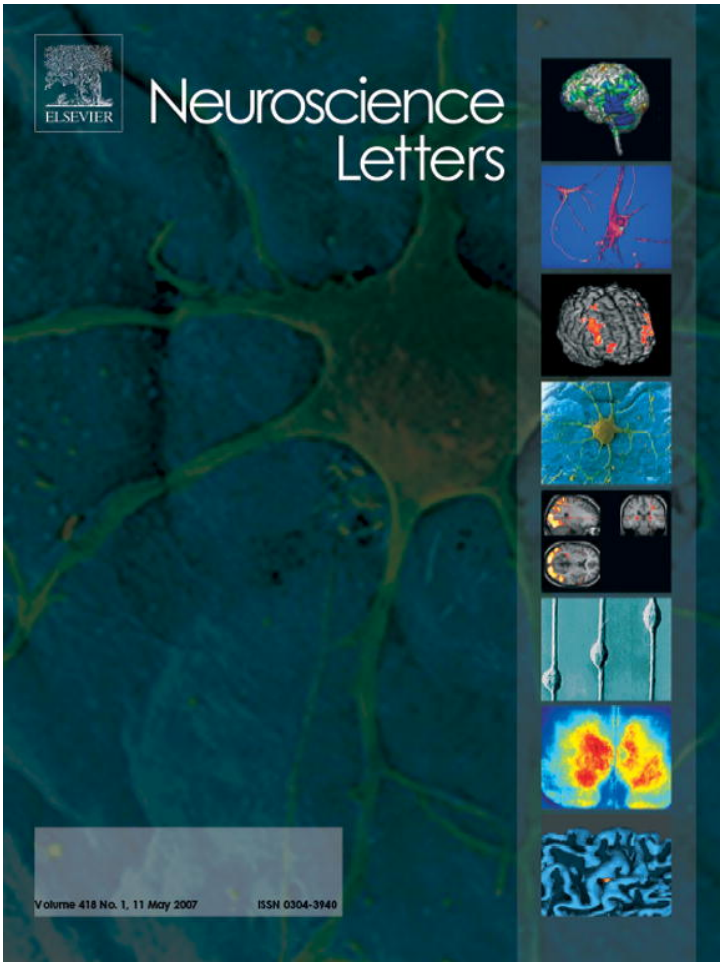

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Bone marrow stromal cells induce changes in pain behavior after sciatic nerve constriction

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Abstract

Peripheral nerve injury, i.e. a single ligature nerve constriction (SLNC), triggers neuropathic pain. Bone marrow stromal cells (MSCs) have been observed to migrate to the injured tissues and mediate functional recovery following brain, spinal cord and peripheral nerve lesions. We have recently shown MSC selective migration to the ipsilateral lumbar (L3–6) dorsal root ganglia (DRGs) after a sciatic nerve SLNC. In this study, we have analyzed the thermal and mechanical sensitivities of animals subjected to a SLNC of the sciatic nerve and an ipsilateral intraganglionic MSC injection, using the von Frey and Choi tests. Control animals were subjected to the nerve lesion either alone or followed by the administration of phosphate-buffered saline (PBS) or bone marrow non-adherent mononuclear cells (BNMCs). All the animals were tested both before surgery and after 1, 3, 7, 14, 21, 28 and 56 days. Animals subjected to the sciatic nerve constriction developed ipsilateral mechanical and thermal allodynia already 3 days after the lesion. The allodynic responses were maintained even after 56 days. MSC administration prevented the generation of mechanical allodynia and reduced the number of allodynic responses to cold stimuli. On the contrary, the injection of either PBS or BNMCs could not counteract allodynia. These results suggest that MSCs may modulate pain generation after sciatic nerve constriction. The underlying mechanisms by which MSCs exert their actions on pain behavior need to be clarified.

Keywords: Mechanical and thermal allodynia; Pain modulation; Peripheral neuropathy; Cellular therapy

The postnatal bone marrow is an organ composed of two main systems rooted in distinct lineages, the hematopoietic tissue proper and the associated supporting stroma. Bone marrow stromal cells (MSCs) are well known as multipotential cells that under specific experimental conditions differentiate into several types of cells, for example, osteoblasts, chondrocytes, adipocytes and myocytes [26,27]. In addition, MSC potential to differentiate into other cell lineages, i.e. those of neurons and astrocytes, has recently been reported, both *in vivo* [2,9,14] and *in vitro* [1,12,28,30]. Moreover, some studies have shown the ability of these cells to migrate to the spinal cord, dorsal root ganglia (DRGs) [9] and brain [20], in either lesioned [20] or control [9] animals, and to differentiate into neuroectodermal and microglial cells.

MSCs administered intravenously to rats subjected to a traumatic brain injury preferentially migrate into the injured hemisphere, where they increase the expression of growth factors [20] and improve functional recovery [18,20]. In an animal model of cerebral ischemia, the intravenous administration of MSCs results in the selective engraftment of the cells in the ischemic hemisphere and in a significant recovery of the somatosensory behavior [5]. Moreover, local MSC implantation in the distal stump of the transected rat sciatic nerve promotes functional recovery assessed by the walking track test [11].

To our knowledge, there are no studies on the effects of MSCs on pain generation after peripheral nerve injury. Single ligature nerve constriction (SLNC) of the sciatic nerve represents an animal model for the study of pain triggered by peripheral nerve injury [3]. Pain behavior appears to be dependent on the degree of nerve constriction, whereby a 'medium' SNLC (mSLNC; 40–80% reduction of the original nerve diameter) has been shown to represent the most effective alternative for induction of mechanical and thermal allodynia-like pain behavior [3].

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We have recently shown that in animals subjected to a sciatic nerve mSNLC and an intraganglionic injection of MSCs, these cells show a selective migratory tropism for the lesioned DRGs [8]. In the ganglia where homing occurs, MSCs acquire a striking perineuronal localization, resembling glial/satellite cells [8]. This characteristic distribution, acquired in an active and time-dependent fashion, suggests an association with a selective role in the injured nervous tissue.

In the present study, we have evaluated the pain behavior of rats subjected to a mSLNC in combination with administration of MSCs directly into the ipsilateral fourth lumbar DRG (L4-DRG).

For MSC and BNMC isolation, Sprague Dawley male rats (200–300 g, Fucal, Buenos Aires, Argentina) were sacrificed using an overdose of chloral hydrate (1.5 g/kg, i.p.), and their tibiae and femurs were dissected out from attached muscle and connective tissue. The epiphyses of the bones were removed, and the marrow was extracted with 3 ml of DMEM (GIBCO) using a 15 G needle and syringe. Red cells were lysed with 0.15 M buffered ammonium chloride solution, and the remaining cells washed twice with phosphate-buffered saline (PBS). The cells were then centrifuged through a density gradient (Ficoll-Paque Plus, 1.077 g/ml, Pharmacia) for 30 min at 400 × *g*. The interface containing mononuclear cells was washed with PBS and centrifuged for 10 min at 250 × *g*. The cells were then suspended at a concentration of 10×10^6 cells/ml in DMEM, 10% fetal bovine serum (GIBCO), 50 μg/ml gentamicine, 2.5 μg/ml anfotericine B, and 50×10^6 cells were plated in 25 cm^2 cell culture flasks. After 3 days, the non-adherent cells (afterwards referred to as bone marrow non-adherent mononuclear cells, BNMCs) were removed by replacing the culture medium. Medium was then changed every 4–5 days until confluence was reached. The cells were then harvested by incubation with 0.25% trypsin–1 mM EDTA (GIBCO), washed with PBS and suspended at a concentration of 50×10^6 cells/ml in PBS.

For MSC transplantation and nerve injury, adult (200–300 g) Sprague Dawley rats were used. Animals were anaesthetized with chloral hydrate (350 mg/kg, i.p.) and their right L4-DRG was exposed using a micro bone rongeurs after dissection of the aponeurotic and the paraspinal muscle group. For MSC transplantation, a suspension of cells (2×10^5 cells in 4 μl PBS) was injected over a 60 s period of time via a drawn glass micropipete (70–100 μm tip diameter) using a micropump syringe injector. Immediatelly after the muscular-aponeurotic and skin individual suture, the sciatic nerve constriction was performed. The right sciatic nerve was exposed and dissected free from the surrounding tissue at the mid-thigh level. It was then wrapped with a thin strip (5 mm long) of polyethylene and constricted to a mSLNC with a reduction of 40–80% of its original diameter [3]. The degree of constriction of each nerve was confirmed after dissection under a surgery microscope using a 10 mm ruler, and also by microscopical observation of 16 μm sections stained with neutral red. Sham animals were subjected to a mSLNC and intraganglionic injection of either PBS (4 μl) or BNMCs (2×10^5 cells in 4 μl PBS). For control purposes, sections of the injected DRG stained with neutral red were microscopically analyzed. Another group of control animals was only subjected to mSNLC. Ten animals were evaluated in each of the studied groups.

Behavioral testing was performed during daytime (9.00–18.00) in all animals before surgery (day 0) and 1, 3, 7, 14, 21, 28 and 56 days after transplantation and nerve constriction. The animals were placed in their acrylic testing chambers for 15 min for adaptation, and mechanical sensitivity was assessed with von Frey hairs (Stoelting, WoodDale, IL, USA). The hairs were applied in ascending order (1, 2, 4, 6, 8, 10, 15, 23 g) onto the plantar surface of both ipsilateral and contralateral hindpaws [4]. Each hair was delivered three times with 5 s intervals. The lowest force at which application elicited a paw withdrawal was taken as the mechanical response threshold. A paw withdrawal reflex obtained with 6 g or less was considered as an allodynic response. Cold sensitivity of the hindpaw to acetone (Choi test) [6] was quantified by foot withdrawal frequency. Hundred microliters of acetone were applied to the plantar surface of the paw using a plastic tubule connected to a 1 ml syringe. Acetone was applied five times to each paw at an interval of at least 5 min. The number of brisk foot withdrawals was recorded.

Statistical analysis was carried out by applying one-way analysis of variance (ANOVA) and Newman–Keuls multiple comparison post-test. Results were expressed as mean ± S.E.M. *p*-values are presented as following: ns $p > 0.05$; $^{*}0.05 > p > 0.01$; $^{**}0.01 > p > 0.001$ and $^{***}p < 0.001$.

MSCs injected into the right L4-DRG prevented the generation of mechanical allodynia and significantly decreased the number of allodynic responses to cold stimuli generated by the sciatic nerve ligature.

Controls: Only rats with with normal behavior before surgery were included in the experiments. Before surgery, the pain threshold for mechanical and thermal sensitivity was normal both on the right (Fig. 1) and left (data not shown) hindpaw footpads and this was also the case for the contralateral footpad after surgery (not shown).

Animals with mSLNC alone: Microscopical observation of constricted nerves showed a reduction of 40–80% of their original diameter. A swelling was observed proximal to the site of constriction. In addition, there was fibrous tissue formation at the site of the ligature, as previously described [3,22]. All rats subjected to mSLNC showed guarding behavior and changes in the posture of the affected hindpaw, including plantar flexion and toe-clenching. Application of von Frey filaments showed a decrease in paw-withdrawal threshold in almost all rats already 3 days post injury with 67% of these animals showing an allodynic mechanical response (less than 6 g) (Fig. 1a). The lowest paw-withdrawal thresholds were obtained 3 days post injury. Moreover, using the Choi test for the evaluation of thermal sensitivity, we found a clear increase in the number of positive nociceptive responses at the same survival time (Fig. 1b). The major number of allodynic responses was observed 3 days after the lesion. There was maintenance of both mechanical and thermal painful responses over time until 56 days post injury (data not shown).

Animals with mSLNC and MSC, BNMC or PBS injection: The injected L4-DRG showed a moderate swelling (increased

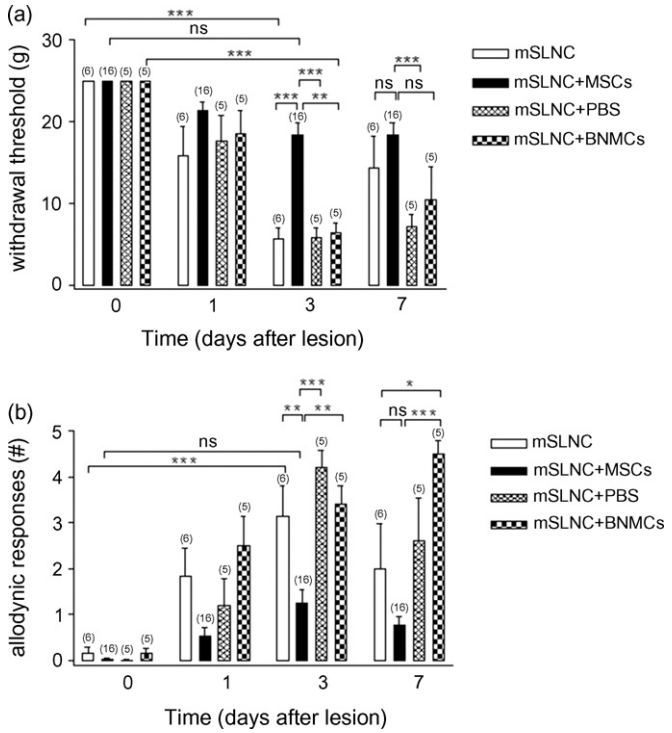

Fig. 1. Effect of a mSLNC of the sciatic nerve on the development of mechanical (a) and thermal (b) allodynia in animals subjected to the lesion alone or followed by an ipsilateral intraganglionic injection of MSCs, PBS or BNMCs. (a) A significant decrease in the paw withdrawal threshold of the von Frey filaments test is induced by the mSLNC 3 days after the lesion. It is noticeable that the injection of MSCs prevents the development of mechanical allodynia while the administration of either PBS or BNMCs has no effect on the response to mechanical stimuli. (b) A significant increase in the number of allodynic responses of the Choi test is induced by the mSLNC 3 days after the lesion. Note that the injection of MSCs drastically reduces the number of nociceptive responses while the administration of PBS has no effect on the response to cold stimuli. BNMC injection induces a significant increase in cold allodynia 7 days post injury. Values show mean ± S.E.M. p-values are presented as following: ns $p > 0.05$; *$0.05 > p > 0.01$; **$0.01 > p > 0.001$ and ***$p < 0.001$.

by 15% when compared to the average size of normal DRGs) caused by the injected solutions, during the first 3 days post surgery. A return to normal size was observed 5 days post injection. Fibrous tissue formation and macrophage infiltration were observed at the site of the injection. In the MSC-treated group, there was a slight decrease in paw-withdrawal threshold with however no generation of allodynia-like behavior even 3 days post injury (Fig. 1a). This non-allodynic pattern was maintained until 56 days after the lesion (data not shown). The injection of either BNMCs or PBS in mSLNC animals did not counteract allodynia (Fig. 1a). Cold stimulation of the hindpaw of MSC-injected animals resulted in a significantly lower number of positive nociceptive responses 3 days post injury, when compared to animals with the lesion alone or with PBS or BNMC administration (Fig. 1b). Moreover, after BNMC injection into the L4-DRG, there was a significant increase in thermal allodynia 7 days post surgery (Fig. 1b), thus showing an opposite effect to that induced by MSC administration, probably due to a mechanical effect. After 56 days, BNMC and PBS-injected animals still presented nociceptive responses to both mechanical and thermal stimuli (data not shown).

The present results show that MSCs have the ability to reduce the allodynic responses generated by a peripheral nerve constriction.

In animal models of brain trauma [17,18] and ischemia [5], rat MSCs have been reported to migrate to the injured brain area after intravascular administration, and promote recovery of the somatosensory behavior [5,17,18]. Local MSC implantation in the distal stump of the transected rat sciatic nerve promotes functional recovery assessed by the walking track test [11]. Furthermore, it has recently been shown that after an intraganglionic injection of MSCs, they preferentially migrate to the lumbar DRGs affected by a peripheral nerve lesion [8]. In the injected ganglia, as well as in the DRGs where homing occurs, MSCs localize perineuronally, resembling glial/satellite cells [8]. This characteristic distribution surrounding neurons is acquired in an active and time-dependent fashion [8] and suggests a selective association and function.

A successful integration of transplanted MSCs, as well as functional improvement, has been evidenced in several other animal models, including Parkinson's disease [19], stroke [29] and contusion [7,25,32] or demyelinating [2] injuries of the spinal cord. Concerning the peripheral nervous system, differentiation of MSCs into functional myelinating Schwann cells and nerve regeneration have been observed after treatment of transected or crushed sciatic nerves with MSCs [10,31]. Here, we show a MSC-induced decrease in neuropathic pain generation after peripheral nerve constriction, using both the von Frey and Choi tests. We observed prevention of the generation of mechanical allodynia and a reduction in the number of cold allodynic responses after MSC administration in animals subjected to a sciatic nerve ligature. These effects were not observed after the administration of BNMCs, showing that the alleviation of pain was inherent to the presence of MSCs and not due to unspecific factors such us mechanical disruption or pathological changes of primary afferent neurons due to the injection.

One important issue to be considered is the fact that MSCs constitutively secrete a diverse spectrum of interleukines (IL), growth factors and chemokines, and express chemokine receptors [13,15,16,21]. These cytokines act as survival, growth or differentiation factors and may modulate primary sensory neurons response to injury and thus influence pain behavior. Furthermore, *in vivo* experiments have reported an increase in the expression of neurotrophins, such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) in animals treated with MSCs after traumatic brain [20] or spinal cord injuries [24], with a parallel functional recovery of the lesioned animals [18,20]. A reciprocal action between BDNF and IL-6 (a chemokine secreted by MSCs) on rat primary sensory neurons has been reported [23]. Moreover, intrathecal infusion of IL-6 increases the concentration of BDNF mRNA in rat lumbar DRGs, and the induction of BDNF in DRG neurons after peripheral nerve injury is severely attenuated in IL-6 knock-out animals [23]. The observed perineuronal localization of MSCs suggests they may be behaving just as glial cells in an injured DRG: producing neurotrophic factors that promote primary sensory neurons survival. Nevertheless, the underlying mechanisms

by which MSCs exert their actions on pain behavior need to be clarified.

The limited success of currently available strategies for the treatment of neuropathic pain suggests the need for new therapeutic approaches. Here we provide data based on an animal pain model, indicating that MSCs could be used as modulators of neuropathic pain. Further studies should be carried out in order to elucidate the molecular mechanisms involved in the observed alleviation of pain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2007.03.001.

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