Neuroprotection and Axonal Regeneration After Lumbar Ventral Root Avulsion by Re-implantation and Mesenchymal Stem Cells Transplant Combined Therapy

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Abstract Ventral spinal root avulsion causes complete denervation of muscles in the limb and also progressive death of segmental motoneurons (MN) leading to permanent paralysis. The chances for functional recovery after ventral root avulsion are very poor owing to the loss of avulsed neurons and the long distance that surviving neurons have to re-grow axons from the spinal cord to the corresponding targets. Following unilateral avulsion of L4, L5 and L6 spinal roots in adult rats, we performed an intraspinal transplant of mesenchymal stem cells (MSC) and surgical re-implantation of the avulsed roots. Four weeks after avulsion the survival of MN in the MSC-treated animals was significantly higher than in vehicle-injected rats (45 % vs 28 %). Re-implantation of the avulsed roots in the injured spinal cord allowed the regeneration of motor axons. By combining root re-implantation and MSC transplant the

number of surviving MN at 28 days post-injury was higher (60 %) than in re-implantation alone animals (46 %). Electromyographic tests showed evidence of functional re-innervation of anterior tibialis and gastrocnemius muscles by the regenerated motor axons only in rats with the combined treatment. These results indicate that MSC are helpful in enhancing neuronal survival and increased the regenerative growth of injured axons. Surgical re-implantation and MSC grafting combined had a synergic neuroprotective effect on MN and on axonal regeneration and muscle re-innervation after spinal root avulsion.

Keywords Ventral root · Motoneuron · Neuroprotection · Axonal regeneration · Stem cells · Surgical repair

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Introduction

Avulsion of the spinal roots causes disconnection between the peripheral and the central nervous systems. The avulsion of dorsal roots leads to anesthesia, sensory dysfunction, and neuropathic pain in the affected limb, while ventral root avulsion (VRA) induces muscular denervation, atrophy, and paralysis. Moreover, the avulsion of the ventral roots results in a marked and progressive death of the axotomized motoneurons (MN) [1–3]. Nevertheless, surviving MN maintain their capacity for axonal re-growth [4–8], so that if the avulsed ventral root is surgically re-implanted into the spinal cord segment, MN can regenerate their axons and also have an increased probability of survival, particularly when re-implantation is performed soon after injury [9, 10]. In patients with brachial plexus avulsion, direct re-implantation of injured roots has allowed some functional recovery [11]. Similarly, in animal models of VRA at cervical and sacral levels, re-implantation of avulsed roots increased MN survival and allowed muscle re-

innervation although with limited functional recovery [10, 12–14]. However, the chances for functional recovery after lumbar VRA and surgery repair are very poor [14, 15]. The lower capacity of lumbar MN to re-innervate distal target muscles may be due to the long distance that surviving neurons have to re-grow [16, 17]. Besides the necessity of axons to re-innervate their target muscles, survival of the avulsed MN is fundamental. In order to prevent MN death most studies have focused on the neuroprotective role of different neurotrophic factors. Thus, exogenous application of brain-derived neurotrophic factor (BDNF) [15, 18] or glial cell line-derived neurotrophic factor (GDNF) [15, 19, 20] has been shown to partially promote survival of neurons and axonal regeneration after spinal root injuries. However, the efficacy of these factors depends on their continuous supply and periodic injections might be necessary. Another possibility is the transplantation of cells that secrete neurotrophic factors. Thus, after VRA local injection of stem cells that express BDNF and GDNF exerted protection against MN death and reduced astrocyte reaction [21].

Mesenchymal stem cells (MSC) are pluripotent stem cells that can differentiate into mesodermal tissue, such as bone, muscle, cartilage, and fat [22]. Transplantation of MSC has been demonstrated to produce beneficial effects in animal models of central and peripheral neural insults. Thus, after spinal cord injury [23–26] and brain stroke [27, 28] focal injection of MSC improves functional recovery and reduces tissue damage and neuronal death. Furthermore, in experimental peripheral nerve injuries, MSC grafts have the capacity to enhance axonal regeneration [29, 30].

As surgical re-implantation of avulsed roots is mandatory for allowing axons to regenerate along the peripheral nerve and reach muscles and then obtain functional recovery, and the results are generally poor or absent after lumbosacral root avulsion, the aim of this work was to complement the surgical repair of avulsed lumbar spinal roots with a MSC transplant into the injured segments of the spinal cord. The re-implantation of avulsed roots will establish reconnection of MN with the spinal nerves, whereas the transplanted MSC will facilitate neuronal survival and axonal regeneration. We demonstrate that either root re-implantation or MSC transplants have beneficial effects regarding MN survival, whereas combination of both strategies showed a synergistic effect and enhanced axonal regeneration.

Materials and Methods

Cell Cultures

MSC Cultures

Primary MSC cultures were obtained from P22 Sprague–Dawley rats. Rats were euthanized with carbon dioxide

(CO₂). Tibias and femurs were placed on cool phosphate buffered saline (PBS), and epiphyses were removed. The diaphyses of bones were flushed with PBS and the marrow was homogenized mechanically. The cell mixture was filtered through a 70- μ m nylon mesh and recovered by centrifugation for 10 min at 1500 rpm (231 g). The pellet was re-suspended in growth medium: alpha modification minimum essential medium (Life Technologies, Grand Island, NY, USA) supplemented with 20 % heat-inactivated fetal bovine serum (Lonza, Verviers, Belgium), 2 mM L-glutamine (Life Technologies) and 100 units/ml penicillin/streptomycin (Life Technologies, 100 $\times$); and plated in 100-mm culture dishes (Iwaki, Asahi Technoglass, Chiba, Japan) at 5×10^6 cells/cm². After 24 h, the supernatant containing non-adherent cells was removed and fresh medium was added. When the culture was near confluence, every 4–5 days, the cells were detached using PBS with 0.05 % trypsin (Life Technologies) and 0.04 % ethylenediaminetetraacetic acid (Sigma, St Louis, MO, USA) and re-plated at 5000 cells/cm². Cells were passaged 3–4 times, and expanded to 80–90 % confluence.

Fibroblast Cultures

Fibroblasts were obtained from P22 rat sciatic nerves. The epineurium was taken with the help of microscissors and fine forceps, and enzymatically dissociated in 1 ml Hank's salt solution (Ca²⁺ and Mg²⁺-free) with the addition of 0.25 % trypsin, 1 mg/ml collagenase A and 1 mg/ml of DNase-I at 37 °C for 1 h. Then, the tissue was dissociated mechanically with a Pasteur pipette, and 13 ml DF10S medium was added to stop the enzymatic reaction. After centrifugation at 900 rpm (83 g) for 10 min, the pellet was re-suspended in 1 ml DF10S medium and cells were counted in a Neubauer chamber. Cells were plated at a concentration of 350–500 cells/mm², and maintained at 37 °C and 5 % CO₂, with changes of the medium every 3 days.

Cell Labeling

For pre-labeling the cells we used a lentiviral vector encoding green fluorescence protein (GFP) under the EF1 α promoter. The cells in passage 2 were plated at 2000 cells/cm² and incubated with the lentivirus at a multiplicity of infection of 10 for 48 h. Then, the medium was changed and the cells cultured as described above. The cells for transplantation were detached and re-suspended in L15 medium (Life Technologies) at 50,000 cells/ μ l—the maximal cell concentration that we found in pilot assays that does not compromise cell survival during injection—and maintained in ice during the surgery.

Spinal Cord Slices Culture

Sprague–Dawley rats at day 7 post-natally were decapitated and the spinal cord lumbar segments were dissected, placed in cold Gey's balanced salt solution (Sigma) enriched with 6 mg/ml glucose, and cleaned from blood and meningeal debris. Spinal cords were then cut with a McIlwain Tissue Chopper (Mickle Laboratory Engineering, Surrey, UK) into 350- μ m thick slices. A volume of 450 μ l of type I collagen solution (BD Biosciences, Erembodegem, Belgium) at a concentration of 3.4 mg/ml supplemented with 10 % fibronectin (BD Biosciences) was mixed with 50 μ l of 10 $\times$ basal Eagle's medium (Life Technologies) and 2 μ l of 7.5 % sodium bicarbonate solution (Life Technologies) [31]. Single 30- μ l drops were deposited on poly-d-lysine- (1 μ g/ml; Sigma) coated coverslips, which were placed in Petri dishes or 24-well multidishes (Iwaki) and kept in the incubator at 37 °C and 5 % CO₂ for 2 hours to induce collagen gel formation. Spinal cord slices were then placed on gelled collagen droplets and covered by a second 30- μ l droplet of the same collagen solution. The embedded samples were incubated with Neurobasal medium (Life Technologies), supplemented with B27 (Life Technologies), glutamine and penicillin/streptomycin (Sigma). After 1 day in culture, the medium of spinal cord cultures was changed by a penicillin/streptomycin-free medium. Spinal cord slices were cultured for 4 days and fixed with 4 % paraformaldehyde at 4 °C for 20 min, and immunostained as described previously [31] to assess neurite growth. For MSC or fibroblast co-culture, an adequate amount of GFP-labeled cell suspension in culture medium was gently mixed into the collagen matrix to get a final density of 5×10^4 in each volume of matrix used to embed the spinal cord slice.

Analysis of Neurite Outgrowth

Spinal cord cultures were stained with anti-neurofilament antibody RT97 (1:200, Developmental Studies Hybridoma Bank) in order to label neurons and growing neurites. Microphotographs for quantitative analysis were taken at 20 $\times$ with a digital camera (Olympus DP50; Olympus, Hamburg Germany) attached to the microscope (Olympus BX51), acquired in Adobe Photoshop CS4 to photomerge them automatically, and analyzed with the aid of ImageJ software (National Institutes of Health; available at <http://rsb.info.nih.gov/ij/>). The conventional Sholl [32, 33] method was adapted for spinal cord slice cultures to count the number of neurites crossing concentric circles at specific lengths from the ventral root exit. The length of the longest neurite in the cultures was also measured for at least 15 samples per condition. For the arborization area, the

microphotographs were transformed to a gray 8-bit image, and the labeled area was assessed after defining a threshold for background correction using ImageJ software.

Surgical Procedure and Cell Transplantation

A total of 75 adult female Sprague–Dawley rats (9 weeks old; 250–300 g) were used in this study and housed with free access to food and water at a room temperature of 22 ± 2 °C under a 12:12 hour light–dark cycle. The experimental protocols were approved by the animal ethics committee of the Universitat Autònoma de Barcelona in accordance with the European Directive 86/609/EEC. In the root avulsion alone study, the animals were distributed randomly in avulsion (AV, $n=15$), avulsion and vehicle injection ($n=12$) or avulsion and MSC transplantation groups (AV-MSC, $n=12$). In the avulsion and surgical repair study, the animals were assigned to avulsion with direct re-implantation and vehicle injection (AV-RE, $n=15$) or avulsion with direct re-implantation and MSC transplantation (AV-RE-MSC, $n=15$). Moreover, to follow the MSC transplanted into the spinal cord, 6 animals were injected with GFP⁺-MSC after VRA.

Under anesthesia with ketamine (90 mg/kg) and xylazine (10 mg/kg), a unilateral intravertebral avulsion of L4, L5, and L6 roots was done as described previously [1, 3]. Briefly, a midline skin incision was made and the paravertebral muscles were retracted to expose the spinal column. A unilateral laminectomy was made at the right side and L4, L5, and L6 ventral and dorsal roots were identified. A moderate traction was applied, detaching both the ventral and dorsal roots from the spinal cord. In the avulsion groups, the roots were withdrawn approximately 5 mm to avoid axonal re-growth. In the avulsion-re-implanted groups, the three ventral and dorsal root stumps were inserted, just after avulsion, in the spinal cord lateral surface of the corresponding segments and secured with a 10–0 suture. In the injected groups, a total of 3 μ l of vehicle (L15) or MSC suspension (50,000 cells/ μ l) was injected into the lateral funiculus of the avulsed cord. Using a glass needle (100 μ m internal diameter; Eppendorf, Hamburg, Germany) coupled to a 10 μ l Hamilton syringe, three 1- μ l injections were performed, each one into each avulsed spinal cord segment. A perfusion speed of 2 μ l/min was controlled by an automatic injector (KDS 310 Plus; Kd Scientific, Holliston, MA, USA), and the needle tip was maintained inside the tissue 3 min after each injection to avoid leaking. The wound was sutured by planes and the animals allowed to recover in a warm environment. At 2 weeks after surgery rats were evaluated by behavioral locomotion and electrophysiological techniques in order to confirm the deficits caused by L4–L6 root avulsion in the hindlimb. If the behavioral-electrophysiological tests

suggested an incomplete injury of L4, L5, and L6 roots, the animal was not included in the study. Thus, a total of 10 animals was excluded from the experiments.

Behavioral Tests

The locomotor behavior of the operated animals was monitored weekly. A subscale of the Basso, Beattie and Bresnahan (BBB) locomotor score [34] relevant for the lumbar root avulsion model was used for evaluating the hindlimb function, as described previously [3, 20]. The voluntary movements of the ankle, knee, and hip joints of the avulsed limb were evaluated, with the scores for each being (0) = no spontaneous movement of the joint, (1) = slight movement of the joint and (2) = normal movement of the joint. The final score for the avulsed limb was the accumulative scores for the 3 joints. The normal score for an intact limb was 6 points. The toe-spreading response was also assessed while lifting the rat by the tail.

Electrophysiological Tests

All the animals were tested at 2 weeks in order to characterize the extent of muscle denervation of the hindlimb muscles after VRA; in addition, rats with root re-implantation repair were also evaluated at 4 and 8 weeks after the injury to assess re-innervation of target muscles. For the electrophysiological tests, animals were anesthetized with pentobarbital (30 mg/kg i.p.), placed prone onto a metal plate and skin temperature maintained above 32 °C. Motor-evoked potentials (MEPs) were elicited by transcranial electrical stimulation of the brain. Two needle electrodes were placed subcutaneously over the skull—the anode over the sensorimotor cortex and the cathode on the hard palate. Single electrical pulses of supramaximal intensity (25 mA, 100 μ s) were applied, and the MEPs were recorded with monopolar needle electrodes from tibialis anterior (TA), gastrocnemius medialis (GM), and interossei plantar (PL) muscles [35, 36]. Peripheral motor nerve conduction tests were performed by stimulating the sciatic nerve with single electrical pulses (100 μ s at supramaximal intensity) delivered by monopolar needles placed at the sciatic notch and recording the compound muscle action potentials (CMAP) of TA, GM, and PL muscles by means of needle electrodes. The active electrode was inserted on the belly of the muscle and the reference at the fourth toe [3, 33]. Signals were amplified, filtered (bandpass 1–5000 Hz), displayed on an oscilloscope (Sapphire 4ME; Medelec-Vickers, Woking, Surrey, UK) and measured.

Retrograde Axonal Tracing

One week before sacrificing the animals of the re-implantation study, the retrotracer FluoroRuby (Life Technologies) was

applied in the sciatic nerve. Under anesthesia with ketamine/xylazine (90/10 mg/kg i.p.) both left and right sciatic nerves were exposed at the mid-thigh. The sciatic nerve was cut and the proximal stump submerged in 5 % FluoroRuby solution for 1 h. Then, the area was flushed with saline and the wound sutured by planes.

Tissue Processing for Histology

The end time point of the avulsion and vehicle injection and AV-MSC groups was at 28 days post-operation (dpo) ($n=10$ for each group), while the animals of groups AV, AV-RE, and AV-RE-MSC were divided and the tissues processed at 28 ($n=8$ for each group) and 52 dpo ($n=5$ for each group). For animals injected with GFP⁺-MSC the end time points were 7, 14 and 28 dpo ($n=2$ for each). The rats were anesthetized deeply (pentobarbital 60 mg/kg i.p.) and intracardially perfused with 4 % paraformaldehyde in PBS. The spinal cord L4, L5, L6, and S1 segments (1 cm total length) and the sciatic nerve from lumbar plexus to sciatic notch were harvested and post-fixed in the same fixative solution for 24 h and cryopreserved in 30 % sucrose. Coronal spinal cord sections 30 μ m thick were cut with a cryostat and distributed in 10 series of 8 sections (separated by 440 μ m) each. The sciatic nerves were cut at 15 μ m and distributed in 5 series of 8 sections (separated by 75 μ m). For morphological evaluation of sciatic nerve cross-sections, a segment of the nerve at the mid-thigh was harvested and post-fixed in glutaraldehyde/paraformaldehyde (3 %/3 %) in cacodylate buffer solution (0.1 M, pH 7.4) overnight at 4 °C, post-fixed in 2 % osmium tetroxide, dehydrated through ethanol series, and embedded in epon (Electron Microscopy Sciences, Hatfield, PA, USA) resin. Transverse semithin sections (0.5 μ m thick) were cut with an ultramicrotome, stained with toluidine blue, and examined by light microscopy.

Motoneuron Number Counts

One series of 8 sections of the spinal cord was incubated for 20 min with fluorescence Nissl labeling solution (Life Technologies) following the manufacturer's protocol. The number of alpha MN present was estimated by the stereological optical dissector method as described elsewhere [2, 3]. MN were identified by their localization in the lateral ventral horn of lumbar spinal cord sections and only MN with diameters higher than 30 μ m, a prominent nucleolus, and polygonal shape were counted. Sequential microphotographs covering the lateral ventral horn were taken at 400 $\times$ and a 30- μ m square grid was superimposed onto each one [37]. Size exclusion was used to selectively count the population of alpha MN avoiding inclusion of gamma MN, interneurons, and glial cells in the counts. In the case of re-

implanted animals, the total number of FluoroRuby retrolabeled neurons in each section was also counted. The mean number of MN per section was calculated. For comparisons, the estimated number of MN present in the ventral horn of the avulsed side was expressed as a percentage of the contralateral side.

Immunohistochemistry and Image Analysis

Sections from animals of the different groups taken at different time points were processed in parallel for immunohistochemistry. Tissue sections were blocked with tris-buffered saline-0.3 % Triton-5 % fetal bovine serum and incubated for 2 days at 4 °C with primary antibodies goat anti-choline acetyltransferase (ChAT) (1/50; Millipore, Billerica, MA, USA) to label motor fibers and mouse anti-glial fibrillary acidic protein (GFAP) (1/1000; Dako, Glostrup, Denmark) to label astrocytes. After washes, sections were incubated for 2 h at room temperature with biotinylated conjugated donkey anti-goat or anti-mouse antibodies (1/200; Vector, Burlingame, CA, USA) and followed by 2 h with AlexaFluor 488 or AlexaFluor 565-conjugated streptavidine (1/200; Jackson ImmunoResearch, West Grove, PA, USA). Slides were dehydrated and mounted with Citoseal 60 (Thermo Fisher Scientific, Madrid, Spain). The sections of GFP⁺-MSC-injected rats were mounted directly without processing after cutting. Images were captured with a digital camera (Olympus DP50) attached to a microscope (Olympus BX51). Analysis of glial reactivity was performed using 10 spinal cord sections (440 µm between pairs) of each animal. Images of the ventral area of the spinal cords were taken at 400× with the same setting. The microphotographs were transformed to a gray scale and analyzed using ImageJ software. Immunoreactivity was assessed by calculating the integrated density of a region of interest measured after defining a threshold for background correction. The regions of interest were selected on the gray matter of the ventral horn and had an area of 0.34 mm². The integrated density is the area above the threshold for the mean density minus the background. To count the number of ChAT + fibers in the nerve sections, images at 400× were taken of 1 series of 8 sections. The mean number of fibers per section was calculated in proximal, medial, and distal parts of the studied nerve segment.

Data Analysis

Data are expressed as mean values and the standard error. For statistical analysis one way analysis of variance was used for parameters measured at only one time point, and two way analysis of variance for repeated measures for time-depending parameters. Post hoc Bonferroni test for comparative pairs of groups was used. In all the comparisons, an α of 5 % was considered as significant.

Results

Spinal Cord Organotypic and Cell Co-cultures

As *in vitro* approximation of MN axotomy, we cultured spinal cord slices in a three-dimensional matrix in the presence of MSC, fibroblasts or control vehicle. Figure 1 shows representative micrographs of the MSC and control cultures where neurites from MN growing outside the spinal cord are observed. After 4 days in culture, the presence of MSC significantly increased the amount of axons that grew a longer distance from the spinal cord exit ($p < 0.05$ between 300 and 550 µm) (Fig. 1g). The maximal length of the growing neurites was also higher in the MSC co-cultures than in controls ($956 \mu\text{m} \pm 87 \mu\text{m}$ vs $648 \mu\text{m} \pm 70 \mu\text{m}$, 149 % ± 15 with respect to control, $p = 0.01$). There was also an increase, although not significant, in the arborization of neurites—a relative measure of the amount and branching of neurites. While the presence of MSC enhanced neurite outgrowth, no significant differences were observed between fibroblast co-culture and control cultures regarding neurites number ($p > 0.05$ for all the distances), maximal length (91 % ± 15 % of control, $p = 0.76$) and arborization (182 % ± 29 % with respect to control, $p = 0.11$). These observations indicate that MSC have the intrinsic capability to enhance regeneration of motor axons, while fibroblasts do not affect motor axons growth. Interestingly, neurites that grew out of the spinal cord slice into the three-dimensional matrix plus MSC regenerated without close association with MSC (Fig. 1c, d), indicating that direct contact between cells and axons is not needed.

MSC Transplantation After VRA

GFP⁺-MSC were found in the injured side of L4, L5, and L6 spinal segments at 7 days after injection (Fig. 2b). Most cells migrated from the injection site and were localized in the ventral root exit zone. At longer times the GFP labeling was reduced inside the spinal cord and no migration out of the injury area was observed. Thus, at 14 days after injury (Fig. 2c) the amount of GFP was less than at 7 days, whereas at 28 days only a few GFP-positive cells were found (Fig. 2d, e).

Using fluorescence Nissl staining we counted the number of MN in coronal sections of L4, L5, and L6 segments of the spinal cord in both the avulsed and the contralateral side. In the intact animals, the number of MN was similar in both sides of the spinal cord. One month after VRA, only about 30 % of the MN were present in the injured spinal cord segments of non-treated rats, while animals transplanted with MSC had a significantly higher number of surviving MN (28.9 % ± 4.4 % and 46.8 % ± 4.3 % for AV and AV-MSC groups respectively) (Fig. 2f–i).

Fig. 1 Organotypic culture of spinal cord slices and co-culture with mesenchymal stem cells (MSC). After 4 days *in vitro* neurites grow longer out of the spinal cord in the three-dimensional collagen matrix (arrows in B and F, immunolabeling of RT97 in red) in green fluorescence protein (GFP) + MSC co-culture (a, b is higher magnification image of the box in a) than in control condition (e, higher magnification in f). (c), (d) (higher magnifications of box in b) show the tip of a regenerating neurite (arrow in d) that grows without contacting the

MSC (arrow in c). Quantification of the number of neurites that grew to specific distances from the spinal cord slice (g, top graph), counted using the Sholl method, the maximal elongation (g, bottom left graph), and arborization (g, bottom right graph) of the neurites shows an increased neurite growth in co-culture of MSC and spinal cord slices with respect to culture alone. Scale bar = 500 μm in (a) and (e), 250 μm in (b) and (f), and 50 μm in (c) and (d)

The immunoreactivity of GFAP was measured in the same lumbar segments to quantify the astrocyte reaction. Because no changes were found between injured segments in each animal, we represent the mean values of the 3 segments. At 28 days after VRA we found an increased GFAP immunoreactivity in the ipsilateral side compared with the contralateral intact side, indicative of hypertrophy and reactivity of astrocytes. The animals treated with MSC showed significantly reduced GFAP immunoreactivity than non-treated rats (Fig. 2j–m), with levels similar to intact animals (200.8 $\% \pm 37.9$ and 96.4 $\% \pm 6.2$, for AV and AV-MSC groups respectively).

MN Survival After Root Re-implantation and MSC Transplant

We used fluorolabeling against Nissl bodies to identify the MN in both re-implantation plus vehicle and re-implantation plus MSC groups at 28 and 56 days after the injury (Fig. 3). Re-implantation of the avulsed roots increased MN survival (46.3 $\% \pm 4.2$ %) compared with animals with avulsion alone at 28 days. The number of MN at the end of the follow-up was reduced significantly compared with the counts at 4 weeks, suggesting a retardation in MN death (Fig. 3f). At this time point, the number of surviving MN with re-

Fig. 2 Mesenchymal stem cell (MSC) transplantation, motoneuron (MN) survival, and astrogliosis after ventral root avulsion. Diagram of the site of intraspinal injection after injury (**a**). Green fluorescence protein (GFP)⁺ MSC were found 1 week after treatment (**b**) in the injection site (box in **a**). A reduction of GFP labeling was observed at 2 weeks (**c**), and at 4 weeks only a few GFP⁺ cells were found (**d** and **e**, arrows). Representative micrographs showing MN labeled with fluorescence Nissl stain of intact (**f**), root avulsed (**g**, group AV), and avulsed plus MSC graft (**h**, group AV-MSC) rats. Number of surviving

MN (**i**), represented as percentage of MN in the avulsed side with respect to the non-injured side, show significant preservation by the MSC transplant. Immunohistochemistry against glial fibrillary acidic protein (GFAP) was used to assess the degree of astrogliosis. GFAP immunoreactivity, calculated as percentage of integrated density in the avulsed side (**k**: group AV; **l**: group AV-MSC) with respect to the non-injured side (**j**), show a significant increase after root avulsion that was prevented by the MSC transplant. * $p < 0.05$ group AV versus group AV-MSC. Scale bar = 200 μ m

implantation ($10.4 \% \pm 0.3 \%$) was similar to that of non-repaired rats ($9.8 \% \pm 1.9 \%$). Thus, early re-implantation of the avulsed roots enhances survival of MN during the first month after surgery, but is not effective to prevent the delayed MN death. The combination of root re-implantation with MSC injection provided a significant increase in MN survival at 4 weeks ($60.9 \% \pm 7.4 \%$) and also at 8 weeks ($26.2 \% \pm 7.1 \%$) in comparison with re-implantation repair alone and non-treated VRA (Fig. 3f).

Motor Axon Regeneration After Root Re-implantation and MSC Transplant

To assess axonal growth in the avulsed and re-implanted roots, immunohistochemistry for ChAT in samples of the

sciatic nerve was performed. We observed ChAT + fibers in the sciatic nerve segment from the lumbar plexus to the sciatic notch level, confirming regeneration of motor fibers. In this segment (length = 2 cm), the numbers of ChAT + fibers per section were counted at 3 points—in the proximal, medial, and distal parts. Contralateral intact sciatic nerves showed a homogeneous distribution and a large number of fibers (Fig. 4a). In the injured nerve, 4 weeks after root re-implantation, the number of ChAT+fibers was lower in both groups, with and without MSC transplant, (Fig. 4b, e) than in contralateral intact nerves. At the same time, in the sciatic nerve of the animals with VRA without re-implantation no ChAT+fibers were found, confirming the inability of axonal regeneration without repair of the avulsed roots. A decrease in the number of ChAT+fibers was observed from proximal

Fig. 3 Motoneuron (MN) survival and axonal projection after root avulsion and re-implantation. Spinal MN were labeled with fluorescence Nissl stain (in green), and MN that were able to project their axons to the sciatic nerve (8 cm distance) were also retro-labeled with FluoroRuby (in red). Co-labeling (in yellow) shows the surviving MN that regenerated their axons in the sciatic nerve. **a** Low magnification micrograph of an injured animal showing the non-injured side (right) and the injured and re-implanted side (left). **b** Magnification of the right-hand box in **(a)** showing the intact MN of the contralateral side. Magnification of the left-hand box in **(a)**, injured side, **c** of a rat of

group avulsion with direct re-implantation (AV-RE), **d** of a rat of group AV-RE-mesenchymal stem cells (MSC), and **e** of a rat of group avulsion (AV). Scale bar = 1 mm in **(a)** and 200 μ m in **(b–e)**. The percentage of MN survival was increased significantly by root re-implantation and further increased when combined with a MSC transplant **(f)**. * $p < 0.05$ AV versus other groups, # $p < 0.05$ AV-RE-MSC versus AV-RE. The number of MN labeled with FluoroRuby was increased in animals of group AV-RE-MSC compared with group AV-RE **(g)**. * $p < 0.05$ AV-RE-MSC versus AV-RE

to distal along the studied nerve segment in both re-implanted groups. Although more axons were found in group AV-RE-MSC than in group AV-RE at 4 weeks at mid and distal levels, the differences were not significant. At 8 weeks the number of ChAT + fibers in both re-implanted groups was similar than at 4 weeks in the 3 distances measured, and no differences between groups were found (Fig. 4f).

To quantify the number of MN that regenerated their axons in the re-implanted root to the sciatic nerve we applied FluoroRuby retrotracer at the sciatic nerve 8 cm from

the spinal cord. In the control re-implanted group, we observed back-labeled MN in the injury side only at 8 weeks, indicating that some motor axons were able to extend 8 cm distal to the spinal cord, whereas the animals of the re-implanted plus MSC group already had some labeled MN at 4 weeks (Fig. 3d), and slightly increased the number at 8 weeks (Fig. 3g). These data indicate that axonal growth was faster in the presence of MSC in the spinal cord, at least during the first 4 weeks. When comparing the number of regenerating MN with that of surviving MN measured by Nissl staining, 20 % of the MN were able to project their

Fig. 4 Choline acetyltransferase-positive (ChAT+) motor fibers regenerated in the segment from lumbar plexus to sciatic notch. In the contralateral non-injured nerve (a) there was a large number of ChAT + motor fibers with a longitudinal and parallel arrangement. After ventral root avulsion and re-implantation some motor fibers were found into the proximal (b) and the distal (c, d) part of the studied nerve segment at 28 days. Quantification of the number of motor axons per section at 28 days (e) and 56 days (f) showed that the number of ChAT + fibers in the injured side decreased with the distance from the spinal cord. In the ventral root avulsion without re-implantation group no motor fibers were observed at 28 days after injury. # $p < 0.05$ avulsion (AV) group versus avulsion with direct re-implantation (AV-RE) and AV-RE-mesenchymal stem cell (AV-RE-MSC) groups. * $p < 0.05$ contralateral (CT) versus avulsed groups. Scale bar=200 μ m

axons at 4 weeks and 43 % at 8 weeks in the MSC transplanted animals, whereas in the re-implantation alone group 60 % of surviving MN (about 2.5 times less in number) were labeled with FluoroRuby at 8 weeks. The higher proportion of regenerating MN in the re-implantation alone group with respect to the MSC-treated animals is a consequence of the

lower MN survival in the former group. These results suggest that the grafted MSC accelerated axonal growth, but at 8 weeks after treatment the effect of the cells was more important on MN survival than on axonal regeneration. Nevertheless, more MN regenerated their axons in the MSC-injected group than in the re-implantation alone group.

In the distal part of the sciatic nerve, taken at 8–9 cm from the spinal cord, a histological study of cross-sections was performed (Fig. 5). After VRA there were areas in the tibial (Fig. 5a, c, e) and peroneal (not shown in detail) branches with a high density of non-injured axons, with morphological appearance and myelin sheaths similar to axons of intact nerves (Fig. 5b, d). In other distinct areas, which corresponded well for the tibial nerve to the location of motor fascicles [38], there were a few axons preserved, many figures of axonal degeneration, and some phagocytizing macrophages (Fig. 5c, f). Similar evidences of denervation were observed in areas of the peroneal nerve, but not in the sural nerve (Fig. 5a, see 'S'), indicative of the preservation of sensory axons. At 4 and 8 weeks after root re-implantation and MSC injection, myelinated fibers of small and medium size, clustered in small fascicles, and frequent small endoneurial vessels were present in the degenerated area corresponding to regenerative units [39, 40] (Fig. 5g), while in the re-implantation alone rats such regenerative units were found only at 8 weeks.

Electrophysiological Outcome After Root Re-implantation and MSC Transplant

We stimulated at the sciatic nerve and at the brain levels to evoke CMAPs and MEPs, respectively, of GM, TA, and PL muscles. After L4–L6 root avulsion, we still recorded a CMAP component with normal latency and small amplitude in the TA and GM muscles, an artefact consequence of cross-linking with non-denervated proximal muscles (Fig. 6, white arrowhead). Four weeks after root avulsion, some animals of the AV-RE-MSC group presented a second CMAP that was polyphasic, with a long latency and very low amplitude in TA and GM muscle recordings (Fig. 6, black arrowhead), indicative of muscle re-innervation [11, 40, 41]. At 8 weeks all the transplanted animals showed responses of re-innervation in the TA and GM muscles, whereas the PL muscle remained completely denervated. In the group AV-RE with re-implantation alone, a few animals showed evidence of re-innervation only at 8 weeks after injury. The amplitudes of polyphasic CMAPs in MSC-treated rats averaged $260 \mu\text{V} \pm 96 \mu\text{V}$ in TA and $236 \mu\text{V} \pm 118 \mu\text{V}$ in GM muscle at 4 weeks, and $263 \mu\text{V} \pm 95 \mu\text{V}$ for TA, and $276 \mu\text{V} \pm 30 \mu\text{V}$ for GM muscle at 8 weeks (Fig. 6), being, at the two time points, higher in the MSC-treated group than in the control re-implanted group.

Fig. 5 Histology of the distal sciatic nerve. **a** Section of the distal sciatic nerve showing the division in tibial (T), peroneal (P), and sural (S) branches. The distribution of myelinated axons in intact tibial nerves was homogeneous (**b** and higher magnification in **d**), while in avulsed nerves (**a**, box magnified in **c**) there were distinct areas regarding the morphological appearance of the myelinated fibers. Some areas were occupied by non-injured axons (right-hand box in **c** and magnification in **e**), similar to an intact nerve (**b** and magnification in **d**). In other areas there were overt signs of axonal degeneration (left-hand box in **c** and magnification in **f**), showing empty endoneurial tubules (black arrowhead in **f**) and degenerating axons (black arrow in **f**). In these areas there were also clusters of small myelinated axons (white arrow in **g**, further magnified in the inset) with typical characteristics of regenerative units. Scale bar=100 μ m in (**a**) 50 μ m in (**b** and **c**), and 10 μ m in (**d**–**g**)

Regarding the MEPs we found a polyphasic response of delayed latency at 4 weeks after injury only in MSC transplanted animals (Fig. 6). At 8 weeks, only 1 animal

of the AV-RE group presented a polyphasic re-innervation potential in both TA and GM muscles. However, MEPs were recorded in most MSC-treated rats. The polyphasic MEPs increased in amplitude from 4 weeks to 8 weeks in the AV-RE-MSC group and were significantly higher than in control re-implanted rats. These results indicate that after root re-implantation and MSC transplantation some motor axons were able to reach their targets and re-innervate the muscles earlier, and in a considerably higher proportion of animals than in non-transplanted rats.

To assess functional recovery after treatments, a score of hindlimb movement was used (Fig. 7). After injury, the animals lost the ability to place the paw of the avulsed limb and toe-spreading responses. Moreover, the mobility of ankle and knee joints were lost and reduced at the hip joint. Despite the electrophysiological outcome found after cell transplant, no significant recovery of the hind paw motion was observed in any group during the follow-up, except for a mild recovery in the capacity of placing the paw on the ground.

Discussion

The avulsion of ventral roots from the spinal cord surface causes massive loss of MN, with 60–80 % death after 3–4 weeks [1, 3]. Strategies aimed to enhance survival of MN are mandatory as a first step in attempts to permit functional recovery after these injuries, which usually have devastating effects in the motor control of the affected territories. In this study we show that a focal transplantation of MSC, without other intervention, increases MN survival by 46 % at least 4 weeks after VRA. Furthermore, when the MSC transplant was combined with direct re-implantation of the avulsed roots, neuronal survival increased and was accompanied by enhancement of axonal regeneration through the re-implanted roots.

Fig. 6 Electrophysiological results after root avulsion and re-implantation. Results of compound muscle action potentials (CAMP) (**a**–**d**) and motor-evoked potentials (MEP) (**e**–**h**) recorded in tibialis anterior (TA) and gastronemius medialis (GM) muscles at 14, 28 and 56 days after injury and re-implantation. In all the recordings a remnant, cross-talk response from proximal muscles was observed (white arrowhead). A second polyphasic component characteristic of re-innervation (black arrowhead) was found in most animals treated with mesenchymal stem cells (MSC) at 28 days after injury (**c**, **g**). At the same time, re-innervation responses were not found in animals without MSC (**a**, **e**). For both CAMP and MEP, the percentage of animals with re-innervation responses (**b**, **f**) and the amplitudes of each response (**d**, **h**) were increased by a MSC transplant. * $p < 0.05$ avulsion with direct re-implantation (AV-RE) versus AV-RE-MSC. In (**a**) and (**c**), vertical bar = 500 μ V, horizontal bar = 20 ms. In (**e**) and (**g**), vertical bar = 100 μ V, horizontal bar = 50 ms

COMPOUND MUSCLE ACTION POTENTIALS

MOTOR EVOKED POTENTIALS

Fig. 7 Functional score of hindlimb movements. Intact animals had 6 points in the score scale (dotted line). After root avulsion and re-implantation there was complete paralysis of the hindlimb. Slight functional improvement was observed 10 days after injury; however, the animals performed around 1 point during the follow-up, without differences between avulsion with direct re-implantation (AV-RE) and AV-RE-mesenchymal stem cell (MSC) groups

MSC Rescue Motoneurons and Reduce Reactive Astrocytosis After VRA

Our results indicate that an MSC graft increases preservation of approximately half of the avulsed MN during the first month after injury. This finding is in concordance with a previous report about 67 % MN survival improvement by BDNF-producing MSC transplants after lumbar VRA [21]. Although the mechanisms through which MSC provide such beneficial effect are still not known, some hypotheses are under investigation. The neuroprotective effect induced by these cells might be mediated by the direct secretion or the induction in the transplanted tissue of some neurotrophic factors, such as BDNF and GDNF [21]. An exogenous application of BDNF protects MN from avulsion injury and sustains regeneration of motor axons, especially when BDNF is administered chronically [15, 18, 42–44]. However, the application of high doses of BDNF after sciatic nerve injury has detrimental effects on axonal regeneration [45]. The expression of the high affinity BDNF receptor, TrkB, is reduced in MN after VRA [46], while the expression of p75, the low affinity receptor for the neurotrophin family including BDNF, is increased [47]. Blockade of p75 with specific antibodies reverts the detrimental outcome induced by high doses of BDNF, and thus demonstrates a role of p75 in this effect [45]. Similarly to BDNF, GDNF can also support MN survival and regeneration [15, 20, 44, 48]. In this case, mRNA levels of both GDNF receptors—high affinity receptor GFRa1 and low affinity receptor c-ret—increase in MN during some weeks after axotomy and avulsion lesions [44].

Besides the neuroprotective role of the MSC transplant, we also observed a reduction in astrocyte reactivity

secondary to the injury. This is in agreement with previous works, that reported reduced astrocyte activation, characterized by GFAP immunoreactivity, 2 weeks after VRA and MSC transplantation [21]. This effect has been described also after spinal cord injury [49] and brain ischemia [27, 50]. MSC are able to modulate the inflammation process [51], which, in turn, can contribute to prevent the astrocyte reaction after injury. Moreover, this effect could also be consequence of the increased neuronal survival and tissue protection induced by the transplanted MSC.

MSC Accelerate Axonal Regeneration Through the Re-implanted Root

MN survival is an important goal after avulsion of ventral roots, but surgical reconnection of the nerve stump with the spinal cord surface is essential to allow axonal regeneration of injured motor axons to target muscles. We observed that acute re-implantation of avulsed roots near the root exit zone increases MN survival and also allows motor axon re-growth. After 4 weeks of re-implantation some motor axons were observed in the lumbar plexus, 4 cm distal to the spinal cord. However, the amount of motor fibers at 8 weeks at the same distance was similar to that at 4 weeks, as described previously [16]. The combination of re-implantation with MSC transplantation further increased MN survival and axonal regeneration compared to the group with re-implantation alone. Although the numbers of motor fibers in the lumbar plexus were similar in both re-implanted group, more axons reached long-distance growth in the MSC-transplanted animals, as observed in the retrotracing study. Thus, we found that some motor axons were able to reach the distal sciatic nerve, approximately 8 cm from the spinal cord at 4 weeks only in MSC transplanted animals. With these results, we can estimate an axonal growth rate of approximately 1.4 mm/day for control re-implanted rats and 2.8 mm/day for the combined therapy group. Therefore, a focal transplant of MSC into the spinal cord enhances the growth rate of motor axons during the first weeks after re-implantation. Moreover, the presence of regenerative axons at the distal sciatic nerve only in re-implanted animals with MSC injection corroborate the longer axonal regeneration induced by these cells. This ability of MSC to potentiate axonal growth has been also described when the cells are transplanted distal to the MN soma. Thus, an increased rate of regeneration by MSC grafts was reported after sciatic nerve transection and repair with synthetic tubes [30] or after facial nerve transection [52]. In our *in vitro* model, we also observed that MSC enhance axonal regeneration, thus confirming the *in vivo* findings.

The electrophysiological results also evidenced faster re-innervation of target muscles in the denervated hindlimb in the group with a MSC transplant after root re-implantation.

Muscle re-innervation by regenerating axons is characterized at the initial stages by polyphasic electromyographic responses, with a long latency and low amplitude, that tend to progress to a monophasic CMAP of increasing amplitude and near normal latency with advancing re-innervation [41]. In the present study, after re-implantation of L4, L5, and L6 roots, some polyphasic responses were recorded in the TA and GM muscles, but not in the more distal PL muscles, in a few animals 8 weeks after injury, indicating functional re-innervation by the regenerated motor axons. Interestingly, the combination of MSC transplantation with re-implantation accelerated the electromyographic responses from 8 to 4 weeks, and increased the amplitude of responses in TA and GM muscles. Nevertheless, the CMAPs did not show much progression during this time, indicating that only a limited number of motor axons were able to re-grow and reach the muscles in the hindlimb.

The capacity of MSC to enhance axonal regeneration may be owing to their ability to increase the expression of neurotrophic factors, such as GDNF and BDNF [21, 53], which are known to promote axonal growth in both *in vitro* [31] and *in vivo* [44] regeneration models. Similarly, application of exogenous GDNF [15, 19, 20] and BDNF [15, 18] in the spinal cord after axotomy improves axonal regeneration. However, excessive levels of BDNF or GDNF, induced by gene vectors, may result in abnormal growth of axons, forming a neuroma at the site of the factor secretion [20]. It is plausible that stem cells provide a better regulation of neurotrophin levels by paracrine and autocrine mechanisms. Nevertheless, our results show that the positive effect was of limited scope, as the number of motor fibers reaching the distal sciatic nerve and the muscles did not increase significantly from 4 to 8 weeks after repair and transplant. This suggests that the early potentiation of axonal regeneration by the MSC decreased from 4 to 8 weeks, likely owing to the low number of transplanted cells found at 4 weeks in the avulsed segments grafted. In fact, the reduction of MN survival from 4 to 8 weeks may be another consequence of the loss of MSC and their trophic action with time.

The inability of transplanted MSC to sustain neuronal survival and regeneration for longer periods of time may be a consequence of their poor survival within the spinal cord. As described previously, the survival of cells transplanted in the spinal cord after injuries is limited independently of cell type, which indicates that the spinal cord environment after damage is hostile to cell transplants in general [54–56]. Despite the immunomodulatory properties of MSC [51], survival of MSC grafted in the injured spinal cord has been reported to decrease with time [49, 54, 57]. Our observations after spinal root avulsion are in agreement with those previous reports. The reduction of the transplanted cells in time, together with the limited proportion of MN that were able to regenerate the axons distally, explain the lack of

functional recovery of hindlimb movements during the 8 weeks of follow-up. In fact, the achieved re-innervation of the hindlimb muscles was still very low in comparison with an intact limb (less than 1 % considering the CMAPs amplitude), and likely insufficient to allow enough muscle contraction for limb movements. In addition, the lack of selective re-innervation of appropriate muscles by regenerating motor axons after severe nerve lesions, such as VRA, often impedes the coordinated muscle contractions needed for an adequate joint movement, despite good muscle re-innervation [58]. Slight functional recovery has been described with re-implantation after cervical spinal roots avulsion [11–13], but is very limited after lumbar root avulsion [15, 48]. Two main factors are determinant of successful muscle re-innervation and functional recovery: the distance from the spinal cord to target muscles and the time-depending capacity of nerves to support axonal regeneration [17]. Following axotomy, acute denervated Schwann cells of the distal nerve stump switch to an immature proliferative state, secrete pro-regenerative neurotrophic factors and sustain axonal growth. In fact, the beneficial effects of root re-implantation would be to provide neurotrophic action from the Schwann cells [17]. However, after prolonged periods of denervation, Schwann cells decrease their pro-regenerative capability, and there is a temporal decrease of neurotrophic factors, especially GDNF and BDNF [16], and an increase of inhibitory molecules in the denervated distal nerve stump [59]. Therefore, the rate of axonal growth declines markedly [16]. Thus, although an MSC transplant into the spinal cord enhances the regeneration of MN close to the transplant site, it can not compensate the reduced potential of long-time denervated nerves to sustain regeneration over longer time and distance. Therefore, a further approach may be needed by manipulating the trophic support in the distal peripheral nerve in addition to extending the time of the focal action provided by the intraspinal cell graft.

Therapeutic Perspectives

Nowadays the repair of spinal root injuries to allow for some degree of functional recovery is challenging. Surgical repair for the reconnection of the detached roots into the correct point of the spinal cord, by direct re-implantation of the root stumps or by nerve grafting [11], seems mandatory. However, owing to the long distance that avulsed axons need to re-grow and the chronic atrophy of the limb muscles when regenerating axons may re-innervate the targets, surgical repair is insufficient. The use of cell therapy combined with surgical repair could be a good approach for these injuries. In this work we demonstrate that focal grafting of MSC extends motoneuron survival and enhances axonal regeneration compared with root re-implantation alone. Although other cell types, such as Schwann cells [60] and olfactory

ensheathing cells [61], have been shown to support axonal regeneration and could also be used, the easier procurement of MSC make them more clinically feasible [62]. Nevertheless, the translation of this cell therapy needs more investigation to define the optimal conditions. More information about the time-window for grafting, the number of injections, the quantity of cells, and the phenotype of the transplanted cells needs to be gathered from pre-clinical studies. The evidences for increased neuronal survival and faster axonal regeneration and muscle re-innervation constitute a proof-of-concept that a MSC transplantation combined with surgical root re-implantation, and later rehabilitation interventions, provides beneficial effects for the treatment of spinal root lesions.

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