds seems to be already maximal after a single 1×10^6 NSC administration; in fact, these were not changed either doubling the cells number or after the re-administration. However, the effect was prolonged in animals treated with the NSC double dose.

None of the treatment schedules elicited any effect on nociceptive thresholds in sham-operated mice.
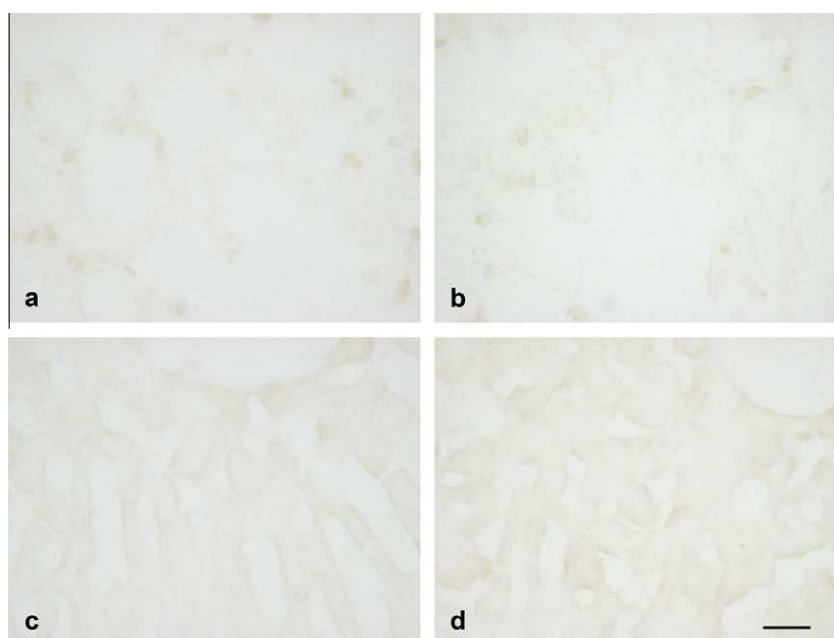

Fig. 2. Absence of neural stem cells (NSCs) in the lung (a,b) and liver (c,d). Chronic constriction injury (CCI) 24 h after vehicle injection (a,c); CCI + NSC 24 h after NSC injection (b,d). Bar 20 μm. Each group analyzed consisted of 3 animals. Representative sections are shown.
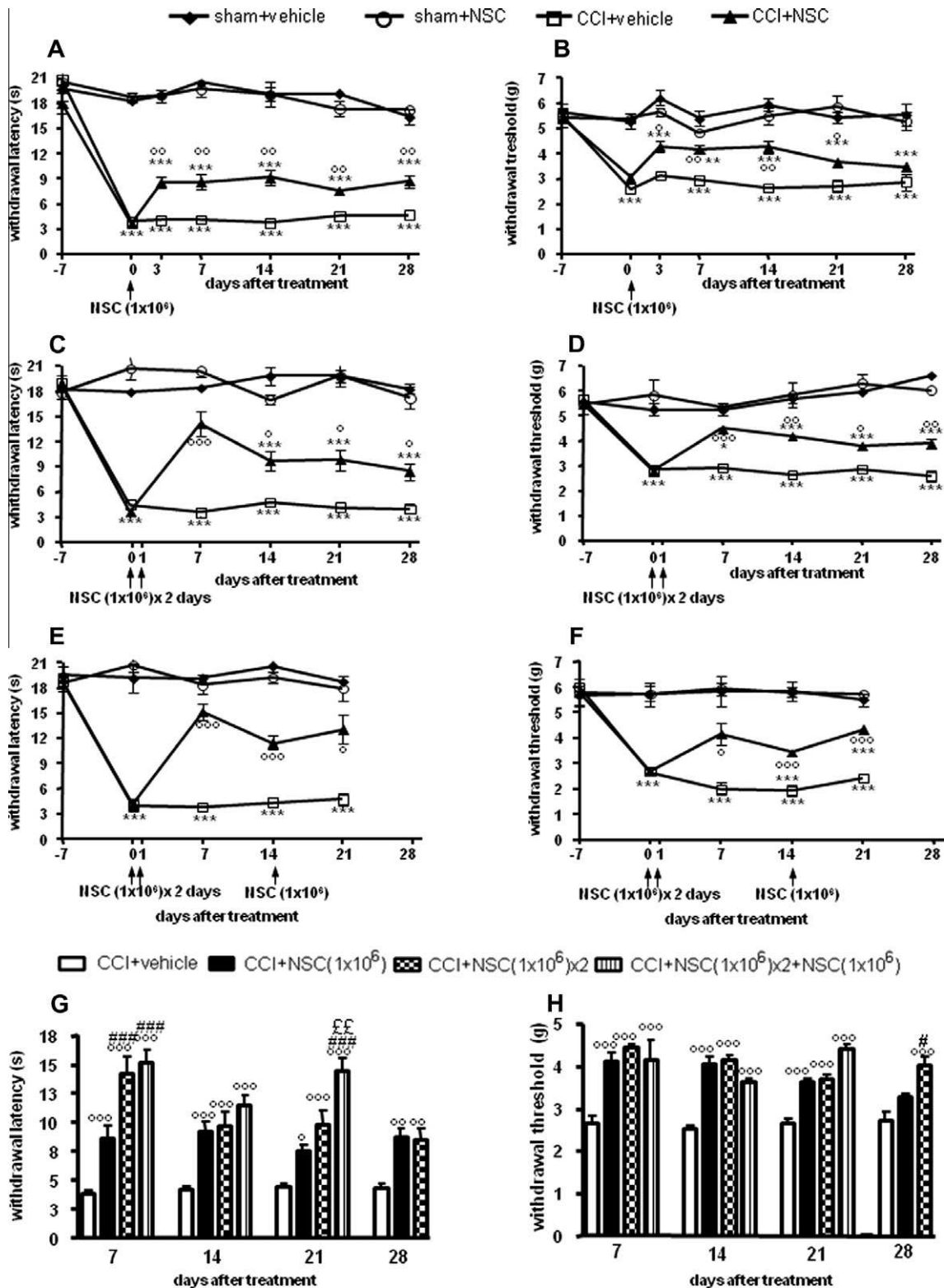
Fig. 3. Time course of the effect of different doses of neural stem cells (NSCs) given intravenously to neuropathic mice (chronic constriction injury [CCI]) starting 7 days after injury on thermal hyperalgesia (A, C, E), measured by Plantar test and mechanical allodynia (B, D, F), measured by Dynamic Plantar Aesthesiometer, expressed as seconds and grams, respectively. (A and B): Mice received a single dose of 1×10^6 NSCs 7 days after surgery (Time 0). (C and D): Mice received double injection of 1×10^6 NSCs at day 7 and 8 after surgery for a total of 2×10^6 NSCs (Time 0, 1). (E and F): Mice received 1×10^6 NSCs at day 7, 8, and 21 after surgery (Time 0, 1, 14) for a total of 3×10^6 NSCs. Data represent means ± SEM of 6–8 mice. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs sham + vehicle mice; °$P < 0.05$; °°$P < 0.01$; °°°$P < 0.001$ vs CCI + vehicle mice. Mean hyperalgesia (G) and allodynia (H) in CCI mice treated with vehicle, 1×10^6, 2×10^6, 3×10^6 cells (see text and panels A–F for details). °$P < 0.05$; °°$P < 0.01$; °°°$P < 0.001$ vs CCI + vehicle mice; #$P < 0.05$; ###$P < 0.001$ vs NSC 1×10^6; ££$P < 0.01$ vs 2×10^6 at days 14 and 21.

In a single experiment, we also confirmed that the non-GFP-transfected NSC cells behaved similarly on hyperalgesia and allodynia to GFP-NSC cells we used in all our experiments (Fig. 4A and B).
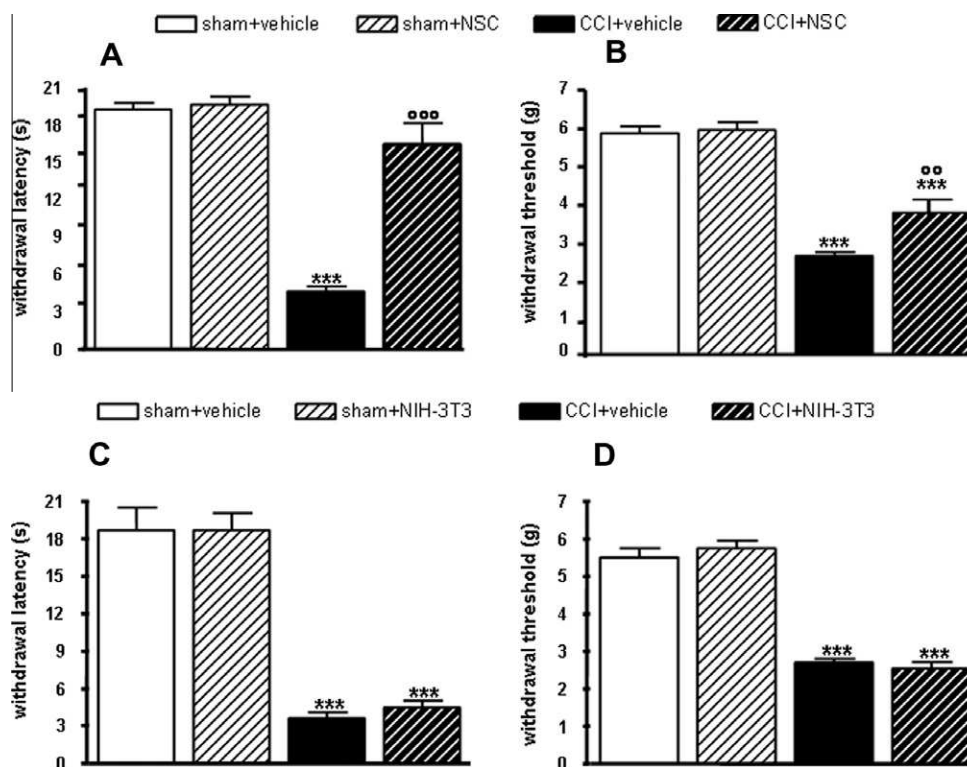
Fig. 4. Effect of Green Fluorescent Protein nontransfected neural stem cell (NSC) and of NIH-3T3 fibroblast intravenous injection on thermal hyperalgesia (A and C) and mechanical allodynia (B and D) measured 7 days after administration. (A and B): Mice received double injection of 1×10^6 non-GFP-transfected NSCs 7 and 8 days after surgery for a total of 2×10^6 cells. (C and D): Mice received double injection of 1×10^6 NIH-3T3 fibroblasts 7 and 8 days after surgery, for a total of 2×10^6 NIH-3T3. Data represent means ± SEM of 6–8 mice. ***$P < 0.001$ vs sham + vehicle mice; °°$P < 0.01$; °°°$P < 0.001$ vs CCI + vehicle mice.

Finally, in order to asses that the amelioration observed was due to stem cells and not to a nonspecific effect of cell injection, we showed that no effect on hyperalgesia or allodynia was present 7 days after the intravenous administration of 2×10^6 NIH 3T3 fibroblasts in CCI or sham mice (Fig. 4C and D).

3.3. NSC effect on spinal cord Fos, SP, CGRP

Although nonspecific, Fos has been considered to be a useful morphological marker in order to identify neuronal activity following noxious stimulation [1,19].

Fourteen days after surgery, in sham mice, Fos expression was present in rare neurons in lamina I-II, and scattered Fos-positive neurons were also found in laminae III-VI (Fig. 5a). Immunopositivity for the pain-related neuropeptides SP (Fig. 5d) and CGRP (Fig. 5g) was present in laminae I-II.

At the same time point, the number of Fos-positive neurons increased in CCI compared to sham mice, in the laminae I and II, and laminae III-VI (Fig. 5b). In the same regions, the immunopositivity for SP decreased (Fig. 5e), while that for CGRP was unaltered (Fig. 5h). Seven days after NSC treatment (2×10^6), the number of Fos-positive neurons was decreased compared with the CCI group (Fig. 5c); the immunopositivity for SP revealed a slight trend to increase (Fig. 5f), while the immunopositivity for CGRP was unaltered (Fig. 5i). Fos expression has been specifically quantified and data are reported in Table 2.

3.4. Effect of NSC on sciatic nerve cytokines

Fig. 6 shows that, as previously reported by us and others [30,42,43], 14 days after CCI, mRNA for the proinflammatory cytokines IL-1 (Fig. 6A) and IL-6 (Fig. 6B) increased at the level of the lesioned sciatic nerve. In 2×10^6 NSC-treated animals, the increase

was significantly reduced 7 days after treatment. Also, mRNA for the antiinflammatory cytokine IL-10 increased after the lesion, but it was further increased after NSC treatment (Fig. 6C). The IL-1 and IL-6 protein profile parallels that of mRNA, as shown in Fig. 6D and E. At difference, IL-10 protein decreased following the lesion and NSC treatment did not significantly change it (Fig. 6F).

3.5. Morphological studies

3.5.1. Sham-operated animals

Fourteen days after treatment, in sham-operated animals, no differences were present in nerve morphology between NSC and control animals (Fig. 7a and b).

3.5.2. Animals with experimental lesion

In CCI mice, the morphology of nerves distal to the site of lesion was consistent with a marked Wallerian degeneration. Severe fiber degeneration with a nearly complete loss of myelinated axons and presence of numerous macrophages in active phagocytosis were evident (Fig. 8).

The mean number of nonneuronal, nucleate cells (Schwann cells, fibroblasts, and possibly blood-derived cells) was significantly higher in NSC animals than in untreated animals both 7 and 14 days after treatment (Fig. 9).

Seven and 14 days after NSC treatment, the mean number of macrophages (8.08 ± 0.54 and 10.17 ± 0.96, respectively) was not significantly different compared to untreated animals (10.54 ± 0.85 and 10.46 ± 0.84). Nevertheless, especially at day 14, macrophage size was often smaller in NSC-treated than in control animals (Fig. 8a and b).

Fourteen days after treatment, despite the persistence of ligature, sprouting of small myelinated axons (mean diameter 2.58 ± 0.13 μm untreated vs. 2.64 ± 0.06 μm treated) was evident.
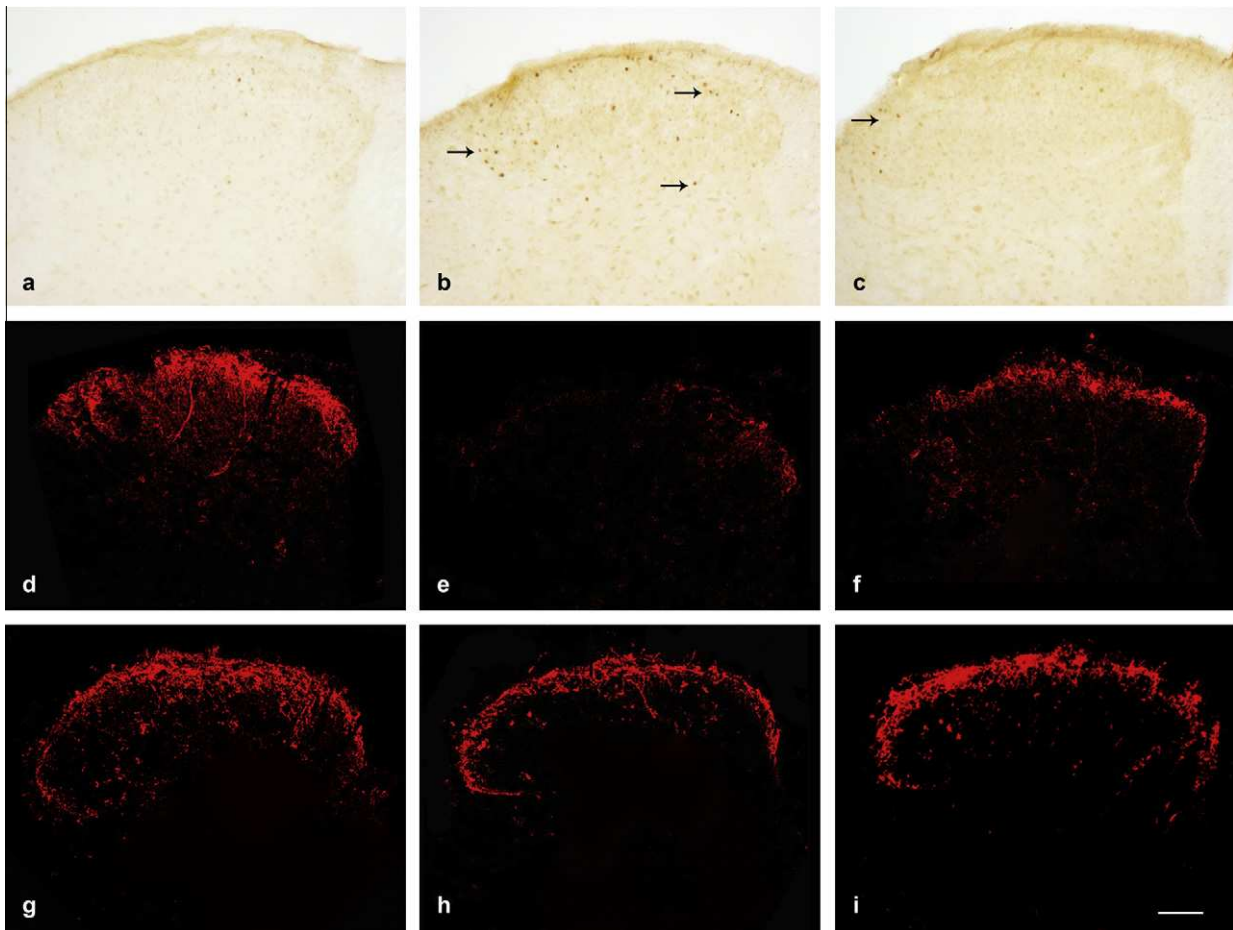
Fig. 5. Fos-positive neurons in the ipsilateral dorsal horn of spinal cord: (a) sham, (b) chronic constriction injury (CCI) 14 days after surgery, (c) CCI + neural stem cell (NSC) 7 days after treatment. Arrows indicate Fos-positive neurons. Confocal images of substance P in the ipsilateral dorsal horn of spinal cord: (d) sham, (e) CCI 14 days after surgery, (f) CCI + NSC 7 days after treatment. Confocal images of calcitonin gene-related peptide (CGRP) in the ipsilateral dorsal horn of spinal cord: (g) sham, (h) CCI 14 days after surgery, (i) CCI + NSC 7 days after treatment. Bar 40 μm. Each group analyzed consisted of 3 animals. Representative sections are shown.

Table 2
Number of Fos-positive neurons/section in spinal cord 7 days after NSC injection.

	Laminae I-II	Laminae III-VI
Sham	2.20 ± 0.1[a]	0.80 ± 0.12
Sham + NSC	1.80 ± 0.22	0.60 ± 0.22
CCI	12.55 ± 0.68[#]	5.03 ± 0.12[#]
CCI + NSC	5.62 ± 0.01[#*]	3.25 ± 0.10[#*]

NSC, neural stem cell; CCI, chronic constriction injury.
  [a] Mean ± SEM. n = 3. For each animal, the mean from its 20 sections was obtained and those means were used to further derive group means.
  [*] $P < 0.01$ vs CCI.
  [#] $P < 0.01$ vs Sham.

In NSC animals, the number of myelinated axons was significantly higher (4.74 ± 1.5) than in untreated mice (1.7 ± 0.8) (Fig. 8c and d).

Electron microscopic observations (Fig. 8e and f) confirmed the presence of degenerating fibers and of macrophages containing myelin debris, but also evidenced less damage of unmyelinated axons. Moreover, the presence of small myelinated axons and some thinly myelinated regenerating fibers was more evident in treated animals 14 days after treatment, when compared with untreated animals.

4. Discussion

Our data are the first evidence that NSC intravenous administration is effective in blunting neuropathic pain originating from a peripheral lesion. The literature has reported the use of stem cells of different origin in order to ameliorate motor performance, regeneration, and neuropathic pain caused by central lesions such as spinal cord injuries, trauma, and chemical lesions. Independently of the cells used, of the experimental model, and of the route of administration, results on neuropathic pain symptoms were generally inconsistent. These were sometimes only partially positive [11,20,33,41]; in other cases, pain was not affected [3,24] or it even worsened [22,27].

In our study we used stem cells with a neural phenotype and looked for an easy route of NSC administration, since in previous studies, cells were administered either intrathecally or directly in the nervous system [20,22,24,27,33,41].

The results presented here show that the acute, intravenous NSC administration ameliorates neuropathic pain symptoms for a significant length of time, and the effect can be potentiated by increasing the dose and can be boosted again by repeating the administration. It can be suggested that, in our hands, NSCs behave very much as one would expect from the use of a drug.

We show that in the CCI model, intravenously administered NSCs preferentially reach the lesioned site, but not other organs such as the lungs or liver. The latter observation is in contrast with other data obtained in a cerebral ischemia model [35]. Stem cell homing is, anyway, a debated issue. We can attempt to explain the difference, arguing that in our experimental setting, we used NSCs in a peripheral nerve lesion model and either NSCs had a preferential homing toward the homologous peripheral tissue, or
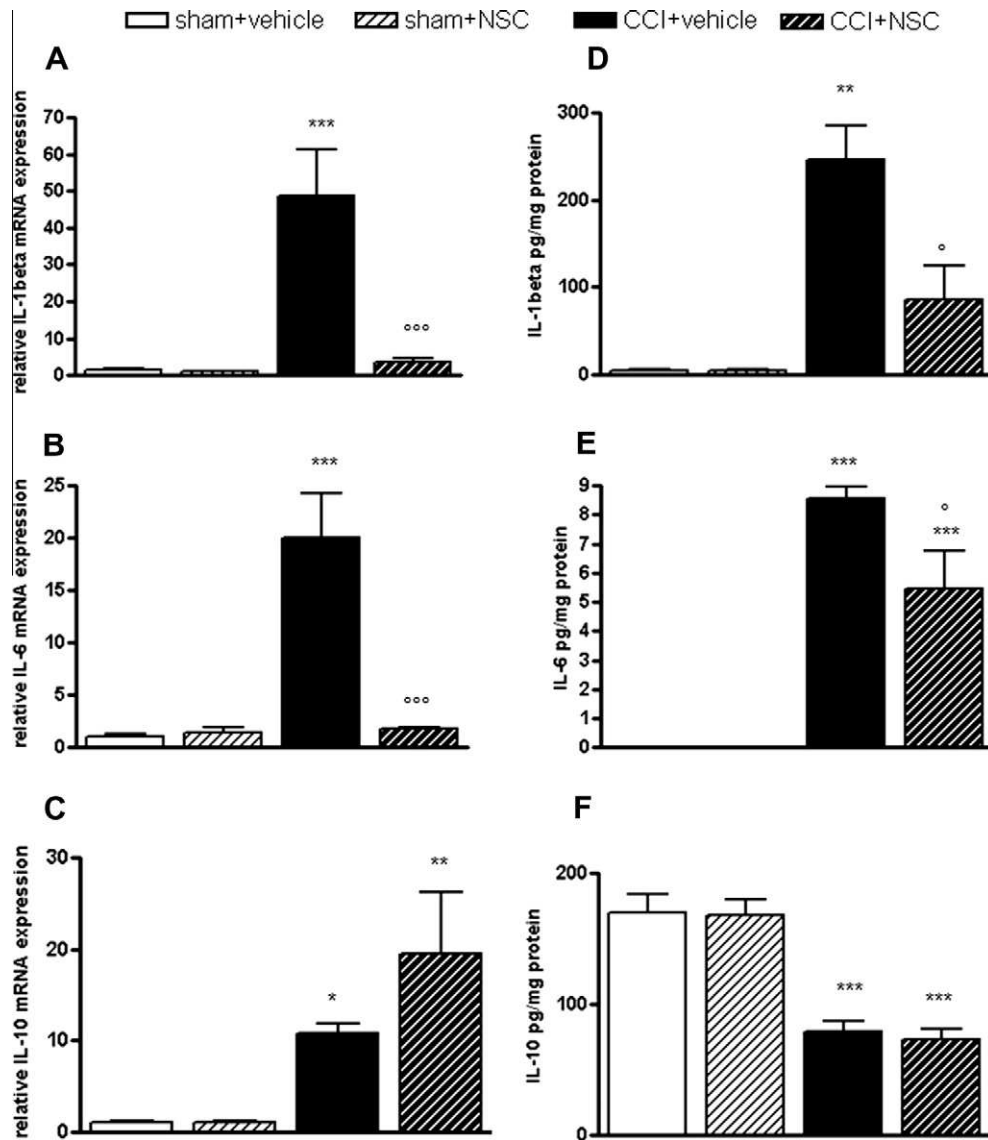
Fig. 6. IL-1 β (A and D), IL-6 (B and E), and IL-10 (C and F) mRNA expression and protein content in ipsilateral sciatic nerve 7 days after 2×10^6 cells. The cytokines mRNA levels, determined by real-time polymerase chain reaction (PCR), were expressed in relation to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and are presented as fold of increase relative to sham animals vehicle treated (A, B, C). Cytokine protein content is normalized to sample total protein (D, E, F). Data are mean ± SEM of 4–5 animals. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs sham + vehicle; $^{\circ}P < 0.05$; $^{\circ\circ\circ}P < 0.001$ vs CCI + vehicle.
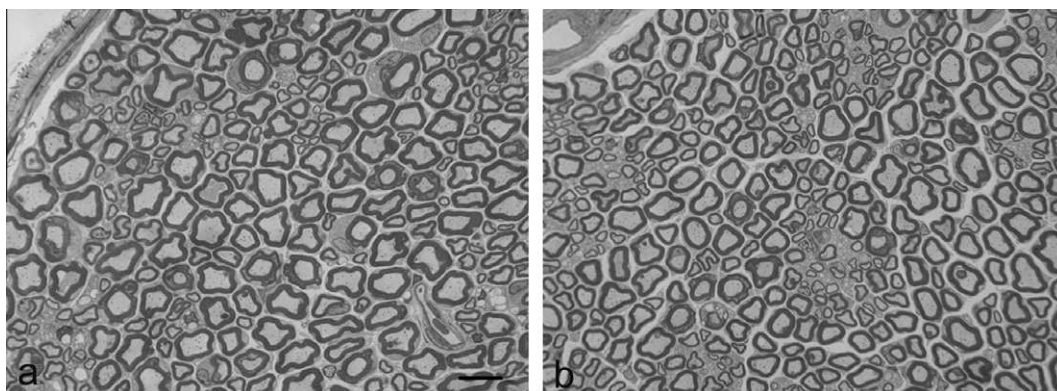

Fig. 7. Representative light micrographs of semi-thin sections of cross-sectioned sciatic nerves of sham-operated mice 14 days after vehicle (a) or 2×10^6 neural stem cell-treated mice (b). Toluidine blue staining. Scale bar = 10 μm.
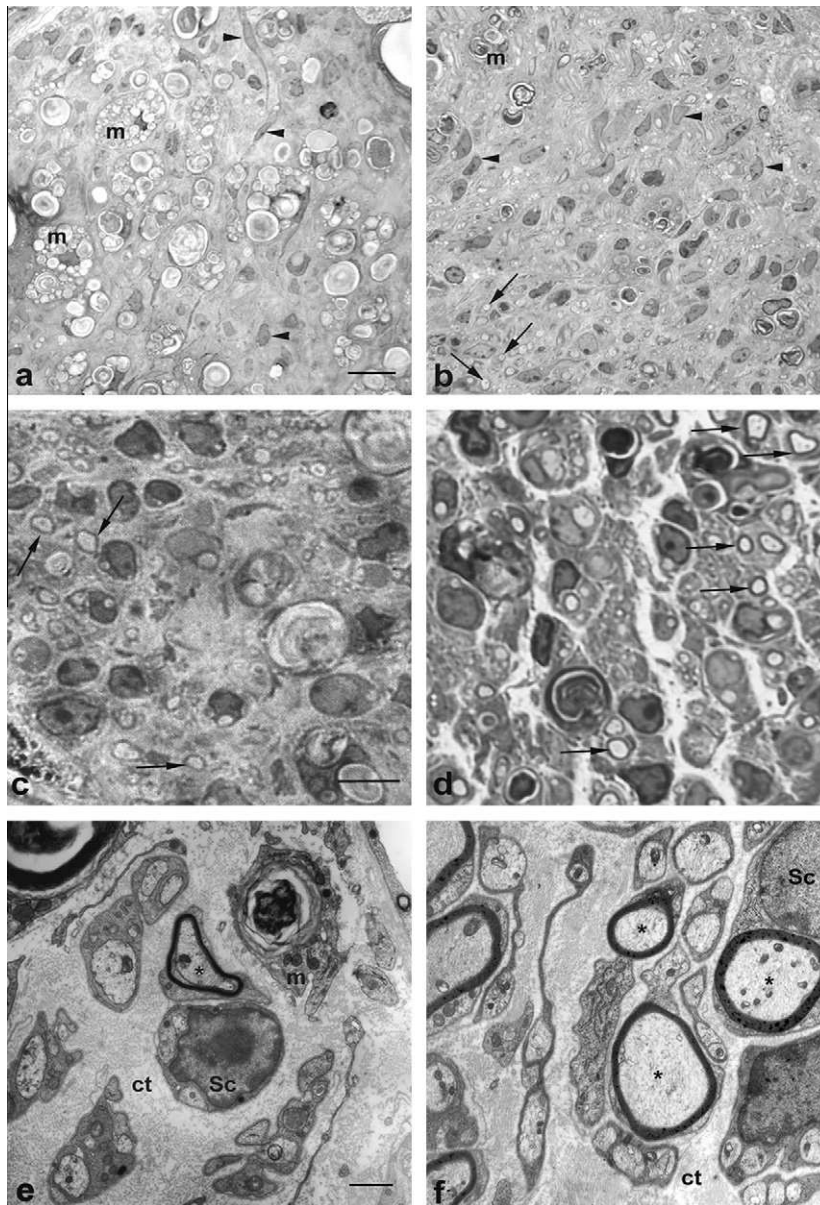
Fig. 8. Light (a and b scale bar = 10 μm; c and d scale bar = 5 μm, toluidine blue staining) and electron micrographs (e and f scale bar = 1 μm) from cross-sectioned sciatic nerves at 21 days after lesion of vehicle (a, c, and e), or neural stem cell (2 × 10⁶)-treated chronic constriction injury mice (b, d, and f). Treated animals show a higher number of nonneuronal cells nuclei (b), smaller macrophages (b), and more evident axonal sprout (d) than untreated (a and c). Arrows indicate some regenerating myelinated fibers; arrowheads point to some nonneuronal cells nuclei. In panels (e and f), some small myelinated axons are indicated with an asterisk. m, macrophage; Sc, Schwann cell nucleus; ct, connective tissue.

a greater number of cells might have been present in other organs at times shorter than 24 h and promptly cleared.

The absence of NSCs in the contralateral intact nerve suggests that NSCs might be facilitated in reaching the lesioned nerve due to the myelin modifications induced in this experimental model, as we and others previously demonstrated [30].

Although in our hands NSCs have an apparently low transplantation efficiency and can be shown locally only for a relatively short time; in the CCI model of neuropathic pain, intravenously administered NSCs counteract hyperalgesia, allodynia, the cytokine changes, and Fos increase for a time span that largely outlasts their presence on site. Moreover, all the effects we observe seem to be dependent on stemness, since non-stem cells do not yield any effect.

It may be interesting to focus attention on the sequence of events we observe. In the first instance, the time course of the

effects on pain definitely anticipates the effect on nerve repair. In fact, the effect on hyperalgesia and allodynia is already present on day 3 after NSC administration, before any nerve repair begins to be present. The timing of the initial effect well correlates with the NSC presence at the lesion site on days 3 and 7 after treatment. However, the NSC effect is maintained while NSC presence at the lesion site decreases. This time course suggests a first moment (days 3 and 7) when the analgesic effect is possibly due to factors locally carried and/or secreted by NSCs and by NSC-stimulated resident cells. The persistence of the effect at later times suggests a trophic and stimulating effect initiated by NSCs on resident cells, which leads toward a reparative process [8] and an improvement of nerve morphology. This effect is shown by the greater number of local nonneural cells such as fibroblast, Schwann cells, and blood-derived cells that can accelerate regeneration and the increased sprouting of myelinated fibers found in lesioned nerves 7
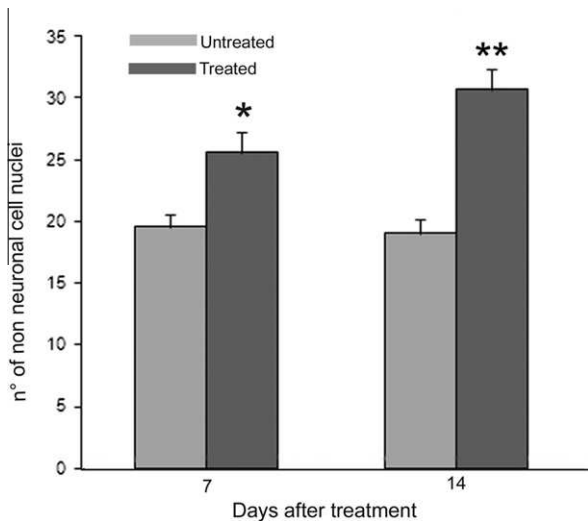
Fig. 9. Mean number ± SEM of nonneuronal cell nuclei (per 600 μm² of cross-sectional nerve area) of treated animals vs untreated animals, respectively, at 7 and 14 days after treatment. *$P < 0.05$; **$P < 0.01$ vs CCI + vehicle (untreated).

and 14 days after treatment. At this time, NSCs are still present, although in a very limited number.

The sequence of events postulated above is also consistent with the profile of NSC effects on Fos, SP, and cytokines. We observe, in fact, a rapid Fos decrease and SP increase after NSC administration that parallels the effect on hyperalgesia and allodynia. Fos decrease suggests a reduction of spinal neurons activation. Consistent with this hypothesis, we and others previously showed a sustained Fos increase in CCI animals that remained comparable at both 7 and 14 days after surgery [7,25]. Also, the effects on cytokines are consistent with a fast NSC modulatory effect due to the transport/secretion of immunomodulatory factors at the lesion site and inhibition of the neuroinflammatory cascade in resident cells, thus contributing to the maintenance of the effect at times when NSCs are not present anymore. We suggest that the role of cytokines and the NSC effects on them is of pivotal importance in explaining our data and the progression toward nerve repair and relief from pain in the CCI model.

It is well known, in fact, that CCI activates a neuroinflammatory cascade characterized by the increase of proinflammatory cytokines (IL-1, IL-6) that plays an important role in the etiopathogenesis and maintenance of neuropathic pain [30,42], and NSCs contribute to counteract this event, decreasing IL-1 and IL-6 concentrations at the lesion site. At difference, the protein concentrations of the antiinflammatory cytokine IL-10 are decreased after the lesion and remain unchanged after NSC treatment; however, its mRNA expression increases after the sciatic nerve injury and is further increased by NSC treatment. The contrasting profile of IL-10 protein and mRNA concentrations can be explained by a rapid protein utilization to counteract the proinflammatory cascade induced by the lesion and the contemporary activation of the synthetic machinery to support a sustained antiinflammatory IL-10 action. NSC treatment induces a further increase of the cytokine mRNA that, it is reasonable to suggest, participates in the therapeutic effect.

In our experiments, consistent with previous data in the literature obtained using the CCI experimental model [23,32], we observe that after the lesion, SP decreases in the spinal cord lamina, while no changes are observed for CGRP. The SP decrease, although well known and reproducible, has never been fully explained. At least 3 possible explanations have been put forward: (1) the degeneration of SP-containing nerve terminals; (2) the downregulation

of SP production in order to potentiate repair-oriented systems after injury; (3) the depletion of SP terminals due to accelerated production and release of the neuropeptide following the lesion. Whatever the cause of SP decrease, our data show for the first time that NSC treatment can partially restore spinal cord SP level. This observation, together with the effect on Fos expression, suggests that NSCs, even if localized only in the peripheral lesioned nerve, also modulate the spinal cord nociceptive signaling pathways.

Consistent with the hypothesis that NSCs act as local modifying agents, in the last few years a good deal of evidence has accumulated showing that exogenously administered stem cells of different origin carry/deliver at the site of action several neurotrophic factors, cytokines, peptides, neurotransmitters, and neuromodulators that contribute the favorable milieu in which they, as well as resident stem cells, can best work at and possibly initiate regeneration [9]. In this context, Caplan pushed himself to define stem cells as "Medicinal Signaling Cells" [8] and Martino et al. [29] proposed the concept of "bystander effect."

Interestingly, and consistent with the role of stem cells as carriers of active factors that modulate behavioral and biochemical neuropathic pain markers, in other experimental models different from pain, microvesicles derived from different types of stem cells have been shown to exert effects similar to those obtained by the respective cells administration [21]. Stem cell-derived microvesicles are a relatively unknown world, yet they have been shown to carry cytokines, neurotrophic factors, miRNA, and to exert potent immunomodulatory effects [13,31,44]. Which specific factors could be delivered by our NSCs is unknown at present, but under investigation. In the literature, stem cells have been shown to contribute antiapoptotic, immunomodulatory, antiscarring, angiogenic, trophic, and chemoattractant factors [12]. Our data suggest at least the presence of immunosuppressive as well as trophic molecules.

This communication between exogenous stem cells and resident cells leads to speculation about the great therapeutic potentials of employing stem cells and/or to the development of new pharmacological approaches to the treatment of neuropathic pain or other diseases, for example, mimicking the stem cell biochemical effects.

In conclusion, although we are aware that at present our results are limited to the CCI model and need to be confirmed in other neuropathic pain models, NSCs are unveiled as a very good tool to control otherwise intractable neuropathic pain. Moreover, they could, in the long term, favor tissue repair and therefore possibly stabilize the therapeutic result.

Conflict of interest statement

The authors have no conflicts of interest to report.

Acknowledgements

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