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Olfactory Ensheathing Cells Transplanted in Lesioned Spinal Cord Prevent Loss of Spinal Cord Parenchyma and Promote Functional Recovery

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ABSTRACT We studied the effects of olfactory ensheathing cells (OECs) transplanted in a photochemical spinal cord injury in adult rats. After dorsal laminectomy at T8 vertebra, subjacent spinal cord was bathed with rose Bengal for 10 min and illuminated with visible light by means of an optic fiber connected to a halogen lamp for 2.5 min at maximal intensity of 8 kLux. Eight injured rats received a suspension of OECs in DMEM, and another eight rats received DMEM alone. Locomotor ability scored by the BBB scale, pain sensibility by the plantar algesimetry test, and motor- and somatosensory-evoked potentials by electrophysiological techniques were evaluated for 3 months postsurgery. Finally, all rats were perfused with paraformaldehyde and transverse sections from the spinal cord segment at the lesion site were immunostained against GFAP. Area of the preserved spinal cord parenchyma was measured from the GFAP-immunolabeled cord sections. The BBB score and the amplitude of motor- and somatosensory-evoked potentials were higher in OECs-transplanted rats than in DMEM-injected animals throughout follow-up, whereas the withdrawal response to heat noxious stimulus was lower in OEC- than in DMEM-injected rats. The area of preserved spinal cord was significantly larger in OECs-transplanted rats than in DMEM-injected animals. These results indicate that OECs promote functional and morphological preservation of the spinal cord after photochemical injury. *GLIA 42:275–286, 2003.*

INTRODUCTION

After lesion to the mammalian spinal cord, injured axons fail to regenerate across the lesion site and do not reestablish synaptic connections with their targets. As a consequence, damage to the spinal cord results in permanent deficits, involving partial or complete paralysis and sensory loss below the level of lesion. In the last years, numerous studies have sought to understand the factors that limit spinal cord regeneration and to assess different strategies for the repair of such injuries (Ramer et al., 2000). Most experimental studies have used techniques that allow us to induce graded spinal cord injuries, such as weight drop (Allen, 1911;

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Basso et al., 1995), aneurysm clip (Fehlings and Tator, 1995; Joshi and Fehlings, 2002a, b), and photochemical (Prado et al., 1987) methods. These experimental paradigms have been used for studying the effects of graded cord injuries on functional recovery and their relationship with histopathological results, and for testing therapies that promote neural protection and axonal regeneration. Pharmacological treatments (Lankhorst et al., 1999; Mu et al., 2000; Yu et al., 2000), infusion of diffusible growth factors (McTigue et al., 1998; Ankeny et al., 2001; Kojima and Tator, 2002), and cellular transplants (Paino and Bunge, 1991; McDonald et al., 1999) have been used for repairing spinal cord lesions.

Olfactory ensheathing cells (OECs) elicit remyelination of demyelinated spinal axons (Barnett et al., 2000; Kato et al., 2000), restore central nerve conduction (Imaizumi et al., 1998, 2000), and promote axonal regeneration (Li et al., 1998; Imaizumi et al., 2000; Ramón-Cueto et al., 2000; Lu et al., 2001, 2002) after transplantation in lesioned spinal cords. Significant recovery of motor and sensory functions was reported in OECs-transplanted rats compared with DMEM-injected animals after complete spinal cord transection (Ramón-Cueto et al., 2000; Lu et al., 2001, 2002). These findings clearly demonstrate that OECs might be used for promoting axonal regeneration and functional recovery after a severe spinal cord injury. In a previous study, we reported that, after severe photochemical cord injury, OECs-transplanted rats showed significantly lower area of cystic cavity and lower expression of proteoglycan and glial fibrillary acidic protein (GFAP) by reactive astrocytes in comparison with non-transplanted animals (Verdú et al., 2001). In vitro studies also demonstrated that OECs modulate GFAP and proteoglycan expression in reactive astrocytes (Lakatos et al., 2000, 2002). These results suggest that OECs create a favorable microenvironment at the lesion site that permits the regeneration of central axons. In the present work, we describe the effects of OECs transplants on functional and electrophysiological recovery after moderate photochemical spinal cord injury during the first 3 months postoperation. Locomotor activity, evaluated using open-field walking, and nociceptive sensitivity of the hindpaw were used as functional tests. Motor- and somatosensory-evoked potentials were used for evaluating the integrity of spinal cord pathways. The area of preserved cord parenchyma was measured from spinal cord sections immunostained against GFAP.

MATERIALS AND METHODS

Culture of OECs, Immunopurification, and Cryopreservation

Primary cultures of OECs from adult (2.5- to 3.0-month-old) female Sprague-Dawley rats were prepared and immunopurified using the method described by Gudiño-Cabrera and Nieto-Sampedro (1996, 2000) with minor modifications. Olfactory bulbs were aseptically removed and stored in Hank's balanced salt solution (HBSS; Sigma) with calcium and magnesium at 0°C. The meningeal layer was stripped off with fine forceps, and the bulbs were treated with 0.25% trypsin, 0.1% collagenase A, and 0.1% Dnase-I (Boehringer Mannheim, Germany) in 1 ml HBSS at 37°C for 45–60 min. After incubation, tissue dissociation was done by gentle trituration using a flame-constricted Pasteur pipette. Then enzymes were inactivated by the addition of 10 ml of Dulbecco's minimum essential medium nutrient mixture F-12 Ham (DMEM; Sigma) with 10% fetal calf serum (FCS, Biological Industries, Israel). The cell mixture was recovered by centrifugation at 900 rpm for 7 min and resuspended in culture medium. Cells were then seeded onto 25 cm^2 flasks coated with poly-L-lysine (10 μg/ml; Sigma), and incubated in 5% CO_2 at 37°C. Culture medium was DMEM with 10% FCS. At 9 days in vitro, this medium was replaced by a defined medium for expanding OECs (Gudiño-Cabrera and Nieto-Sampedro, 2000).

After reaching confluency (about 15 days in culture), the cell monolayer was rinsed twice with HBSS without calcium and magnesium, at 4°C, and detached by incubation with trypsin (0.05% w/v)-EDTA (0.02%; Sigma). Trypsin action was arrested by the addition of medium and the cell mixture was recovered by centrifugation at 900 rpm for 7 min. OECs were immunopurified by mean of magnetic Dynabeads M-450 (DBM-450, Dynal, Oslo) precoated with goat antimouse IgG and attached with mouse monoclonal 192 IgG antibodies that recognize the p75-NGF receptor (Chemicon). The cell mixture was resuspended in DMEM medium and incubated with DBM-coated magnetic beads, for 40 min, with mixing. The cells selectively attached to the magnetic beads were separated from the cells that did not express p75-NGF receptor with the help of a potent magnet. The magnet retained the complexes of magnetic beads-OECs on the side of the polypropylene tube. These complexes were washed with DMEM for removing the unattached p75 negative cells. The p75-positive cells were resuspended in freezing medium (Sigma) and cooled at 4°C, transferred to a −20°C freezer for 10 min, then to a deep freezer at −80°C. For transplantation, frozen cell vials were thawed at 37°C, washed two times by centrifugation (900 rpm, 7 min) in DMEM, and the pellet was resuspended in the same medium. The cells were transplanted within 2 h of thawing.

Under our conditions, more than 90% of cells in primary cultures show spindle-like morphology with two or three processes, while less than 10% of cells had flatten appearance. A few macrophages/microglial cells were also identified in our cultures. In accordance with previous studies (Gudiño-Cabrera and Nieto-Sampedro, 1996), more than 97% of the immunopurified cells were p75-positive. Consequently, the majority of transplanted OECs are spindle-shaped p75-positive cells, described as Schwann-cell-like phenotype (Franceschini and Barnett, 1996; Li et al., 1998;

Gudiño-Cabrera and Nieto-Sampedro, 2000; Wewetzer et al., 2002).

Animal Surgery

Sixteen adult female Sprague-Dawley rats (250–300 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed prone on a heating pad to maintain body temperature constant. The back of the animals was shaved and disinfected with povidone iodine, and a longitudinal incision of the skin and muscles extending from mid to low thoracic vertebrae was done. The spinal cord was exposed by a dorsal laminectomy at T8 vertebra. The dura was carefully cut to facilitate penetration of solutions. Rose Bengal (RB; 1.5% in saline; Sigma) was applied directly on the exposed spinal cord for 10 min. The excess dye was removed by a double saline rinse. The lesion was induced by illuminating the exposed dorsal surface of the spinal cord by means of one optic fiber positioned 10 mm on top of the cord. The optic fiber was connected to a halogen lamp (Raypa, Barcelona, Spain). The spinal cord was illuminated at maximal intensity of 8 kLux for 2.5 min. To prevent damage by the calorific source of the halogen lamp, during the illumination time the spinal cord was bathed with saline solution (García-Alías et al., 2002; Verdú et al., 2002).

Thirty minutes after injury, eight animals received four injections of a suspension of OECs (45,000 in 3 μl DMEM) into the exposed spinal cord (group OEC). Each animal received a total of 180,000 OECs in DMEM. Another eight rats received four injections of an equal volume of vehicle alone (group DMEM). The suspension was applied through a glass micropipette by means of repeated 20-ms pulses of 10 psi (Picospitzer II, General Valve, Fairfield, NJ) (Verdú et al., 2001). Finally, the wound was closed and the animals were kept in a warm environment until full recovery. Amitriptyline (150 μg/ml) was administered in the drinking water to prevent neuropathic pain (Navarro et al., 1994). The experimental procedures adhered to the recommendations of the European Union and the U.S. Department of Health for the care and use of laboratory animals and were approved by the ethics committee of our institution.

Functional Methods

Functional tests were performed before operation and at 7, 14, 21, 30, 45, 60, and 90 days postoperation. Locomotor activity was evaluated using the open-field walking scoring system and measuring the locomotor ability for 5 min. One animal at a time was allowed to move freely inside a circular plastic tray (90 cm diameter × 24 cm wall height). Two independent examiners observed the hindlimb movements of the rat and scored the locomotor function according to the BBB scale (Basso et al., 1995). The final score of each animal was the mean value of both examiners. The BBB scale ranges from 0 (no hindlimb movement) to 21 (normal movement-coordinated gait).

Animals were also subjected to testing by the inclined-plane method (Rivlin and Tator, 1977). The maximum angle at which the animal could maintain a stable position for 5 s on the inclined plane was recorded.

Nociceptive sensitivity was evaluated by a heat radiation method using a plantar algesimeter (Hargreaves et al., 1988). Briefly, the rat was placed into a plastic box with an elevated glass floor. From the bottom of the box, the light of a projection lamp (150 W) was focused directly onto the plantar surface of the hindpaw. The time to withdrawal of the heated paw (withdrawal latency) was measured through a time meter coupled with infrared detectors directed to the plantar surface. The maximal time of stimulation was limited to 40 s to avoid skin damage. The value for a test was the mean of three trials separated by 5-min resting periods.

Electrophysiological Methods

Motor- and somatosensory-evoked potentials were tested to evaluate the integrity of spinal cord descending and ascending tracts. Animals were anesthetized with sodium pentobarbital (25 mg/kg, i.p.) and placed in a prone position over a warmed flat coil controlled by a hot-water circulating pump to maintain skin temperature above 32°C. Motor-evoked potentials (MEPs) were recorded using monopolar needle electrodes placed at the tibialis anterior muscle, while the contralateral sensorimotor cortex was stimulated by single rectangular pulses of 0.1-ms duration, delivered through needle electrodes inserted subcutaneously, the cathode over the skull overlying the sensorimotor cortex and the anode at the nose. This configuration produces transcranial stimulation that activates subcortical efferent pathways (Zappulla et al 1988; Adamson et al 1989). For somatosensory-evoked potentials (SSEPs), electrical stimuli 0.1 ms in duration and 4 mA in intensity were applied at 5 Hz to the tibial nerve by means of two needle electrodes inserted at the ankle. Evoked responses were recorded using two needle electrodes placed subcutaneously on the skull (same sites as stimulation needles for MEPs). A total of 256 responses were recorded during 100 ms following each stimulus and averaged. The signals were amplified, filtered (bandpass 1 Hz/5 kHz for MEPs and 10 Hz/1 kHz for SSEPs), and displayed on the oscilloscope (Sapphire 4ME, Vickers) to measure the amplitude and the latency of the evoked potentials (Nuwer, 1998). To ensure reproducibility, the recording and stimulating needles were placed under a microscope to secure the same placement on all animals guided by anatomical landmarks. All electrophysiological tests were performed before operation and at 14, 30, 60, and 90 days postoperation.

Immunohistochemical Methods

At the end of functional and electrophysiological tests, the animals were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate-buffer saline solution (PBS, 0.1 M, pH 7.4). The spinal cords were removed, fixed in the same solution for 24 h, and divided in several segments. All spinal cord segments were cryoprotected in PBS containing 30% sucrose and stored at 4°C. Serial cryotome transverse sections 40 μm thick from the spinal cord segment subjacent to T8 vertebra were washed free floating in PBS with 0.3% Triton-X-100 (Fluka) and 1% fetal calf serum (Biological Industries, Israel) for 1 h and incubated for 24 h at 4°C with rabbit antisera to GFAP (1:1,000, Chemicon). After washes, sections were incubated with secondary antiserum, donkey antirabbit Cy3-labeled immunoglobulin G (1:200, Jackson Immunoresearch) overnight at 4°C. Following additional washes, sections were mounted on gelatin-coated slides, dehydrated in ethanol, and mounted with DPX. As a specificity control, some spinal cord sections were incubated without primary antibody and processed as described above. Samples were viewed under an Olympus BX-51 microscope equipped with epifluorescence using appropriate filters.

Low-power magnification (×4) images of spinal cord sections were taken with the aid of a digital camera (Olympus DP50) attached to the microscope and analyzed using NIH Image software. Spinal cord sections stained against GFAP were used for measuring the area of preserved cord parenchyma, because high-intensity staining was observed in reactive astrocytes surrounding and delimiting the destroyed parenchyma. Two independent subjects made these measurements over the same images, and the final area of preserved cord parenchyma of each transverse sections analyzed was the mean value of both subjects. Between 8 and 10 transverse spinal cord sections per animal were studied.

Statistical Analysis

All functional, electrophysiological, and histological measurements were performed in a blinded manner. A code of rats and histological slices was used. Data are shown as mean ± SEM. Statistical comparisons between groups were made by analysis of variance (ANOVA) with a posthoc Scheffé test for multiple comparisons. To examine specific differences between pre- and postoperative times, a paired *t*-test was used. Differences were considered significant if $P < 0.05$.

RESULTS

During the first week postoperation, rats transplanted with OECs maintained their body weight, while rats injected with DMEM alone showed a decrease of their body weight. From 14 to 90 days postoperation, all animals of both experimental groups began to gain weight.

Functional Results

Before the spinal cord lesions, all animals showed normal locomotor behavior (scored 21). At 7 days postoperation, in both experimental groups the BBB score decreased with respect to preoperative values; the mean score value was about 11 and 16 in the DMEM and the OEC group ($P < 0.05$), respectively. During the following weeks, the BBB score increased progressively, up to values about 18 and 19 in DMEM and OEC rats, respectively, at 90 days postoperation (Fig. 1A). In spite of the fact that the BBB score values were slightly higher in OEC rats with respect to DMEM animals, no significant differences were seen between the two groups from 14 to 90 days postoperation.

The preoperative mean values of the maximum angle in the inclined plane were about 47°. At 7 days postoperation, the mean maximum angle decreased to 36° and 39° in DMEM and OECs rats, then increased progressively to reach mean values of 43° and 45° in DMEM and OEC groups at 90 days postoperation. During all postoperative days, the mean maximum angle was slightly but not significantly higher in OEC than in DMEM animals (Fig. 1B).

Nociceptive withdrawal responses evaluated by plantar algesimetry had a latency of about 15 s in both experimental groups before lesion. At 7 days postoperation, the time of withdrawal of the heated paw increased in group DMEM with respect to preoperative values, whereas it was similar to preoperative values in OEC rats. During the next 3 weeks, the withdrawal latency increased to values about 24 and 19 s in groups DMEM and OEC. Significant differences were seen between groups OEC and DMEM for all postoperative days tested (Fig. 1C).

Electrophysiological Results

The MEPs recorded prior to operation showed a single wave with a mean latency of about 6 ms and mean amplitude of about 15 mV in both groups (Fig. 2A). After photochemical injury, no changes in the latency were observed during the 90 days postoperation, while the amplitude decreased markedly in group DMEM. At 14 days postoperation, MEPs were recorded in five of eight DMEM rats, showing a mean amplitude of about 1 mV, and in all OEC rats with a mean value amplitude of about 12 mV. At 90 days postoperation, MEPs were recorded only in three of eight DMEM rats with a mean amplitude value of about 0.03 mV, while all rats from the OEC experimental group showed MEP responses with a mean amplitude of about 15 mV. Significant differences were found between DMEM and OEC groups at all the postoperative days studied (Fig. 3).

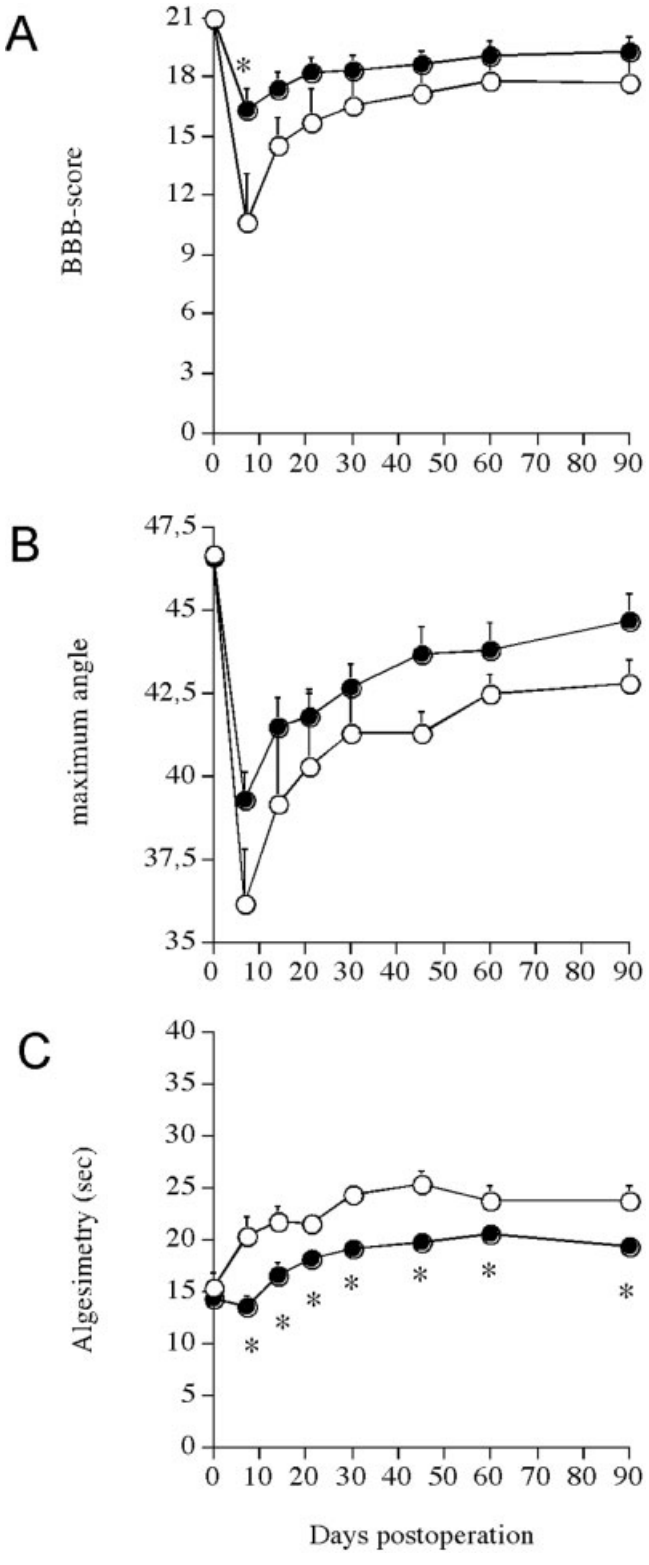


Fig. 1. Evolution of (**A**) the locomotor activity evaluated by the BBB score, (**B**) the maximum angle in the inclined plane test, and (**C**) the time to withdrawal of the heated paw in the plantar algesimetry test over time in the DMEM (opened circle) and OECs (filled circle) experimental groups. $P < 0.05$ (asterisk) vs. DMEM group.

Control SSEPs showed a series of well-defined negative peaks at latencies of 14, 21, and 30 ms, named N1, N2, and N3 waves, with mean amplitudes of about 13, 16, and 12 μV, respectively (Fig. 2B). At 14 days postoperation, the amplitude of all three waves of the SSEPs decreased significantly with respect to preoperative values in both experimental groups. However, the amplitude of SSEPs was significantly higher in group OEC than in group DMEM from 14 to 90 days postoperation (Fig. 4). After injury, no changes in the latency of the SSEPs were observed.

Immunohistochemical Results

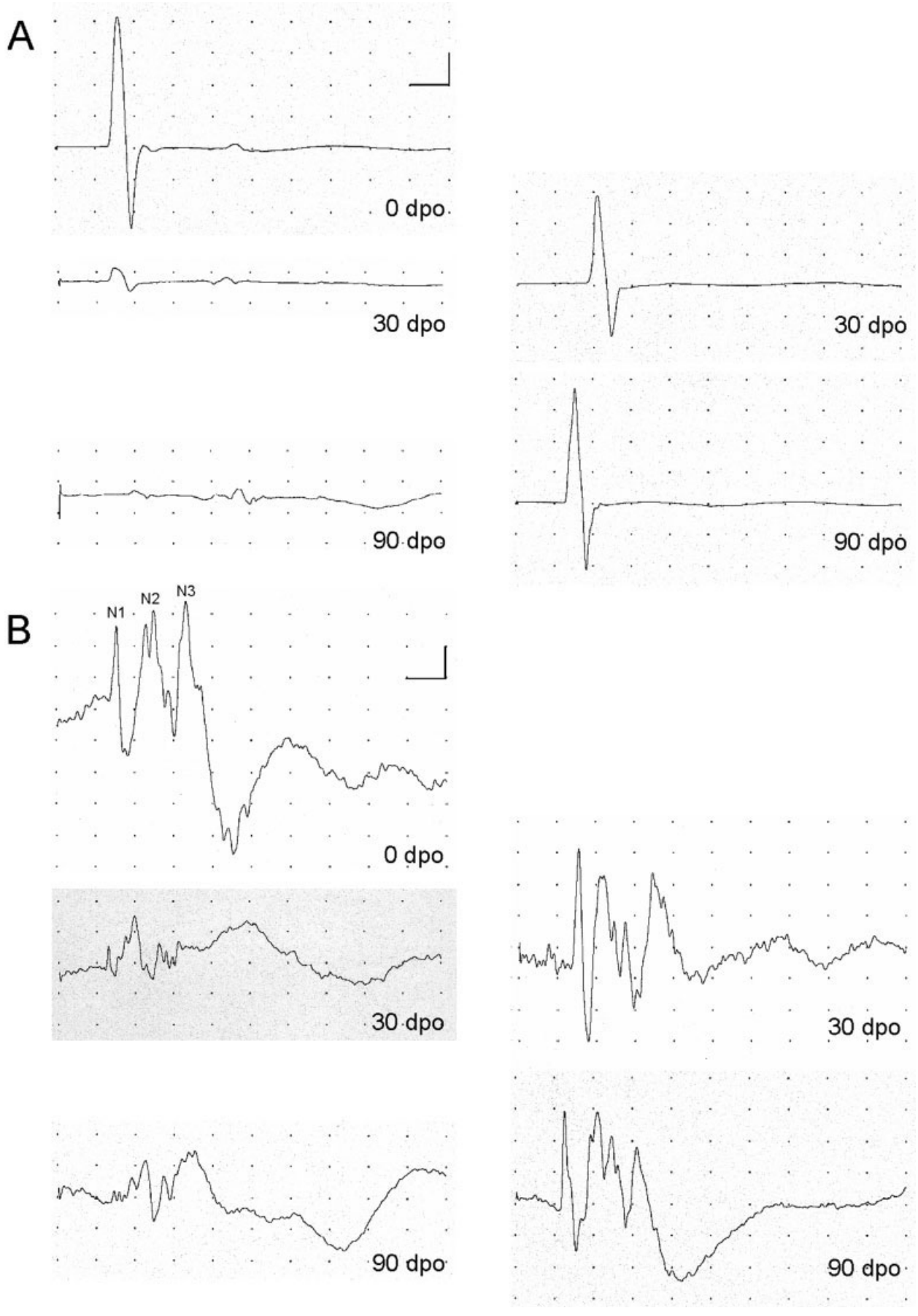
In group DMEM, transverse GFAP-immunostained spinal cord sections from T8 spinal cord segment showed an open cavity affecting the dorsal, dorsolateral, and ventrolateral funiculi up to canal central, while ventral funiculi, including gray matter of the ventral horns, remained intact. Destroyed spinal cord parenchyma remains were seen in dorsal areas (Fig. 5A). In animals of group OEC, desestructuration of the dorsal cord parenchyma was seen, with microcavities affecting the white and gray matter and enlargement of the central canal (Fig. 5B). The averaged area of preserved spinal cord was 4.15 ± 0.12 and 4.85 ± 0.12 mm^2 in DMEM and OEC groups, respectively ($P < 0.001$).

In all rats, hypertrophied astrocytes, heavily stained for GFAP, were seen around the cavity or the desestructured cord parenchyma. The spinal cord parenchyma from DMEM rats showed higher number of reactive hypertrophied astrocytes, with long and robust processes extending in all directions and heavily stained for GFAP (Fig. 5C). In OECs-transplanted rats, few astrocytes showed a hypertrophied appearance, and most showed slightly GFAP-stained cell bodies with thin processes (Fig. 5D).

DISCUSSION

The results of this study show that transplantation of OECs in the site of a moderate photochemical injury to the rat spinal cord significantly prevented the loss of cord parenchyma and improved the functional outcome during 3 months postinjury. In comparison with rats that received an injection of medium, OECs-transplanted rats showed better locomotor function and closer to normal nociceptive responses. Electrophysiological results revealed that the amplitude of the motor- and somatosensory-evoked potentials was significantly higher in OECs- than in DMEM-injected rats, providing evidence of functional preservation of descending and ascending spinal pathways.

In the majority of human spinal cord injuries, the mechanism of the primary injury is acute compression or laceration of the spinal cord due to displacement of bone or disk into the spinal cord during fracture-dislo-


A
0 dpo
30 dpo
90 dpo
30 dpo
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B
N1
N2
N3
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Figure 2.

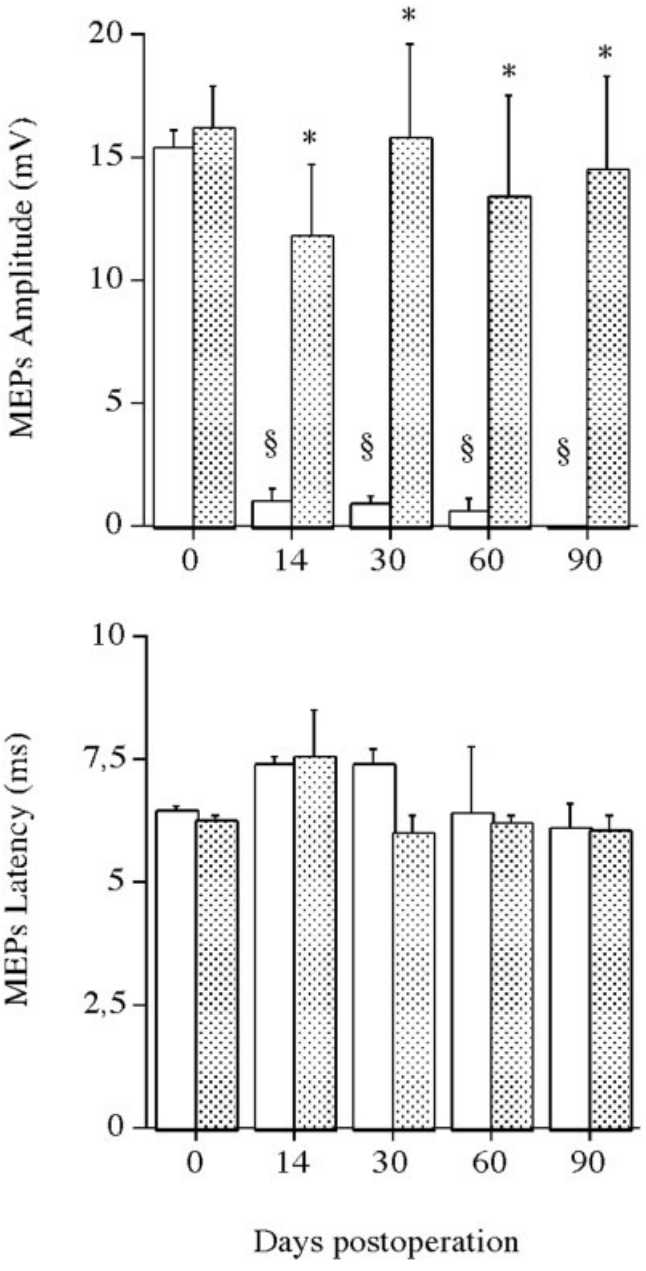
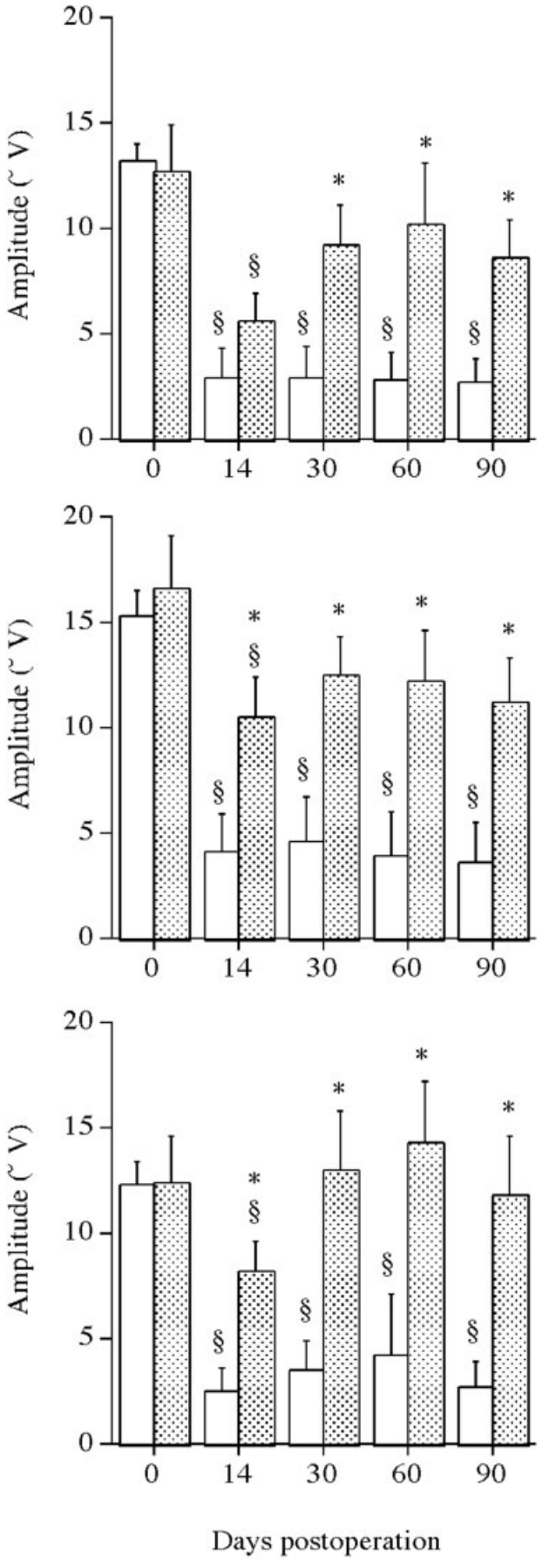


Fig. 3. Histogram representation of the amplitude (**top**) and latency (**bottom**) over time of the MEPs recorded at the tibialis anterior muscle in the DMEM (opened bar) and OECs (filled bar) groups. $P < 0.05$ (asterisk) vs. DMEM group; (section symbol) vs. preoperative values from the same experimental group.

cation or burst fracture of the spine (Tator, 1983). In patients with acute spinal cord injury, the mechanical injury rarely transects the cord completely, even when there is complete loss of neural functions caudal to the level of injury (Kakulas, 1984; Geisler et al., 1991). For this reason, several experimental models have been developed for producing graded spinal cord lesions and studying regeneration in partially lesioned spinal cords. Experimental models developed to simulate the compression type of acute cord trauma include the weight-dropping technique (Allen, 1911; Basso et al., 1995, 1996), extradural inflatable balloon compression (Tarlov et al., 1953), and aneurysm clip compression (Rivlin and Tator, 1978; Fehlings and Tator, 1995). After contusion or compression trauma to the spinal cord, sequential pathological changes including hemorrhage, edema, axonal and neuronal necrosis, and demyelination followed by cystic formation are described in the lesion site (Schwab and Bartholdi, 1996; Taoka and Okajima, 1998; McGraw et al., 2001). Between 15 and 30 min posttrauma, small hemorrhages with extravasation of erythrocytes and plasma into the perivascular spaces were seen. Between 2 to 7 days, massive number of macrophages and other polymorphonucleate cells invade the lesion site. Swollen axons with or without myelin are typically observed during the first few days after injury. Several days later, the hemorrhagic zone shows cavitation and the adjacent areas exhibit patchy necrosis, often with sharply defined margins. Within 3–5 days after injury, reactive astrocytes begin to wall off the lesioned region. Astrocytes accomplish this by increasing the expression of the structural protein GFAP, migrating from the adjacent undamaged parenchyma toward the injured site, and finally interdigitating their processes. Together with the reactive astrocytes around the lesioned area, leptomeningeal cells, which are normally found on the surface of the CNS, as well as fibroblasts, migrate into the wound cavity and aid in the reformation of the basal lamina. All these cells constitute the named gliotic scar that isolates the necrotic cord parenchyma from the rest of undamaged spinal cord.

The photochemical method of spinal cord damage has histopathological similarities with contusion and compression experimental lesions. In previous studies, we reported that during the first 60 min posttrauma, light and electron microscopy showed an important cytotoxic edema with astrocytic swelling, hemorrhagic extravasation, and myelin degradation in the lesion site. After 15 days, a cavity was observed in the necrotic area, and hypertrophied, heavily GFAP- and proteoglycan-stained astrocytes were seen delimiting this cavity (Verdú et al., 2001, 2002). A photochemical lesion in adult rats can induce graded severity of spinal cord injury. By increasing the illumination time, functional, electrophysiological, and morphological consequences are progressively more severe. Animals irradiated for 1 min showed small spinal cavities involving the dorsal funiculi. The cavity was progressively larger, involving dorsal horns in animals irradiated for 2.5 min, the dorsolateral funiculi also in animals irradiated 5 min and even the ventrolateral funiculi in those irradiated for 10 min (Verdú et al., 2002). A

Fig. 2. Sample electrophysiological recordings of (**A**) MEPs and (**B**) SSEPs in rats of groups DMEM (left) and OECs (right) at 0, 30, and 90 days postinjury. N1, first negative wave complex; N2, second negative wave complex; N3, third negative wave complex. Horizontal scale, 5 ms; vertical scale, 4 mV for all MEP recordings. Horizontal scale, 10 ms; vertical scale, 3 μV for all SSEP recordings.



significant negative relationship was found between the area of cavity of the spinal cord and the functional and electrophysiological impairment (García-Alías et al., 2002). This experimental model therefore appears to be adequate for investigating different therapeutic strategies to promote neuroprotection and central regeneration.

The ability of the OECs to promote axonal regeneration has been demonstrated in several regions of the central nervous system. Transplants of cultured OECs mediate the reentry of regenerating dorsal root axons into the dorsal gray matter (Ramón-Cueto and Nieto-Sampedro, 1994) and the synapses formed by these regenerating sensory fibers allowed functional restitution of spinal reflex arcs (Navarro et al., 1999; Taylor et al., 2001). When the corticospinal tract was focally lesioned, OECs transplanted into the lesion site enhanced the elongation of corticospinal axons inside of the transplanted area (Li et al., 1998).

Functional recovery in OECs-transplanted rats in comparison with vehicle-injected animals has also been reported after complete transection of the adult rat spinal cord. Ramón-Cueto et al. (2000) reported that 7 months after surgery, about 20% of OECs-transplanted rats climbed by a grid with a slope of 90°, whereas none of the DMEM-injected rats passed this test. In addition, OECs-transplanted rats showed positive responses to light skin contact and propioceptive stimuli 7 months postsurgery. Of interest is that the functional recovery observed in the OECs-transplanted group compared to that in the DMEM group was significant after 3 months postoperation (Ramón-Cueto et al., 2000). Lu et al. (2001) also reported that, after complete spinal cord transection at T10, OECs-transplanted rats improved hindlimb usage. Using the open-field walking test, they found that at 8–10 weeks postsurgery, OECs-transplanted rats reached a BBB score of about 6–8, whereas the BBB score of the nontransplanted rats averaged only 2. Recently, Shen et al. (2002) reported that at 10 weeks after spinal cord transection, 5 of 12 OECs-transplanted rats showed recordable motor-evoked potentials, whereas none of the rats that received DMEM alone had MEPs. All these findings demonstrate that OECs transplanted in severe spinal cord injuries promote functional recovery.

Several factors may explain the abilities of OECs to promote regeneration of central axons and functional recovery after spinal cord injuries. First, in vitro OECs express nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glia cell line-derived neurotrophic factor (GDNF), and their receptors (Woodhall

Fig. 4. Histogram representation of the amplitudes of the N1 (**top**), N2 (**middle**), and N3 (**bottom**) waves of SSEPs over time in DMEM (opened bar) and OECs (filled bar) groups. $P < 0.05$ (asterisk) vs. DMEM group; (section symbol) vs. preoperative values from the same experimental group.

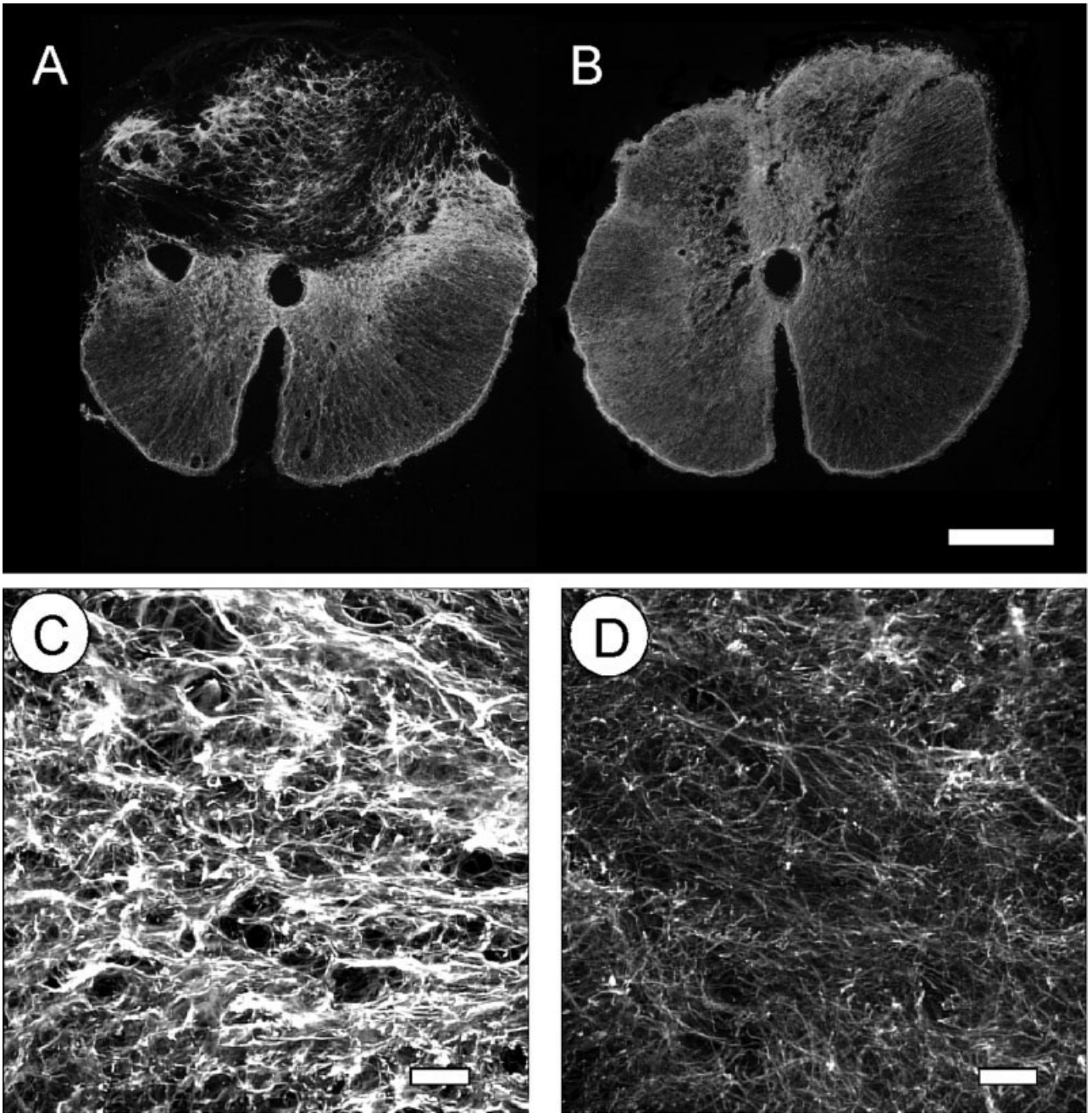


Fig. 5. Spinal cord sections immunostained against GFAP taken at the lesion site segment from the DMEM (**A**) and OECs (**B**) groups. Confocal images of astrocytes immunolabeled against GFAP in the gray matter of DMEM (**C**) and OECs (**D**) experimental groups. Bar: A and B, 1,000 μm; C and D, 25 μm.

et al., 2001). OECs also express neurotrophin 4/5 (Boruch et al., 2001) and p-75 low-affinity NGF receptor (Ramón-Cueto et al., 1993). It is well known that all these neurotrophic factors promote sprouting and regeneration of central axons. Injured ascending sensory axons are responsive to NGF, BDNF, and neurotrophin 3 (NT-3) (Bradbury et al., 1999; Oudega and Hagg, 1999). Lesioned rubrospinal and corticospinal axons are also responsive to BDNF, which promotes regeneration of rubrospinal axons (Liu et al., 1999; Plunet et al., 2002) and survival but not regrowth of lesioned corticospinal axons (Lu et al., 2001). NT-4/5 prevents atrophy of rat rubrospinal neurons after axotomy and promotes rubrospinal regeneration (Kobayashi et al., 1997). GDNF promotes survival and axonal regeneration of noradrenergic neurons of the locus coeruleus (Arenas et al., 1995; Holm et al 2002). In addition, OECs produce molecules known to be involved in neurite extension such as L1, laminin, N-CAM, PSA-N-CAM, and fibronectin (Ramón-Cueto and Avila, 1998).

These findings suggest that OECs are capable of promoting the survival and regeneration of the main ascending and descending pathways in the adult spinal cord. However, it is unknown if OECs transplanted into the lesioned cord also express and secrete all these neurotrophic factors.

Secondly, OECs transplanted in lesioned spinal cords induce a downregulation of GFAP and proteoglycan expression by the reactive astrocytes (Verdú et al., 2001). In the present study, we confirm these results at a longer time, 3 months postlesion, when the injury process has reached a steady state. Studies that have used cocultures with astrocytes showed that OECs did not induce overexpression of chondroitin sulfate proteoglycans (CSPGs) in astrocytes, whereas cocultured Schwann cells increased CSPG expression in astrocytes (Lakatos et al., 2000, 2002). In vitro studies demonstrated that proteoglycan inhibits neurite outgrowth, and that a monoclonal antibody against proteoglycan neutralized this inhibitory activity (Bovolenta et al., 1997). Recently, Plant et al. (2001) reported that CSPG immunoreactivity was increased at the graft-host cord interface, and it was associated with astrocytes and fibroblasts. These findings suggest that accumulation of proteoglycan compounds near the wound may impair the elongation of injured central axons. In fact, chondroitinase-ABC treatment, which degrades CSPGs, allows neurite elongation (Zuo et al., 1998) and promotes axonal regeneration after spinal cord injury (Yick et al., 2000; Bradbury et al., 2002). Our results (Verdú et al., 2001) suggest that OECs directly or indirectly modulate reactive astrocytes, reducing their reactivity and creating a protective environment. We cannot exclude that OECs might also influence other cells, such as microglia and leukocytes, which infiltrate the cord parenchyma during the first hours after injury, modulating their proinflammatory response. The preservation of more cord parenchyma by transplanted OECs after the photochemical spinal cord injury (Verdú et al., 2001), confirmed in the present work, may also be explained by the proregenerative and promodulating inflammatory properties of OECs.

Thirdly, the effects of OECs might be potentiated by their ability to migrate inside the spinal cord parenchyma. In a previous study, we found that when prelabeled OECs were injected at T12-L1 spinal cord levels, OECs could be observed at rostral (T9–T10) and caudal (L4–L6) spinal cord levels 15 days after spinal cord injury and transplantation (Verdú et al., 2001). Boruch et al. (2001) also reported that 1 month after dorsal hemisection, p75-positive OECs were able to migrate within the injured dorsal aspect but did not enter the uninjured ventral portion of the spinal cord. In addition, OECs migration was reported after transplantation in the hippocampus (Gudiño-Cabrera and Nieto-Sampedro, 1996) or within the transected medial longitudinal fascicle (Gudiño-Cabrera et al., 2000). On the other hand, previous studies have reported that transplanted OECs survived for 2 (Gudiño-Cabrera et al., 2000) or 7 months (Ramón-Cueto et al., 2000) after transplantation. All these findings strongly suggest that OECs are able to survive and migrate large distances when transplanted in the central nervous system.

In conclusion, OEC transplantation constitutes a promising therapeutic procedure for repairing injured spinal cords. The results reported in the present study provide the first evidence that transplants of OECs prevent loss of cord parenchyma and improve functional recovery after incomplete spinal cord injury, the main type of human spinal cord injury. However, the clinical use of this therapeutic procedure requires further studies in animal models.

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ACUTE TRANSPLANTATION OF OLFACTORY ENSHEATHING CELLS OR SCHWANN CELLS PROMOTES RECOVERY AFTER SPINAL CORD INJURY IN THE RAT

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We compared the neurological and electrophysiological outcome, glial reactivity and spared spinal cord connectivity promoted by acute transplantation of olfactory ensheathing cells (group OEC) or Schwann cells (group SC) after a mild injury to the rat spinal cord. Animals were subjected to a photochemical injury of 2.5 minutes irradiation at the T8 spinal cord segment. Thirty minutes after lesion, a suspension containing 180000 OECs or SCs was injected into the injury site. A control group (group DM) received injection of the vehicle medium alone. During 3 months postsurgery, behavioral skills were assessed with open field-BBB scale, inclined plane and thermal algesimetry tests. Motor (MEPs) and somatosensory evoked potentials (SSEPs) were performed to evaluate the integrity of spinal cord pathways, whereas lumbar spinal reflexes were evaluated by the H reflex responses. Glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycan (CSPG) expressions were quantified immunohistochemically at the injured spinal segments, and the preservation of corticospinal and raphespinal tracts caudal to the lesion was evaluated. Both OEC and SC transplanted groups showed significantly better results in all the behavioral tests than the DM group. Furthermore, the OEC group had higher MEP amplitudes and lower H responses than the other two groups. At the injury site, the area of spared parenchyma was larger in transplanted than in control injured rats. OEC-transplanted animals had reduced astrocytic reactivity and CSPG expression in comparison to SC-transplanted and DM rats. Taken together, these results indicate that transplantation of both OEC and SC has potential for restoration of injured spinal cords. OEC grafts showed superior capability to reduce glial reactivity and improve functional recovery.

INTRODUCTION

The inherent failure of the mammalian central nervous system (CNS) axons to regenerate after an injury and the chronic functional deficits caused to the patients have encouraged many laboratories to investigate possible therapies to achieve CNS repair. Traumatic spinal cord injuries result both in the death of grey matter neurons and in the disruption of ascending and descending white matter tracts at the site of the injury, leading to the loss of motor, sensory and autonomic control below the site of injury. From the different therapies assayed (Olson 1997, Gage and Horner 2000), cell grafting has emerged as a powerful and promising tool for enhancing restitution of the lost functions. In several experimental injury models, transplantation of different types of cells, including Schwann cells (SCs) (Paino and Bunge 1991; Xu et al., 1995), macrophages and microglia (Rapalino et al., 1998, Prewitt et al., 1997), oligodendrocyte precursors (Franklin et al., 1996), olfactory ensheathing cells (OECs) (Li et al., 1997; Ramón-Cueto et al., 2000, Verdú et al., 2003b), tanycytes (Prieto et al., 2000) and stem cells (McDonald et al., 1999; Tang et al. 2001) partially improved the functional capabilities of the animals, by promoting survival, regeneration and remyelination of spinal axons.

SCs and OECs have specially focused the attention for bridging the gap after spinal cord injuries. Several experiments demonstrated regeneration of central axons into peripheral nerve or SC grafts (Richardson et al., 1980; Paino and Bunge 1991; Xu et al.,

1995; Guest et al., 1997), suggesting that the rich trophic environment produced by the SCs promotes the elongation of propiospinal and supraspinal axons within the graft. However, it has been reported that central axons failed to re-enter into the host cord from the distal interface of the graft (Xu et al., 1997; Plant et al., 2001). On the other hand, the particular regenerative properties of the adult mammalian olfactory system, which allow continuous regeneration of peripheral olfactory neurons throughout the lifespan of the animal, is believed to be supported by the OECs (Doucette 1984). Grafts of OECs into complete or partial spinal cord lesions promote axonal regeneration (Li et al., 1997, Ramón-Cueto et al., 2000, Liu et al., 2002) and functional recovery (Ramón-Cueto et al., 2000, Liu et al., 2002, Verdú et al., 2003b).

On designing future therapeutical strategies spinal cord injury, the important question arises as to whether OECs and Schwann cells are comparable cell types or whether they, in fact, mediate specific effects making the one of them most suitable for special applications (Wewetzer et al., 2002). There are only a few studies in the literature comparing the effects of SC and OEC transplants in injured spinal cords. After spinal cord focal demyelination, Akiyama et al. (2002) reported that following intravenous injection of bone marrow cells, SCs or OECs, only the former were able to remyelinate the injured spinal cords. In a dorsal hemisection model of the spinal cord, Imaizumi et al. (2000) showed that both SC and OEC transplants restore spinal cord conduction and promote axonal regeneration across the transection site. Other recent study showed that delayed transplantation of SCs but not of OECs improves locomotor recovery after a moderate contusion to the spinal cord (Takami et al., 2002). The aim of the present work was to compare the functional outcome of animals with OEC or SC grafts acutely transplanted after a photochemical injury to the rat spinal cord, and to evaluate the neuroprotection properties of these grafts by studying glial reactivity and spinal cord connectivity.

MATERIALS AND METHODS

SC and OEC culture

Primary cultures of SCs and OECs from adult Sprague-Dawley rats were prepared, immunopurified and cryopreserved as described in detail elsewhere (Verdú et al 2000; 2003b). For transplantation, frozen cell vials were thawed at 37º C, washed two times by centrifugation (900 rpm, 7 min) in Dulbecco's Modified Eagle's medium nutrient mixture F-12 Ham (DMEM; D8900, Sigma, St Louis, MI, USA), and the pellet resuspended in the same medium. The cells were transplanted within 2 hours of thawing. Under our conditions, the majority of transplanted OECs are spindle-shaped p75-positive cells, described as Schwann-cell-like phenotype (Franceschini and Barnett, 1996; Gudiño-Cabrera and Nieto-Sampedro, 2000; Wewetzer et al., 2002). The purity of transplanted Schwann cells was above 80%.

Spinal cord injury and cell transplants

Twenty-four adult female Sprague-Dawley rats (200-250g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed prone on a heating pad to maintain the body temperature constant. The thoracic back area was shaved and disinfected with povidone iodine, and a longitudinal incision of the skin and muscles extending from mid to low thoracic vertebrae was done. The spinal cord was exposed by a dorsal laminectomy at T8 vertebra. Rose Bengal (RB; 1.5% in saline solution; Sigma) was applied topically over the spinal cord dorsal surface for 10 minutes, and the excess of dye removed with saline solution rinses. Then, the spinal cord was illuminated with an optic fiber, positioned 10 mm above the spinal cord, connected to a cold light source equipped with a halogen bulb. All the spinal cords were illuminated with an intensity of 80 kLux for 2.5 minutes. To prevent damage caused by the calorific source of the lamp, the spinal was bathed with saline solution during the illumination time (Verdú et al., 2003a, 2003b).

Thirty minutes after injury, the animals received four injections of a suspension of OECs (45000 in 3 µl DMEM each) (group OEC, n= 8) or a suspension of SCs (45000 in 3 µl DMEM each) (group SC,

n= 8) into the exposed spinal cord. Each animal received a total of 180000 OECs or SCs. The control group consisted of animals that received four injections of an equal volume of DMEM alone (group DM, n= 8). The injections were done at the rostral and caudal edges and laterally to the mid plane of the exposed spinal cord segment. The suspensions were applied through a glass micropipette (80 µm i.d.) by means of repeated pulses of 20 ms duration and 10 psi (Picospritzer II, General Valve, Fairfield, NJ). To prevent leakage, the micropipette was removed from the spinal parenchyma two minutes after the end of each injection. Finally, the wound was sutured with 5-0 silk thread in the muscular plane and small clips in the skin and disinfected. Rats were kept in a warm environment until full recovery. Animals were housed two per cage, exposed to a 12 h light/dark cycle, and had free access to food and water. The experimental procedures were approved by the Ethics Committee of our institution.

Neurological evaluation

All the neurological tests were performed before the operation and at 7, 14, 21, 30, 60 and 90 days postoperation (dpo). Locomotor behavior was evaluated with the open field test and scored by the BBB scale (Basso et al., 1995). The animals were placed individually in a circular enclosure and allowed to move freely for 5 minutes. Two independent examiners observed the hindlimb movements of the rat and scored the locomotor function according to the BBB-scale ranging from 0 (no movement) to 21 (normal movement). The animal ability to maintain postural stability was evaluated with the inclined plane test. The rats were placed on the inclined plane, and the maximum inclination at which the rat could maintain its position for 5 seconds was recorded and described as the "capacity angle" (Rivlin et al., 1977).

Nociceptive sensibility was evaluated with a heat-radiation method using a plantar algesimeter (Hargreaves et al., 1988). The rats were placed individually inside a plexiglass box on an elevated glass floor. A radiant heat stimulus was applied by focusing a light beam directly onto the plantar surface of the hindpaw. The time to withdrawal of the heated paw was measured through a timemeter coupled with infrared detectors directed to the plantar surface. The maximal time of stimulation was limited to 40 seconds to avoid skin damage. The value for a test was the mean of three trials separated by 5 minutes resting periods.

Electrophysiological evaluation

Electrophysiological tests were performed bilaterally on both hindlimbs before the injury and at 14, 30, 60 and 90 dpo. The animals were anaesthetized with pentobarbital and placed prone over a warmed flat coil controlled by a hot water circulating pump to maintain skin temperature above 32° C. To ensure reproducibility in all the electrophysiological tests, the stimulation and recording needles were placed under microscope magnification to secure the same placement in all animals guided by anatomical landmarks.

Central conduction studies were performed to evaluate the integrity of spinal cord ascending and descending pathways. Motor evoked potentials (MEPs) were elicited by transcranial stimulation with two needle electrodes placed subcutaneously over the skull, delivering single electrical pulses of 25 mA intensity and 100 µs duration, and recorded from the tibialis anterior muscle (García-Alías et al., 2003). The signals were amplified, filtered (bandpass 1-5000 Hz) and displayed on the oscilloscope (Sapphyre 4ME, Vickers) to measure the latency to the onset and the amplitude from the onset to the peak of the negative deflection. To ensure reproducibility, five consecutive responses were recorded, with a time interval of 30 seconds between stimuli, and the recording with the highest amplitude used for analysis. Somatosensory evoked potentials (SSEPs) were elicited by applying stimuli (3 mA, 100 µs at 6 Hz) to the tibial nerve at the ankle. The active recording needle electrode was placed subcutaneously over the contralateral sensorimotor area, with a reference electrode at the base of the nose and a ground electrode at the base of the tail. A total of 256 signals were amplified, filtered (bandpass 20-1000

Hz) and averaged. The latency and the peak-to-peak amplitude of the three main negative waves were measured (García-Alías et al., 2003).

Motor nerve conduction studies were performed to evaluate the lumbar H-reflex. The sciatic nerve was stimulated with single electrical pulses (100 μs duration and supramaximal intensity) delivered by monopolar needles inserted percutaneously at the sciatic notch. The compound muscle action potentials (CMAPs) of the tibialis anterior muscle were recorded by means of needle electrodes, and displayed in the oscilloscope. The latency from stimulus to the negative peak and the amplitude from the onset to the peak of the M and H waves were measured (Valero-Cabré and Navarro 2002). The ratio of H/M maximal amplitudes was calculated, as it provides an index of the proportion of motoneurons recruited via monosynaptic reflex relative to the total motoneuronal pool (Thompson et al., 1992).

Anterograde tracing of the costicospinal tract

After the last electrophysiological evaluation, the animals were anaesthetized (pentobarbital 40 mg/kg i.p.) and placed in a stereotaxic frame. A bilateral craniotomy centered at a point 2 mm lateral to the Bregma was made, the dura was cut and a stab, 1.5 mm deep, was made to the sensorimotor cortex. Using a fine forceps (Dumont # 5), two to four DiI crystals (D-3911, Molecular Probes Europe, Leiden, The Netherlands) were inserted into the depth of the cortical incision such that the cortical edges became approximated and covered the crystals (Tsai et al., 2001). Finally, the skin was sutured and disinfected. Fifteen days after DiI application, rats were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate-buffered solution (PBS; 0.1M, pH 7.4). The T1, T8 and T13 spinal cord segments were identified, removed, post-fixed overnight in the same solution and stored in a cryoprotection solution (30% sucrose in 0.1M PBS) at 4°C. Using a cryostat the spinal cord segments were cut serially into 40 μm thick transverse sections, and stored free-floating in De Olmos antifreeze buffer. Five intact control rats were anesthetized and perfused transcardially and the same spinal cord segments removed and processed as above (group CNT). Spinal cord sections were washed in PBS, mounted on gelatin coated slides and examined under a fluorescent microscope (Olympus BX-51) using appropriate filters (Tsai et al., 2001).

Immunohistochemical procedures

Cryostat sections from the T8 segment were washed free-floating in PBS with 0.3% Triton-X-100 (Fluka) and 1% fetal calf serum (Biological Industries, Israel) for 1 h. Then, they were incubated with rabbit antisera to glial fibrillary acidic protein (GFAP; 1:1000, Chemicon, Temecula, CA), mouse antisera to nerve growth factor low affinity receptor (p75, 1:200, MAB365, Chemicon), and chondroitin sulfate clone CS-56 (CSPG; 1:50, C-8035, Sigma) for 48 hours at 4°C. After several washes, the sections were incubated with secondary antisera, donkey anti-rabbit or goat anti-mouse Cy3 labeled immunoglobulin G (1:200; Jackson Immunoresearch, West Grove, PA, USA), overnight at 4°C. Following additional washes, sections were mounted on gelatin coated slides, dehydrated in ethanol and mounted with dextropropoxyphene (DPX, Fluka). For evaluating antibody specificity, some sections were processed as described, but primary antisera were not added. The samples were viewed under an Olympus BX-51 microscope equipped with epifluorescence using appropriate filters. In order to visualize the serotoninergic descending pathways from brainstem raphe nuclei, spinal cord sections from T13 segments were immunostained against serotonin (5-HT, 1:200, Diasorin, Stillwater, MN).

Low-power magnification (x4) images of spinal cord sections were taken with the aid of a digital camera (Olympus DP50) attached to the microscope and analyzed using NIH Image software. GFAP immunostained sections were used to quantify the preserved spinal cord parenchyma at the lesion site (T8), because strong immunostaining was seen in reactive astrocytes surrounding and delimiting the destroyed parenchyma. Two independent subjects made these measurements over the same images (8-10 sections for each animal),

and the final area of preserved cord of each section analyzed was the mean value of both subjects (Verdú et al., 2001, 2003b). Confocal images of GFAP-immunostained sections from the lesion level (T8) were used for measuring the GFAP staining. By means of a confocal imaging system (Leica TCS-4D) images representing an area of 0.07 mm^2 from both ventral spinal cord horns of at least three sections per animal were collected (a total of 6 images/rat) using x40 lens. The grade of GFAP immunostaining was quantified by the method described by Acarín et al. (1997). The measurement, referred as the "reactive grade", was defined as the ratio between immunolabeling density in spinal cord sections of injured animals versus that of unlesioned rats.

Spinal cord sections immunolabeled against CSPG were used for measuring the intensity of CSPG fluorescence using a similar method to that described by Plant et al (2001). Spinal cord images (x4) were analyzed using NIH Image software. Fluorescence intensity was ranged in a scale between 0 (maximal fluorescence) to 250 (no fluorescence). At least 8 sections per animal were collected and measured.

Statistical analysis

All neurological, electrophysiological and histological measurements were performed in a blinded manner. A code of rats and histological slices was used. Data are shown as the mean ± SEM. Electrophysiological results are expressed as the percentage with respect to preoperative values for each rat. Statistical comparisons between groups were made with Kruskal-Wallis and Mann-Whitney U tests. Differences were considered significant if $p< 0.05$.

RESULTS

After spinal cord injury, no signs of infection or bladder dysfunction were noted. The body weight of the animals increased over time, and at the end of the evaluation their weights increased 124± 4 %, 127 ± 3 % and 117 ± 9 % in the OEC, SC and DM groups respectively.

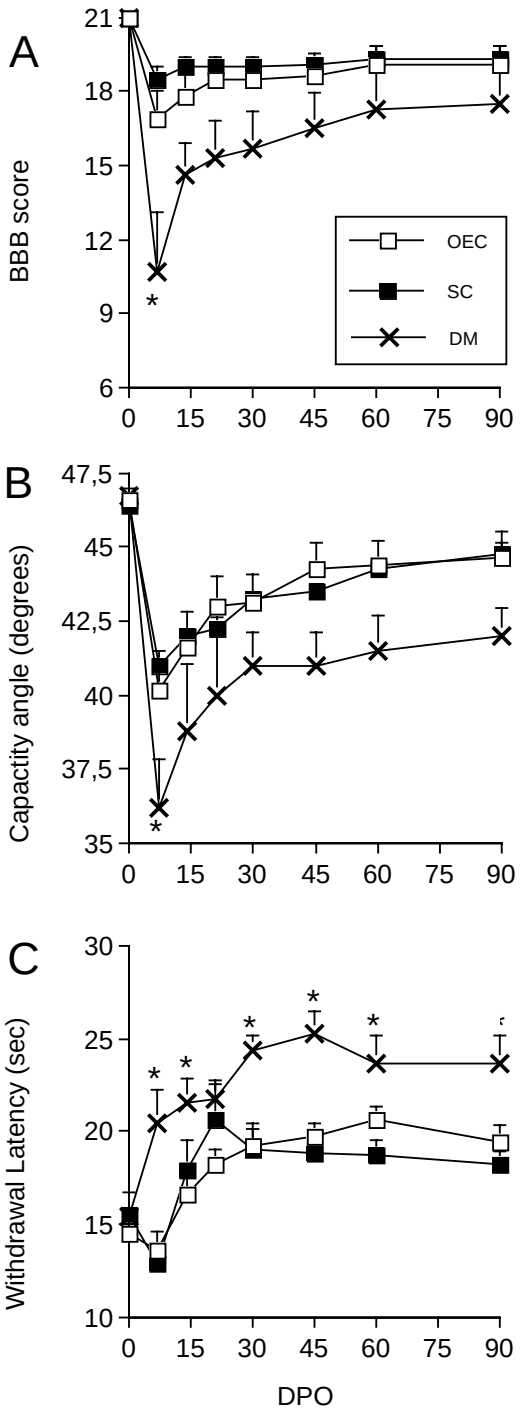

Figure 1. Evolution of (A) the open field locomotor activity evaluated by the BBB-scale, (B) the maximum angle in the inclined plane test, and (C) the time to withdrawal of the heated paw in the plantar algesimetry test over time after photochemical injury to the spinal cord. * $p< 0.05$ vs group DM.

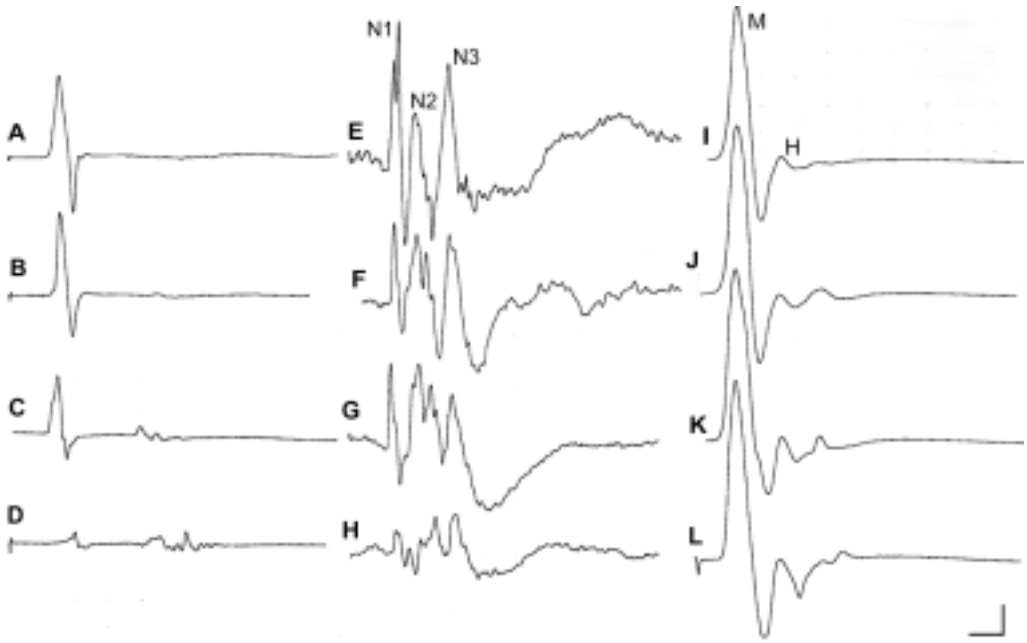

Figure 2. Representative electrophysiological recordings of (A-D) motor evoked potentials, (E-H) somatosensory evoked potentials and (I-L) compound muscle action potentials from an animal prior to injury (A, E, I), and from rats of group OEC (B, F, J), group SC (C, G, K) and group DM (D, H, L) at 90 dpo. Horizontal bar = 5 ms, vertical bar = 5 mV in A-D; horizontal bar = 10 ms, vertical bar = 4 μV in E-H; horizontal bar = 2 ms, vertical bar = 10 mV in I-L.

Functional results

At 7 dpo the animals of all the groups presented moderate locomotor deficits. Rats of OEC and SC groups showed coordinated and balanced stepping, with irregularity of fine details of locomotion, scoring 16 ± 1 and 18 ± 0.5 respectively, while DM animals had gross locomotion with unbalanced stepping and a mean score of 10 ± 1 ($p < 0.05$ with respect to groups OEC and SC) (Fig. 1A). All the animals exhibited gradual improvement in hindlimb locomotion reaching at 90 dpo mean scores of 19 ± 0,5, 19 ± 0,7 and 15 ± 2 in OEC, SC and DM groups respectively. OEC and SC grafted animals won fluidness and velocity in their movements and maintained only mild residual deficits, whereas animals from group DM recovered stability and balanced locomotion. No significant differences were found at the end of follow-up (Fig. 1A).

The mean values of the capacity angle for each experimental group along follow-up are shown in Figure 1B. At 7 dpo, the mean capacity angle values decreased with respect to pre-operative values, and were 40 ± 0.9, 41 ± 0.5 and 36 ± 1.6, in OEC, SC and DM groups, respectively. Significant differences were found between group DM and the two transplanted groups. With time, the animals increased slightly their ability for maintaining stance at higher angles, without significant differences between groups (Fig 1B).

Withdrawal latencies obtained with the plantar algesimeter test ranged between 14 and 16 seconds in preoperative controls. After injury, limb withdrawal occurred at higher temperatures and consequently at longer latencies. By 90 dpo the mean withdrawal latencies were 19 ± 1, 18 ± 1 and 23 ± 2 in OEC, SC and DM groups, respectively. Significant differences were found between group DM and the glial transplanted groups along all the follow-up (Fig. 1C).

Electrophysiological results

The MEP baseline recordings on the tibialis anterior muscle consisted in a short latency, negative deflection signal with an amplitude between 14 and 20 mV and an onset latency of about 6 ms (Fig. 2A). No changes in latency were observed after spinal

cord injury in any experimental group. At 14 dpo, the amplitude of MEPs was about 80%, 79% and 7% with respect to preoperative values in OEC, SC and DM groups, respectively. During follow-up the amplitude of MEPs increased in group OEC, decreased slightly in group SC and markedly in group DM. At 90 dpo the mean percentage amplitudes were 109%, 63% and 0.1%, in OEC, SC and DM groups, respectively (Fig. 2B-D, Fig 3A), being significantly lower in group DM than in the other two groups.

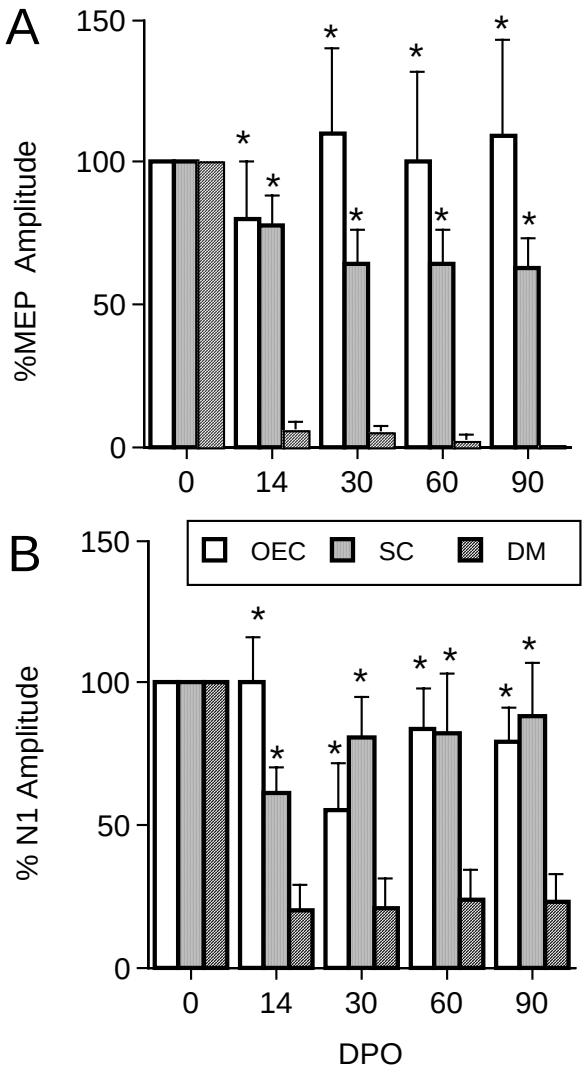

Figure 3. Mean percentage of (A) the amplitudes of the MEP, and (B) the N1 wave obtained in the SSEP tests with respect to preoperative values of each animal along the 3-month follow-up.

In the preoperative tests, the SSEPs consisted in a sequence of negative and positive peaks (Fig. 2E). Three complexes of negative peaks, named N1, N2 and N3, were consistently recorded in all the animals. After injury, no changes in the latency of the SSEPs complexes were observed, whereas the amplitude of all three complexes decreased with respect to preoperative values, being the N1 wave the most affected (Fig. 2 F-H, Fig 3B). Along follow-up the amplitude of the three peaks remained invariable in group DM rats, while it recovered partially in OEC and SC animals. Significant differences were found between the DM group and the SC and OEC groups.

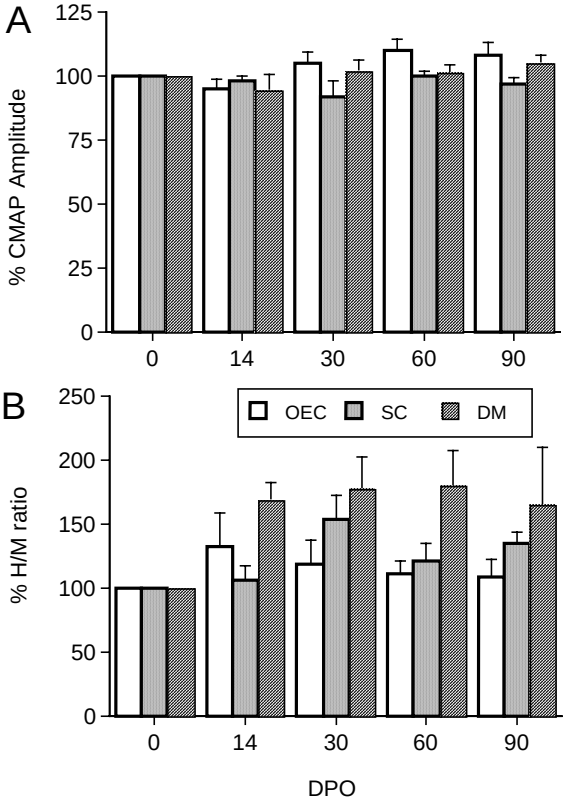

Figure 4. Mean percentage of (A) the amplitudes of the M wave, and (B) the H/M ratio obtained in motor nerve conduction tests with respect to preoperative values of each animal along the 3-month follow-up.

Electrical stimulation of the sciatic nerve gave rise to two negative deflections, the direct M wave and the late H reflex wave, recorded in the tibialis anterior muscle (Fig. 2I). After thoracic spinal cord injury, the M wave latency and amplitude remained almost unchanged during the 90 dpo in all the animals (Fig. 4A). In contrast, the amplitude of the H wave tended to increase (Fig. 2J-L), and in consequence the H/M ratio increased after injury. The increase in the H/M ratio was

marked and sustained over time in the control DM group, whereas it was lower and tended to return to preoperative values in the OEC and SC groups (Fig. 4B), although no significant differences were found due to the large interindividual variability.

Immunohistochemical results

Transverse sections of the T8 spinal cord segments of the DM animals showed that the photochemical injury produced a cavity, which mainly affected the dorsal aspect of the spinal cord, including the dorsal funiculus and the dorsal and mid aspects of the lateral funiculi, as previously described (Verdú et al 2003a). The cavity was surrounded by scarring tissue, strongly immunostained against GFAP. The OEC and SC-transplanted animals presented an open cavity on the dorsal aspect of the spinal cord, but with a lesser extension than in the non-transplanted DM group, with partial preservation of the dorsolateral funiculi (Fig. 5A-C). The average area of preserved spinal cord parenchyma was 3.28 ± 0.15 mm^2 in group OEC and 2.97 ± 0.22 mm^2 in group SC, both significantly higher than the 2.50 ± 0.22 mm^2 found in group DM.

Hypertrophied astrocytes, heavily stained for GFAP were present in the spinal cord parenchyma. In the SC and DM groups, these astrocytes had longer and thicker processes than in the OEC animals. The reactive grade of GFAP was significantly lower in group OEC (17.9 ± 5.6) than in group SC (28.8 ± 4.4) and in group DM (28.6 ± 2.2). At the lesion site, CSPG immunostaining was seen in cord sections of all the experimental groups. The strongest labeling was found surrounding the cavity (Fig. 5D-F). The immunostaining intensity was 189.7 ± 17.7, 177.7 ± 8.6 and 158.8 ± 11.5, in groups OEC, SC and DM, respectively. Significant differences were found between group DM and the other two groups ($p < 0.05$). In addition, p75 positive cells were also seen in spinal cord sections from all the injured rats, forming a dense meshwork within the dorsal area. No p75 positive cells were detectable within ventral areas of the injured spinal cord. Some of the p75 positive cells had spherical shape morphology, while others adopted a long bipolar morphology with large and thin or thick processes. Qualitatively, more p75 cells were found in glial transplanted groups than in group DM

In transverse spinal cord sections at T13, serotonin fibers were seen in the lateral and ventral grey matter and in the white matter tracts. This pattern was similarly observed in the spinal cord sections of all groups (Fig. 6D-F).

Anterograde tracing of the costicospinal tract

In spinal cord sections taken at T1 level from all rats strong DiI staining was seen in the ventral aspect of the dorsal columns, corresponding to the corticospinal tract. In contrast, at T13 spinal cord segment, DiI staining was seen in spinal cord sections of only 3 of 8 rats of group OEC, and in none of groups SC and DM (Fig. 6A-C).

DISCUSSION

The results of this study show that an acute transplantation of OECs or SCs exerts neuroprotective effects on the spinal cord parenchyma in animals subjected to a photochemical injury of the thoracic spinal cord. In concordance with the increased tissue preservation, better restoration of behavioral skills was achieved in OEC and SC transplanted rats than in control DM rats. Furthermore, the animals with OEC transplantation presented a better electrophysiological outcome than SC and DM injected animals. The histological results showed reduced glial reactivity and CSPG expression at the site of injury in the OEC animals compared to SC and DM animals.

The mild photochemical lesion induced (illumination for 2.5 minutes; see Verdú et al., 2003a) mainly compromised the dorsal and lateral funiculi and the dorsal horns, whereas the ventral and ventrolateral funiculi and ventral horn grey matter remained unaffected. The locomotor function in control injured rats was mildly involved (scores 15-16 in the BBB scale). It has been shown that lesions of the ventrolateral funiculus but not of the dorsal funiculus produce severe locomotion deficits, resulting

in poor scores in the open field test (Schut et al., 2002).

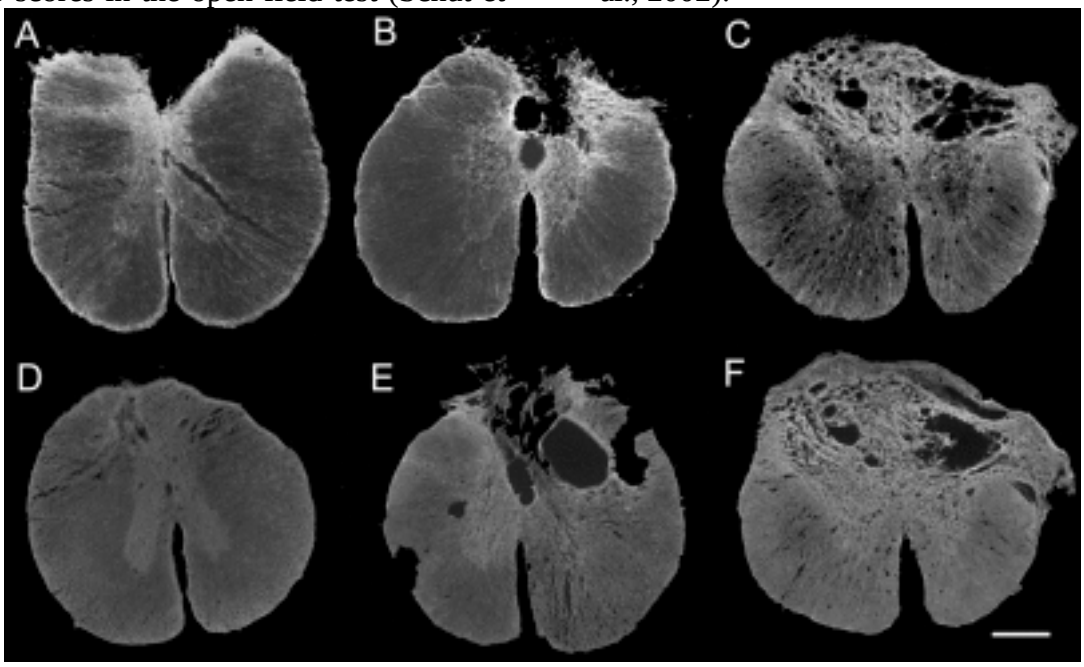

Figure 5. Spinal cord sections immunostained against GFAP (A-C) and CSPG (D-F) taken at the lesion site from animals of group OEC (A, D), group SC (B, E) and group DM (C, F). Glial grafts promote sparing of the injured dorso-lateral areas of the spinal cord.

As a result of the increased sparing of lateral funiculi induced by cell grafts, the transplanted groups had moderate but significantly improved neurological outcome.

In contrast, the amplitude of MEPs and SSEPs was markedly higher in OEC and SC transplanted groups than in the medium injected group. The use of electrophysiological techniques allows detection of functional differences between spinal cord injured groups, that may be masked in gross neurological evaluations of locomotor behavior (Loy et al., 2002; García-Alías et al., 2003). Supramaximal transcranial stimulation produces depolarization of cortical and brainstem motor neurons, which propagate the impulse along the spinal cord to synapse and depolarize lumbar motoneurons (Konrad et al., 1990). Injuries of the spinal cord compromise the preservation of MEPs, mainly when the lateral funiculi are lesioned (Adamson et al., 1989). The maintenance of MEPs in OEC and SC grafted groups is thus attributable to the larger sparing of the lateral funiculi in these animals compared to DM animals . On the other hand, the low intensity stimuli applied to the tibial nerve to evoke the SSEPs produce excitation of large myelinated afferent fibers, which mainly correspond to the dorsal column spinal pathway (Fehlings et al., 1988). However, other afferent spinal tracts are involved, mainly the spinocerebellar tracts which run along the dorsal and mid aspect of the lateral funiculi (York 1985). The presence of SSEPs in all our injured groups is suggestive of multiple spinal pathways contributing to the recorded signals. Again, the different preservation of dorsolateral funiculi would explain the maintenance of SSEPs after injury with amplitudes that were significantly higher in OEC and SC groups than in the DM group. The amplitude values of MEPs and SSEPs remained without noticeable changes in group DM during follow-up, whereas they increased slightly from 14 dpo to later time tests in grafted groups. This can be due to regeneration of a population of injured axons spanning the lesion site or to remyelination of demyelinated axons by the grafted glial cells.

After spinal cord injury the facilitation of spinal reflexes leads to the development of hyperreflexia. This phenomenon is electrophysiologically detected by an increase of the H/M ratio. The increase of reflex excitability is due to the interruption of descending input from supraspinal nuclei, leading to diminished presynaptic inhibition of Ia muscle afferents by spinal interneurons

(Calancie et al., 1993) and to changes in motoneuron excitability (Chen et al., 2000).

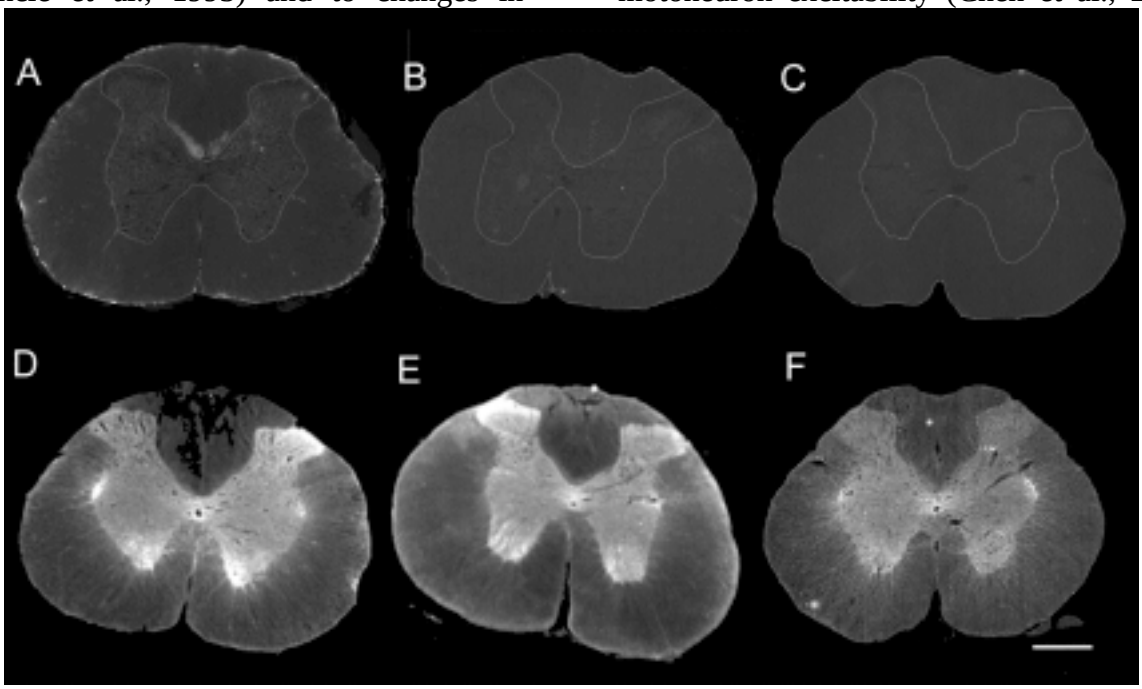

Figure 6. Spinal cord sections at T13 showing the dorsal corticospinal tract labeled with DiI in rats of group OEC (A), group SC (B), and group DM (C). Spinal cord section at T13 immunostained against 5-HT from rats of group OEC (D), group SC (E) and group DM (E).

The three experimental groups presented a high H/M ratio due to an increase in the H reflex wave amplitude at early stages of follow up. The ratio tended to return to normal values with time in the two grafted groups, more in the OEC group than in the SC group, whereas it remained elevated in the control group. The normalization of the H reflex response in the grafted groups is attributable to preservation of the spinal descending pathways, and therefore partially recovered supraspinal control. The tracing evaluation of the corticospinal tract showed spared/regenerated axons only in three OEC transplanted animals. On the other hand, immunostaining of 5-HT positive fibers at T13 showed no appreciable differences between groups. Brainstem serotoninergic neurons have a high capability for collateral sprouting, and this might be the cause for the lack of differences between our injured groups. This would also explain the ability of all the animals to walk after injury (Jacobs et al., 2002).

Our histological results showed that the three experimental groups had p75-positive cells at the injury site. Both SCs and OECs are stained by p75 antibody labeling. Spontaneous and local infiltration of SCs from the dorsal roots occurs after photochemical, contusion and compression injuries of the spinal cord (Felts and Smith 1992, Bunge et al., 1994, Beattie et al., 1997). However, both glial transplanted groups had more p75-positive cells than the DM group. This finding is suggestive of survival of transplanted cells at the injury site, although further studies with specific labels for OECs and for SCs should be preformed in order to determine the differential survival and migration of each type of glial cell transplanted in vivo (Takami et al., 2002).

The OEC grafted animals presented lower GFAP and proteoglycan immunoreactivity in astrocytes than SC and DM groups. In previous studies, we showed that OEC transplantation into spinal cords subjected to photochemical injury reduced astrocytic reactivity, diminishing their hypertrophy and the expression of GFAP and proteoglycans, compared to the control injured spinal cord (Verdú et al., 2001, 2003b). In vitro studies demonstrated that proteoglycan inhibits neurite outgrowth (Bovolenta et al., 1997). Recently, Plant et al. (2001) reported that CSPG-immunoreactivity was increased at the

distal SC graft-host cord interface, and it was associated not only with astrocytes and fibroblasts but also with transplanted SCs. These findings suggest that accumulation of proteoglycan compounds near the wound may impair the elongation of injured central axons. The modulation of astrocyte reactivity by the presence of OEC but not by SCs raises the question of the possible communication, either by cell contact or by secretion of soluble factors, between cell types. These phenomena could explain the transient hypertrophy and up regulation of GFAP in olfactory bulb astrocytes after a transection of the olfactory nerve, while transplantation of mature glial scars of preinjured optic nerve into transected olfactory nerve, maintains astrocyte reactiveness and impedes axonal regeneration (Anders and Hurlock 1996).

Previous studies reported that both OEC and SC grafts may enhance functional recovery after spinal cord injuries in the adult rat (Guest et al., 1997, Xu et al., 1997; Pinzón et al., 2001, Ramón-Cueto et al., 2000, Plant et al., 2003; Verdú et al., 2003b). However, limited information exists comparing the effects of OEC or SC grafts in the same spinal cord injury model. Imaizumi et al (1998, 2000) found that either OECs or SCs similarly facilitated long distance regeneration of functionally remyelinated axons within the spinal cord after dorsal columns transection. On another hand, Takami et al. (2002) recently reported that SC grafts were more effective in promoting axonal sparing/regeneration than OEC grafts, and only SC grafts improved the hindlimb locomotor performance in animals subjected to moderate contusion of the thoracic spinal cord. Our results differ from those of Takami et al (2002) in that both OEC and SC transplants promoted tissue preservation and functional recovery after a mild photochemical injury, but OEC grafts gave the best results. Several reasons may explain the differences found between these studies. First, the effects of glial cell grafts may differ depending on the type and severity of the spinal cord injury; comparatively the contusion injury induced in the work of Takami et al (2002) was more severe than the lesion induced by our photochemical method. Second, the time interval between injury and cell grafting was also different; Takami et al (2002) grafted the cells 7 days after the injury while we transplanted only 30 minutes after. In this regard, Martin et al (1996) demonstrated that immediate (30 minutes) or 10-days delayed SC grafts, but not intermediate (4 days) grafts reduced the size of cystic cavitation in mild injured spinal cords. They suggested that release of cytotoxic metabolites could be prevented by acute transplantation, and that 10 days after injury these metabolites could have been cleared out but not at an intermediate stage where they could be harmful to the grafted cells (Martin et al., 1996). On another hand, immediate transplantation of OECs did not improve locomotor behavior after moderate contusion (Plant et al 2003, Resnick et al 2003) whereas 7 days delayed transplantation produced a slight improvement; despite that tissue preservation was similar with both timings (Plant et al 2003). Thirdly, the purity and phenotype of the cultured cells for the transplant (Imaizumi et al., 2000; Rubio et al., 2002), the amount of cells and the precise site of transplantation may strongly affect the results.

Both SCs and OECs are known to express a number of different molecular markers in common, display a similar morphological phenotype in culture and respond to the same growth-promoting molecules (Ramón-Cueto and Valverde 1995; Wewetzer et al., 2002), synthesize and secrete several growth factors that promote the survival and regeneration of different types of neurons (Woodhall et al., 2001, Lipson et al., 2003, Boruch et al., 2001). In addition, transplanted SCs and OECs remyelinate injured spinal axons with a peripheral type of myelin, similarly restoring the conduction properties, although they display morphological differences (Imaizumi et al., 2000). With regard to the differential effects, OECs but not SCs are able to migrate in the host spinal cord parenchyma (Iwashita et al., 2000; Boruch et al., 2001), and OECs differ from SCs in their interaction with astrocytes (Lakatos et al., 2000; Verdú et al., 2001), thereby being capable of creating a favorable microenvironment for central regeneration.

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El transplante de células de la glía envolvente del bulbo olfatorio favorece la preservación de parénquima medular y evita una pérdida de función motora y sensorial *

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Objetivo. Evaluar el efecto a largo plazo del transplante de células de la glía envolvente (GE) del bulbo olfatorio tras lesión de la médula espinal.

Material y métodos. A 16 ratas adultas Sprague-Dawley se practicó una laminectomía dorsal en la vértebra T8, dejando al descubierto la médula espinal subyacente, la cual se bañó con rosa de Bengala durante 10 minutos y posteriormente se lesionó, por iluminación con una fibra óptica acoplada a una lámpara halógena, durante 2,5 minutos. A la mitad de los animales se les inyectó, con una micropipeta acoplada a una jeringa Hamilton, 180.000 células de GE en 10 µl de DMEM (grupo GE), y a la otra mitad sólo 10 µl de DMEM (grupo DM). Se evaluó la recuperación locomotora según la escala BBB y la sensibilidad nociceptiva según la prueba de la algesimentría plantar. A los 90 días postoperación, tras perfusión se extrajo la médula lesionada que se procesó inmunohistoquimicamente para GFAP y p75. Con las secciones inmunomarcadas a GFAP se evaluó el área de médula espinal preservada.

Resultados. Los animales del grupo GE mostraron un nivel de locomoción superior y retiraron antes la pata al estímulo nociceptivo que los del grupo DM. Tambíen hubo una mayor preservación de parénquima medular, y más células p75 positivas en el grupo GE que en el DM.

Conclusiones. El transplante de GE favorece la preservación de parénquima medular y evita la pérdida de funciones motoras y sensoriales.

Palabras clave: Algesimetría, Glía envolvente, Lesión fotoquímica, Locomoción, Médula espinal.

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La lesión de la médula espinal se caracteriza por una interrupción inmediata y severa de las vías nerviosas ascendentes y descendentes medulares, dejando pacientes tetra- o parapléjicos para el resto de sus vidas. En el mundo son millares de personas las que sufren los efectos de una lesión medular, y muchos de ellos necesitan ayuda asistencial y psicológica durante años [27]. A parte del tratamiento farmacológico con agentes anti-inflamatorios, como la metilprednisolona, que mitigan los efectos del daño medular secundario [2], no existe, hoy por hoy, una cura efectiva para las lesiones medulares, lo que deja a los pacientes con unas bajas perspectivas clínicas de solución a su problema.

El uso de animales de laboratorio con los que poder generar lesiones medulares, es esencial para obtener información de los procesos fisiopatológicos que suceden durante la lesión medular, así como ensayar métodos reparativos de la médula espinal, que favorezcan la preservación de tejido medular, que minimicen los efectos de la lesión primaria y secundaria, y que promuevan la supervicencia, regeneración y reinervación funcional de las neuronas lesionadas. La contusión por la caída de un peso ("weight-drop"), la compresión por medio de pinzas ocluyentes, la sección y/o hemisección, y la lesión fotoquímica son diferentes modelos experimentales de lesionar la médula espinal [1, 5, 18, 21, 35, 43, 51]. Todos ellos se caracterizan por causar disrupción de las membranas celulares, de las vías axonales ascendentes y descendentes, y lesiones vasculares. Las hemorragias y el edema medular, conllevan a un estado de deprivación de oxígeno (isquemia-hipoxia) y de glucosa (hipoglucemia) en la zona de lesión, así como cambios en los niveles extracelulares de electrolitos, responsables de un bloqueo de la conducción de potenciales de acción por los axones lesionados (shock neurogénico), y en un aumento de la liberación de aminoácidos excitatorios (glutamato) causantes de la excitotoxicidad neuronal [44, 47]. Junto a todo ello, también se produce una reactivación de los astrocitos y de la microglía. Los primeros se transforman a células hipertróficas, con robustas prolongaciones citoplasmáticas rellenas de proteína ácida fibrilar de la glía (GFAP). Estos astrocitos reactivos se acumulan alrededor de la zona de lesión primaria, generando una cicatriz glial, que sella y separa la zona necrótica del resto de sistema nervioso central intacto. Asimismo, también contribuyen a la formación de matriz extracelular, con la síntesis y liberación de proteoglicanos, los cuales son inhibitorios para la elongación axonal, pues causan la retracción de los conos de crecimiento de las neuronas regenerativas supervivientes [7]. La disrupción vascular, favorece la entrada de elementos vasculares, inicialmente de neutrófilos y posteriormente de monocitos que se transformaran a macrófagos. Estas células vasculares, sintetizan y liberan un conjunto de citoquinas que inducen la reactivación de la microglía residente. Todos estos tipos celulares tienen como misión la limpieza de las cavidades císticas de los restos celulares muertos. Realizan su papel mediante la liberación de radicales libres de oxígeno, de óxido nítrico y de enzimas lisosomales, factores que no sólo degradan los restos de las cavidades, sino que potencialemente también pueden causar lesiones en regiones adyacentes intactas [26, 44].

Si bien las repuestas neurales postlesión son conducentes al aislamiento de la zona lesionada, dichos mecanismos generan un microambiente completamente desfavorecedor para la elongación de los axones de las neuronas supervivientes, y en todos los casos tienden a magnificar y extender la lesión más allá de la zona estrictamente lesionada. Son varios los tratamientos reparativos ensayados en modelos experimentales de lesión medular, entre los que se incluyen: (i) administración de diversos fármacos, como metilprednisolona, MK-801, o gaciclidina que intentan reducir la infiltración de neutrófilos y monocitos, o bien evitar la muerte neuronal por exitotoxidad [11, 29, 32, 52]; (ii) la infusión de factores de crecimiento, como son las neurotrofinas, que promueven la supervivencia, regeneración y remielinización de las neuronas lesionadas [8, 10, 19, 28]; (iii) la neutralización de factores inhibitorios, como

los anticuerpos contra MAG, que inhiben el efecto de esta proteína de la mielina de los oligodendrocitos sobre los conos de crecimiento, o el tratamiento con condroitinasa ABC que degrada el proteoglicano [3, 36, 55]; y (iv) el transplante de células gliales, especialmente de células de la glía envolvente del bulbo olfatorio (GE), las cuales promueven la regeneración de los axones lesionados y la restitución parcial de funciones perdidas [21, 38].

Una de las ventajas de la terapia mediante transplante de GE es que sus efectos no se limitan a la zona del transplante, pues dicha glía tiene una alta capacidad de migración a través de la médula espinal lesionada [6, 21, 40, 49]. Los estudios experimentales previos en los que se ha transplantado GE son contradictorios, pues hay investigadores que indican que en los animales transplantados existe mayor recuperación funcional respecto a animales sin transplante o con transplante de vehículo [17, 24, 38, 50], mientras que otros investigadores no obtienen diferencias entre el grupo con y sin transplante de GE [40, 46]. Sin embargo, en los animales transplantados con GE se reduce la cavitación [33, 49, 50] y aumenta el número de fibras regenerativas a través de la lesión [21, 33, 38] . Estos resultados sugieren que el transplante GE en lesiones de la médula espinal puede tener un claro papel beneficioso, reduciendo la extensión de la lesión primaria y/o secundaria, y generando un microambiente favorecedor de la regeneración de los axones de las neuronas supervivientes, aunque ello no se traduzca, en todos los casos, en una mayor recuperación funcional.

Recientemente se ha demostrado que, a largo plazo, el transplante de GE mediante inyección con pulsos de aire, favorece la preservación de tejido medular y ello contribuye a una mayor recuperación funcional [50]. Se desconoce si, la técnica de transplante de células puede influir en una mayor o menor preservación de tejido y ello en los resultados funcionales. Los objetivos del presente estudio son, en primer lugar determinar si el transplante de GE puede tener un efecto reparador en lesiones de la médula espinal, y en segundo lugar evaluar si, el transplante de GE mediante microinyecciones con jeringa Hamilton, permite obtener resultados similares a los reportados mediante inyecciones con pulsos de aire.

MATERIAL Y METODOS

Cultivo, purificación y criopreservación de las células gliales

Las células de la glía envolvente se obtuvieron de bulbos olfactorios de ratas Sprague-Dawley adultas (250-300 gr). Tras anestesia profunda con pentobarbital sódico (70 mg/kg, i.p.), y bajo condiciones de asepsia se procedió de la decapitación y extracción de los bulbos olfatorios que se recogieron en solución salina balanceada de Hank (HBBS, Sigma) con calcio y magnesio a 0º C. Bajo campana de flujo laminar se procedió a eliminar las meninges y seguidamente a la digestión enzimática de los bulbos en una mezcla enzimática de tripsina-colagenasa-DNAsa, durante 30-45 minutos a 37º C. Posteriormente se disoció el tejido mecánicamente por medio de una pipeta Pasteur a la que previamente se estrechó su punta. A continuación, la suspensión celular se centrifugó (900 rpm, 7 minutos), se eliminó el sobrenadante, y el precipitado celular se resuspendió en medio DF10S, y que sembró en frascos de cultivo (25 cm^2) previamente poli-L-lisinados, utilizando como medio de cultivo DF10S. El medio de cultivo se cambió cada 3 días. A los 9-10 días se utilizó un medio quimicamente definido para expandir las células de la glía envolvente [13]. Tras la confluencia del cultivo (15 días in vitro), se procedió a levantar las células del frasco mediante el uso de tripsina-EDTA en HBBS sin calcio y magnesio. Tras centrifugación y eliminación del sobrenadante, se purificaron las células de la glía envolvente mediante bolitas magnéticas recubiertas con anticuerpos anti-p75. Las células p75 positivas fueron seleccionadas del resto mediante el uso de un potente imán, que retenía las células p75 positivas a las paredes del tubo de ensayo. Posteriormente, tras varios centrifugados, la suspensión celular se incluyó en medio crioprotector y se congeló a – 80º C hasta su uso [12].

Procedimiento quirúrgico y transplante celular

En condiciones de asepsia y anestesia con pentobarbital (60mg/kg, i.p.), bajo un microscopio estereoscópico, y fijando el animal en un sistema estereotáxico, se practicó una laminectomía dorsal en la vértebra T8, dejando al descubierto la médula espinal subyacente. La médula se bañó con una solución de rosa de Bengala (RB, 1.5% en solución salina) durante 10 minutos. El exceso de solución se eliminó con varios baños de solución salina y la zona bañada se iluminó con una fibra óptica acoplada a una lámpara halógena de 100 W (OSRAM, Germany) durante 2,5 minutos a una intesidad lumínica de 80 kLux, controlada por medio de un luxómetro (TES-1332, Electrical Electronic Corp, Taiwan). La fibra óptica se colocó a 10 mm de la superficie dorsal de la médula, y durante todo el tiempo de iluminación, la médula espinal estuvo bañada con solución salina para evitar el resecamiento de la misma [51]. Tras la lesión medular se procedió al transplante de 180.000 células de glía envolvente en 10 µl de DMEM (Grupo GE; n= 8), o bien al transplante de sólo 10 µl de DMEM (Grupo DM; n= 8). La suspensión celular o el vehículo se inyectaron, bajo microscopio estereoscópico, mediante el uso de una micropipeta de vidrio (d.i. 70-90 µm) acoplada a una jeringa Hamilton montada en un micromanipulador. Las inyecciones se realizaron muy lentamente en dos puntos de la médula espinal lesionada (rostral y caudal), a una profundidad de 1 mm, lo que corresponde aproximadamente a las columnas dorsales de la médula espinal. Finalmente se procedió a suturar los planos musculares y cutáneos, y a desinfectar la zona intervenida con povidona iodada. Los animales se mantuvieron en observación hasta que despertaron de la anestesia.

Métodos funcionales

Todas las pruebas funcionales se realizaron antes de la operación y a los 7, 14, 30, 60 y 90 días postlesión. La capacidad de locomoción de los animales se evaluó colocando al animal en un campo abierto circular (90 cm de diámetro x 24 cm de pared) y valorando el movimiento de la extremidades posteriores según la escala desarrollada por Basso y colaboradores [4]. Dos observadores independientes realizaron esta valoración y el resultado final fue el promedio de ambos observadores. La escala BBB oscila entre 0 puntos (parálisis completa) hasta 21 puntos (locomoción normal).

La sensibilidad nociceptiva se evaluó mediante la prueba de la algesimetría plantar [15]. Los animales se colocaron en una caja transparente alta, cuyo suelo estaba formado por una placa de vidrio, a través de la cual se hacía incidir, sobre la superficie de las patas posteriores del animal, un rayo de luz incandescente que surgía de una lampara halógena de 150W. Mediante un detector de infrarrojos, se valoró el tiempo que el animal tardaba en retirar la pata posterior al estímulo nociceptivo. Para evitar lesiones cutáneas se limitó el tiempo máximo de exposición a la luz incandescente a 40 segundos. La prueba se repitió tres veces, a intérvalos de descanso de 5 minutos. El valor final fue el promedio de las tres repeticiones.

Métodos inmunohistoquímicos

A los 90 días postoperación, los animales fueron anestesiados profundamente y perfundidos transcardiacamente con 4% de paraformaldehido en tampón fosfato salino (PBS; 0,1M; pH: 7.4). Posteriormente se extrajeron las médulas espinales, y el segmento medular lesionado fue postfijado en la misma solución durante 24 horas a 4º C. Posteriormente fue incluido en un medio crioprotector (30% de sacarosa en PBS) durante 48 horas a 4ºC. De dicho bloque medular, se realizaron secciones transversales seriadas de 30 µm de grosor mediante un criostato, que se recogieron en un tampón crioprotector De Olmos y fueron guardadas a – 18º C hasta su uso.

Por medio de la técnica de flotación, las secciones fueron lavadas con PBS para eliminar el exceso de De Olmos, posteriormente fueron incubadas con 0,3% de Triton-X-100 y 1% de suero fetal bovino en PBS, durante 1 hora a 4º C y en constante agitación, y finalmente se incubaron con el anticuerpo primario policlonal de conejo contra la proteína ácida fibrilar de la glía (GFAP; 1:1000; Chemicon) y monoclonal de

ratón anti- receptor de baja afinidad del factor de crecimiento nervioso (p75; 1:200; Chemicon) durante 24 horas a 4º C y en agitación. Posteriormente, y tras varios lavados con 1% de suero fetal bovino en PBS, las secciones medulares se incubaron con el anticuerpo secundario IgG de asno anti-conejo o anti-ratón conjugados a la cianina 3,18 (Cy3; 1:200; JacksonImmunoresearch) durante toda la noche a 4º C y en agitación. A día siguiente, tras varios lavados con PBS, las secciones se motaron en portaobjetos pre-gelatinados, se deshidrataron en baños crecientes de etanol (50º , 70º, 90º , 96º , absoluto) y finalmente el cubreobjetos se montó con DPX (Fluka). Como control, algunas secciones fueron incubadas sólo con anticuerpo secundario. Todas las secciones fueron observadas con un microscopio de epifluorescencia (Olympus BX51) usando filtros apropiados.

Mediante el uso de una cámara digital (Olympus DP50) acoplada al microscopio, se captaron imágenes completas de las médulas espinales inmunomarcadas para GFAP (x4), que fueron analizadas por medio del software NIH-image (sofware público). Estas imágenes fueron utilizadas para medir el área de médula espinal preservada, pues el marcaje GFAP delimita muy bien las regiones de parénquima medular lesionado del preservado. Dos investigadores independientes efectuaron dichas mediciones sobre las mismas imágenes capturadas, y el área final de médula espinal preservada, de cada sección analizada, fue el promedio del valor obtenido de ambos investigadores. Para cada animal se analizaron entre 8-10 secciones medulares [50].

Análisis estadístico

Todas las valoraciones funcionales, electrofisiológicas e histológicas se han realizado a ciegas, mediante el uso de un código tanto en animales como en muestras histológicas. Los datos se muestran como media ± error estándar. Las comparaciones estadísticas entre grupos se han realizado mediante el análisis de la varianza (ANOVA), usando la prueba de Scheffé para múltiples comparaciones. Se han considerado diferencias significativas cuando $p< 0,05$.

Todos los procedimientos y protocolos experimentales del presente estudio han sido aprobados por el Comité de Ética en Experimentación Animal y Humana de nuestra institución.

RESULTADOS

Antes de la operación todos los animales de ambos grupos experimentales mostraron un patron normal de locomoción, puntuado como 21 en la escala BBB. A los 7 días postoperación, la habilidad locomotora de los animales de ambos grupos experimentales disminuyó, siendo de aproximadamente 14 y 19 puntos en la escala BBB , para los grupos DM y GE, respectivamente. Posteriormente, la puntuación de la locomoción según la escala BBB aumentó progresivamente en los animales de ambos grupos, alcanzando al final del seguimiento un valor de aproximadamente 16 y 20 puntos de la escala BBB en los grupos DM y GE, respectivamente. A lo largo de todo el seguimiento postoperatorio el grado de locomoción de los animales del grupo GE fué significativamente superior al de los animales del grupo DM (Tabla 1).

El tiempo de retirada de la pata posterior, a un estímulo térmico doloroso, fue de aproximadamente 15 segundos en todos los animales de ambos grupos experimentales antes de la operación. Tras la lesión medular, el tiempo de retirada al estímulo doloroso aumentó en los animales de ambos grupos experimentales, siendo en promedio de aproximadamente 19 y 17 segundos en los grupos DM y GE, respectivamente a los 7 días postoperación. Enre los 7 y 90 días postlesión, hay una tendencia a aumentar ligeramente el tiempo de retirada en los animales del grupo DM, mientras que en los del grupo GE tiende a disminuir. A lo largo del seguimiento, el valor promedio del tiempo de retirada en los animales del grupo DM fue igual o superior a 18 segundos, mientras que en los animales del grupo GE nunca superó los 18 segundos. Durante todo el seguimiento, el tiempo de retirada en los animales del grupo GE fue significativamente inferior al de los animales del grupo DM (Tabla 1).

Tabla 1. Resultados funcionales obtenidos a lo largo del seguimiento después de una lesión fotoquímica de la médula espinal en los grupos experimentales estudiados.

Prueba	**dpo**	**Grupo DM**	**Grupo GE**
Locomoción (escala BBB)	0	21,00 ± 0,00	21,00 ± 0,00
	7	13,62 ± 1,14	18,81 ± 0,69 * *
	14	17,08 ± 0,90	19,82 ± 0,46 *
	30	16,75 ± 0,77	19,94 ± 0,49 * *
	60	16,38 ± 0,70	20,03 ± 0,47 * * *
	90	16,13 ± 0,51	20,19 ± 0,40 * * *
Algesimetría (segundos)	0	15,42 ± 0,10	15,41 ± 0,09
	7	16,64 ± 0,30	17,42 ± 0,12 * *
	14	17,90 ± 0,21	16,60 ± 0,08 * * *
	30	18,87 ± 0,36	16,61 ± 0,14 * * *
	60	18,42 ± 0,40	16,31 ± 0,17 * *
	90	18,40 ± 0,24	16,07 ± 0,15 * * *

* p< 0,05; * * p< 0,01; * * * p< 0,001 respecto al grupo DM; dpo: días postoperación.

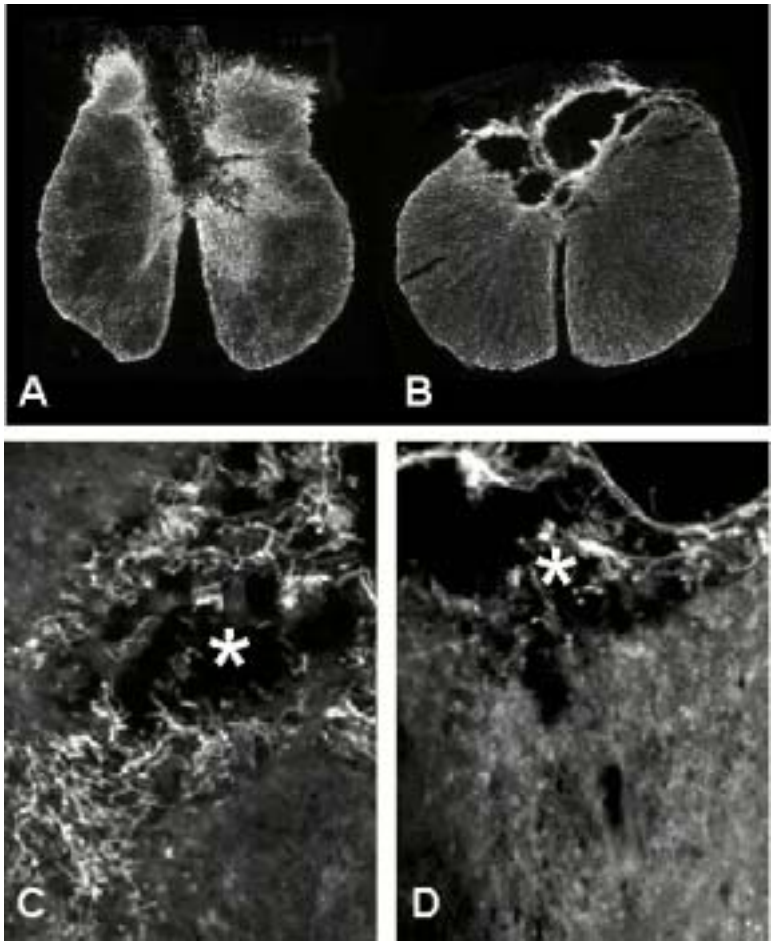

Figura 1. *Secciones transversales de la médula espinal inmunomarcadas contra GFAP de las ratas con transplante de glía envolvente del bulbo olfatorio (A) o con transplante de DMEM (B). Imágenes de microscopía óptica con epifluorescencia de campos próximos a la zona lesionada (asterisco) de secciones medulares inmunomarcadas para p75 de ratas con transplante de glía envolvente (C) o con inyección de DMEM (D). Todas las manchas blancas y filamentosas de las imágenes C y D corresponden a células inmunoreactivas al p75. Nótese que existen más en la imagen correspondiente al grupo GE que al DM. Imágenes A y B capturadas a x4; imágenes C y D capturadas a x20.*

En todos los animales de ambos grupos experimentales la lesión afecta principalmente la región dorsal de la médula espinal. En los animales del grupo DM, la lesión incluye el funículo dorsal, comprometiendo, en la mayoría de los animales, el tracto corticoespinal e incluso el canal central. Asimismo, en varios animales de este grupo experimental también se observan lesionadas las regiones dorsolaterales de la médula espinal. Un patrón similar se observó en las secciones de los animales del grupo GE, aunque en la mayoría de ellos la lesión se circunscribe casi exclusivamente al funículo dorsal y en alguno se extiende hacia regiones dorsolaterales (Fig 1A-B). El promedio del área de médula espinal preservada fue de 2,85 ± 0,09 y 3,12 ± 0,07 mm^2 en los grupos DM y GE, respectivamente; observándose diferencias significativas entre ambos grupos experimentales ($p< 0,05$). Cualitativamente, se observaron más células positivas para p75 en las secciones medulares de los animales con transplante de GE que en los animales con inyección de DMEM (Fig 1C-D).

DISCUSIÓN

Los resultados del presente estudio demuestran que, el transplante agudo de células de la glía envolvente del bulbo olfatorio mediante microinjección con jeringa Hamilton, en una lesión moderada de la médula espinal, evita una pérdida significativa de las funciones motoras y sensoriales. Asimismo, en los animales con transplante de células gliales, el área de médula espinal preservada es significativamente superior a la de los animales con transplante de DMEM. Cualitativamente, se observa un mayor número de células positivas a p75 en las secciones medulares del grupo GE que en las del grupo DM. Todos estos resultados son similares a los observados recientemente cuando se transplanta glía envolvente o DMEM mediante micropipetas de vidrio acopladas a un inyector de pulsos de aire [50], lo cual sugiere que el método de transplante celular no es un factor que influya directamente en la recuperación funcional, sino que es la terapia celular la que repercute sobre la preservación de tejido y de las funciones neurológicas.

Los mecanismos por los cuales, las células de la glía envolvente del bulbo olfatorio, pueden realizar los efectos observados en el presente estudio son múltiples. Diversos estudios “in vitro” han demostrado que las células de la glía envolvente son capaces de sintetizar y liberar al medio de cultivo diversos factores neurotróficos, tales como el factor neurotrófico derivado de la glía (GDNF), el factor neurotrófico derivado del cerebro (BDNF), el factor de crecimiento nervioso (NGF) [53], y la neurotrofina 4/5 [6]. La supervivencia y regeneración de los axones de las neuronas rubroespinales y corticoespinales es dependiente de BDNF [22, 25, 34], mientras que la elongación de las neuronas noradrenérgicas del locus ceroleus son dependientes de GDNF [16]. A pesar de que no exiten estudios experimentales que demuestren que las células de GE transplantadas sigan sintetizando y liberando factores tróficos, tampoco existen estudios que demuestren lo contrario, por lo que potencialmente las células transplantadas “in vivo” siguen manteniendo las mismas propiedades que las “in vitro”, y ello hace pensar que las células de GE son capaces de promover la supervivencia y regeneración de axones espinales. En especial, los tipos axonales anteriormente citados, participan muy directamente en el control motor y de locomoción en la rata. De hecho, diversos investigadores han demostrado la regeneración de axones espinales, a través de la lesión, en aquellos animales que habían recibido un transplante de GE [23, 24, 30, 38].

Un segundo factor es que la GE transplantada pueda facilitar una mayor migración de células promotoras de la regeneración, tales como las células de Schwann del sistema nervioso periférico. Tras una lesión medular sin reparación se produce, a los pocos días de la lesión, una entrada de células de Schwann desde la periferia que invaden el parénquima medular, tendiendo a localizarse en las proximidades de las cavidades císticas [9, 20]. Estas células de Schwann infiltrates, pueden contribuir a la supervivencia y regeneración de los axones

lesionados. De hecho se ha comprobado que injertos de nervios periféricos [41, 42, 48] o implantes de cámaras tubulares rellenas con células de Schwann [14, 31, 39, 54], promueven la regeneración de los axones centrales por el interior de dichos implantes, lo cual se atribuye al conjunto de factores neurotróficos secretados por las células de Schwann implantadas. Tanto la célula de la glía envolvente como la célula de Schwann son positivas para p75 [37], por lo que la presencia de células p75 en las secciones medulares de ambos grupos experimentales, no nos permite discernir si todas ellas son de un sólo tipo, y sobretodo que tipo son. Sin embargo, cualitativamente detectamos más células p75 positivas en las secciones histológicas de los animales con transplante de GE que en las de los animales con DMEM, lo que sugiere que la GE transplantada puede favorecer la migración de células de Schwann, las cuales también contribuyen a generar un microambiente favorecedor de supervivencia y/o elongación de los axones espinales lesionados.

En tercer lugar, las células de la glía envolvente también tienen la capacidad de sintetizar y secretar moléculas de la matriz extracelular, tales como laminina y fibronectina [37], componentes ambos del parénquima medular. De los resultados del presente estudio, no se puede concluir que la GE transplantada sintetice estos componentes y que, por tanto, contribuya a la generación de parenquima medular destruido. Tan sólo se confirma que, en los animales con transplante glial, el área de parenquima medular preservado es significativamente superior a los animales con transplante de DMEM. A pesar de ello, la posibilidad de que la glía envolvente participe en la sintesis “de novo” de elementos de la matriz extracelular del parénquima medular, puede explicar, en parte, los resultados histológicos obtenidos en el presente estudio. Por otra parte, es bien sabido, que dichos componentes de la matriz extracelular favorecen la elongación de las neuritas “in vitro” [45], a la vez que los axones regenerativos necesitan de un sustrato sobre el que elongarse, por lo que siempre intentan rodear las cavidades císticas que se generan tras una lesión medular [9]. Finalmente, no podemos olvidar que todas estas propiedades de la glía envolvente pueden estar potenciadas por su capacidad de migrar a lo largo de la médula espinal lesionada.

En resumen, el presente estudio confirma que el transplante de GE del bulbo olfatorio promueve, directa o indirectamente, una mayor preservación de tejido medular, lo que puede facilitar la regeneración de axones espinales supervivientes y mantener así una mayor respuesta motora y sensorial. La inyección de la GE mediante micropipeta acoplada a una jeringa Hamilton no ofrece resultados funcionales e histológicos diferentes a los observados cuando estas células son transplantadas mediante micropipeta acoplada a un impulsor de aire [50]. Las células de GE constituyen un buen método terapéutico para la reparación de la médula espinal lesionada, sin embargo, su aplicación clínica requiere de muchos más experimentos en modelos animales, que permitan dilucidar algunas de las lagunas conceptuales anteriormente reseñadas.

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DISCUSIÓN

Los resultados obtenidos en la presente tesis muestran la utilidad del modelo de lesión fotoquímica para el estudio de las lesiones traumáticas de la médula espinal, y el rol neuroprotector ejercido por el transplante de glía envolvente del bulbo olfatorio en animales sujetos a una lesión fotoquímica.

Estos datos sugieren que el transplante celular es una estrategia terapéutica prometedora para ser utilizada en un futuro en pacientes con lesiones medulares, y aun más, que de todas las posibles poblaciones celulares, la GE es una buena candidata para ser transplantada ya que promueve la protección del tejido medular, reduce la reactividad glial y favorece el restablecimiento funcional de la médula espinal lesionada.

1. La lesión fotoquímica como modelo experimental de lesión traumática

Es imprescindible, para la futura recuperación del lesionado medular, la aplicación de terapias que consigan aumentar la supervivencia neural y potenciar la regeneración axonal. El primer paso para poder abordar con éxito un proyecto tan ambicioso es desarrollar un modelo de lesión experimental que reproduzca fielmente los efectos producidos en las lesiones traumáticas y que permita estudiar no sólo la dinámica de la patología medular, sino además la evaluación de las diferentes aproximaciones terapéuticas con las que conseguir el restablecimiento funcional del SNC.

Los modelos de compresión, contusión, sección, lesión fotoquímica, criolesión o lesión por cambios electrolíticos del parénquima medular son en la actualidad los métodos más utilizados en el laboratorio para inducir una lesión experimental de la médula espinal. A pesar de este amplio abanico de modelos, no hay ninguno que mimetice fielmente los efectos producidos en el traumatismo humano. Sobretodo porqué en la mayoría de los modelos animales es necesaria la cirugía de la columna vertebral que ponga al descubierto la médula espinal a la que lesionar, mientras que en los traumatismos humanos la lesión medular se genera por la contusión o compresión de la médula encerrada en el interior de su cavidad ósea. Esta singularidad es de especial relevancia ya que la resultante final de las fuerzas aplicadas en la médula descubierta es distinta a la que se produce si la médula se encuentra encerrada en el interior del canal vertebral. A pesar de esta diferencia, de momento insalvable ya que hasta la fecha no hay ningún modelo de compresión o contusión que evite la laminectomía vertebral, los distintos procedimientos experimentales ejercen unos efectos similares sobre la médula espinal que conllevan una estereotipada alteración morfológica y funcional. No obstante, las características propias de cada modelo permiten al investigador escoger aquel que se

adecue a sus necesidades y objetivos experimentales, sopesando en cada caso las ventajas y desventajas de cada modelo en concreto.

Los modelos de contusión son en la actualidad los más utilizados ya que, entre varios motivos fueron escogidos para desarrollar los programas NASCIS (Basso et al., 1996b). El impacto del proyectil sobre la médula espinal genera una cavidad necrótica central que se extiende tanto radialmente como a lo largo del eje rostrocaudal. Esta cavidad está envuelta por tejido gliótico, y en su interior se encuentran tanto restos celulares como leucocitos (Bresnahan et al., 1987). Estudios recientes han confirmado que la lesión ejercida por el modelo de contusión es la que más se asemeja a los traumatismos medulares en humanos (Metz et al., 2000). Sin embargo, no está absente de críticas, sobretodo por ser un modelo que presenta una gran variabilidad entre animales, y porqué durante mucho tiempo se ha cuestionado si la lesión que producía era puramente debida a la distorsión tisular o si además desataba las lesiones secundarias medulares (Chehrazi et al., 1989).

Los modelos de compresión, tanto el clip de aneurismo como los balones hinchables, presentan dos inconvenientes principales. En primer lugar, ambos métodos requieren un abordaje quirúrgico más complejo de la médula espinal que la simple laminectomía, ya sea para introducir los balones en el interior del canal vertebral, o para colocar parte del clip compresor en la zona ventral de la médula espinal, y en segundo lugar, por el desconocimiento de la presión real ejercida sobre la médula, ya que parte de la presión es amortiguada por las propias paredes de la columna vertebral (Taoka y Okajima, 1998).

El modelo de sección tiene la ventaja de ser fácil de realizar y de conocer la naturaleza de los tractos espinales lesionados. No obstante, la sección completa de la médula espinal humana ocurre en escasas ocasiones, ya que, aun habiendo la pérdida funcional completa de los niveles por debajo de la lesión, es común la preservación de un porcentaje variable de parénquima medular (Geisler, 1991). Además, la sección completa de la médula espinal y de las meninges que la envuelven presenta un inconveniente añadido, y es que su rotura reduce la tensión natural entre los extremos medulares, por lo que se produce un hueco físico, que representa "per se" un obstáculo para la regeneración axonal. Finalmente, la rotura de las meninges puede favorecer la infiltración local de células exógenas al SNC alterando el ambiente celular (Taoka y Okajima, 1998).

En lo referente a la lesión fotoquímica, los resultados obtenidos en esta tesis evidencian la utilidad de este modelo para el estudio de las lesiones de la médula espinal. En primer lugar, las alteraciones morfológicas y los déficits funcionales causados en la médula espinal se asemejan a los producidos por otros modelos experimentales y a las descritas en humanos, lo que permite extrapolar y comparar los resultados obtenidos con los trabajos de otros investigadores y englobarla como un

modelo de lesión traumática experimental. En segundo lugar, la lesión producida es graduable y poco variable, y consiste en en la reproducción de las alteraciones conductuales y anatómicas. Por último, hemos comprobado que es compatible con el desarrollo de terapias reparadoras, y que es un modelo fácil de realizar, por lo que lo hace interesante para el estudio de la lesión y protección de la médula espinal.

No obstante, el modelo de lesión fotoquímica presenta dos grandes diferencias respecto a los modelos de contusión y compresión. La primera de ellas es la propia naturaleza de la lesión. Mientras que en la contusión/compresión la lesión primaria es la alteración biomecánica del tejido, en la lesión fotoquímica son los radicales libres de oxígeno, producidos tras la fotoactivación del rosa de Bengala, los responsables de inducir la lesión. En ambos modelos se desencadenan las cascadas de lesiones secundarias que desatan una respuesta caracerística de la médula espinal. Es por ello que, a medio y a largo plazo los efectos producidos en la médula son similares y por ello comparables. No obstante, no se sabe cómo afecta al desarrollo de la lesión la propia naturaleza de la lesión primaria, y cómo los primeros instantes de la lesión pueden determinar su futura evolución.

La segunda diferencia es la morfología de la cavidad generada. Las lesiones por contusión generan una cavidad cística en la zona donde el proyectil ha impactado. Esta cavidad se ubica en el centro de la médula espinal, afectando principalmente a la sustancia gris y las zonas más concéntricas de la sustancia blanca, de tal forma que la cavidad está rodeada de parénquima medular, incluso por la parte dorsal que es por donde el proyectil ha impactado. En función del peso o de la distancia recorrida por el proyectil, la lesión es más o menos severa, y la cavidad resultante será de mayor o menor tamaño (Wrathall et al., 1985, Gale et al., 1985, Basso et al., 1996a, Khan et al., 1999). En cambio, la cavidad producida por la lesión fotoquímica puede ser abierta o cerrada en función de la severidad de la lesión. En lesiones muy poco severas se produce una cavidad cerrada que se asemeja a la generada con el modelo de contusión, mientras que en lesiones más severas se forma una lesión abierta. En estas últimas, la lesión se inicia desde el extremo dorsal de la médula espinal, de tal forma que la acción de los radicales libres así como el desarrollo de los procesos de lesión secundaria afectan inicialmente a la zona más dorsal de la médula espinal, y a medida que aumenta gradualmente la severidad de la lesión van afectando a áreas medulares más ventrales. Esta expansión dorso-ventral de la lesión puede ser una ventaja, ya que debido a la anatomía y a la distribución concreta de los distintos tractos espinales así como la función que desempeña por cada uno de ellos, pueden ser utilizados como “marcadores funcionales” de la integridad de la médula espinal. De este modo, la expansión dorso-ventral de la lesión va acompañada de una pérdida de las funciones medulares que se ubican en las distintas áreas de la médula espinal. En este sentido, la exclusiva afectación del funículo dorsal abole la propagación de

los potenciales somatosensoriales, la afectación del funículo dorsal y la parte dorsal del funículo lateral abole los potenciales somatosensoriales y motores evocados, mientras que la afectación del funículo dorsal y la totalidad del lateral abole los potenciales evocados espinales e induce pérdidas notorias en el control de la marcha y en la mayoría de las pruebas neurológicas. Por último, la alteración de todo el parénquima medular conduce a la completa paraplejia del animal. En este sentido hay una clara correlación anatómico-funcional y el daño tisular de un tracto o grupo neuronal se pone de relieve con la aparición de déficits funcionales.

Además, el trabajo realizado pone de manifiesto que el uso de ciertas técnicas permiten una discriminación más clara de una función respecto de otras. En este sentido, el uso de técnicas electrofisiológicas, tanto para la conducción central como periférica, permite realizar un análisis más sensible de la actividad funcional de la médula espinal de los animales operados, encontrando diferencias entre grupos que con otras pruebas neurológicas no se encuentran. Estas evidencias concuerdan con las propuestas por Loy y colaboradores (2002) quienes también observan diferencias electrofisiológicas pero no conductuales en animales con lesiones desmielinizantes de los funículos ventrales o laterales. Estos autores atribuyen este fenómeno a la redundancia funcional de los tractos espinales. En tal caso, la afectación de una vía concreta sería compensada por la actividad de las demás, o de forma alternativa, la lesión espinal podría desenmascarar funciones de un tracto que en estado fisiológico no desempeña (Loy et al., 2002). Así pues, el estudio electrofisiológico, mediante el cual se puede estimular tractos espinales concretos, ofrece una herramienta experimental muy útil para el estudio de la función de la médula espinal.

2. Efecto del transplante de glía envolvente en la médula espinal lesionada

Los resultados obtenidos en los diferentes experimentos realizados, tanto a corto como a largo plazo, muestran que el transplante de GE promueve la preservación del tejido medular. En el modelo severo de lesión, en el que se ha afectado la circuitería del patrón central de la locomoción, no se ha observado ninguna mejora conductual ni electrofisiológica de los animales. En cambio en los estudios a largo plazo, el menor tiempo de irradiación añadido a que la lesión se realizó en un segmento torácico medio, ha permitido evaluar el progreso funcional de los animales. De este modo, los animales con transplante de GE han presentado mejoras funcionales en comparación con los animales con inyección de vehículo o con transplante de células de Schwann.

2.1 Propiedades de la glía envolvente

El éxito en la regeneración y/o elongación de las neuronas olfatorias se ha atribuido, en gran parte, a las funciones desarrolladas por la GE, responsable de generar un ambiente permisivo para el crecimiento de los axones tanto por la rama periférica como por la parte central del sistema nervioso (Doucette, 1984). La glía envolvente es un tipo celular propio del sistema olfatorio y que no se encuentra en ninguna otra región del SNC. Son células que envuelven a los axones sensoriales amielínicos desde su origen en el epitelio nasal hasta su terminación en las láminas más externas del bulbo olfatorio. El hecho que sean células presentes tanto en el SNP, formando parte del nervio olfatorio, como del SNC formando parte de la lámina más superficiales del bulbo, las hace poseedoras de una propiedades únicas, ya que la GE comparte características morfológicas y funcionales con las células de Schwann y con los astrocitos. La GE son células muy plásticas, tanto en su morfología como en la expresión de marcadores celulares. El cultivo de GE ha permitido subdividir a la glía en dos subgrupos, las "Schwann-like" y las "astrocyte-like", es decir las células de la GE similares a células de Schwann y las similares a astrocitos, respectivamente (Pixley, 1992). Las células de GE similares a astrocitos ("astrocyte like") se caracterizan por ser células de morfología aplanada, inmunopositivas para el GFAP y PSA-NCAM, pero que no expresan el receptor p75 NTR. En cambio, las similares a células de Schwann ("Schwann-like") son células fusiformes que expresan el receptor p75 NTR, pequeñas cantidades GFAP, pero no las moléculas de adhesión PSA-NCAM (Barber y Lindsay 1982, Pixley 1992, Franceschini y Barnett 1996). Además, la ontogenia de los astrocitos, glía envolvente y células de Schwann es muy diferente, ya que mientras los astrocitos derivan del tubo neural, las células de Schwann derivan de la cresta neural y las células de la GE lo hacen de las placodas olfatorias (Chuah, 1991).

Las funciones que desempeñan son también muy heterogéneas. Como los astrocitos, las células de la GE forman parte de la *glía limitante* del bulbo olfatorio (Barber y Lindsay, 1982), un papel que es exclusivamente desarrollado por los astrocitos en el resto del SNC (Peters et al., 1990). Además, las células de la GE son capaces de envolver a los axones amielínicos, con la particularidad de que una misma célula envuelve a varios haces axonales a la vez. Y se asemejan a las células de Schwann en que pueden adoptar un papel mielinizador similar al desempeñado por estas células, cuando se cultivan con neuronas (Devon y Doucette, 1992) o cuando son transplantadas *in vivo* (Franklin et al., 1996, Imaizumi et al., 1998). Estos resultados sugieren que el fenotipo mielinizador o amielínico que las células de la GE adoptan puede estar determinado por factores sintetizados por los axones (Barber, 1982).

Tanto la presencia de factores de adhesión en la matriz extracelular como la síntesis de factores tróficos y quimiotrópicos son imprescindibles para la correcta elongación de los axones. Estudios realizados tanto *in vivo* como *in vitro*, han mostrado que en el trayecto desde el epitelio nasal hasta las láminas más superficiales del bulbo olfatorio, la GE secreta toda una combinación de estos factores, que la hacen, en gran medida, responsables de guiar y promover el crecimiento de los axones sensoriales (Chuah y West, 2002). De este modo, la GE sintetiza laminina (Treolar et al., 1996), NCAM y L1 (Chuah et al., 1991) y un conjunto de proteoglicanos que están implicados en la fasciculación de los axones (Treolar et al., 1996). La GE juega un papel importante determinando la orientación del crecimiento axonal. En este sentido, estudios recientes han demostrado que las células de la GE expresan semaforina 3A, una molécula que ejerce un papel repulsivo sobre las neuropilinas presentes en los axones sensoriales olfativos (Schwarting et al., 2000). Asimismo, muestran en sus membranas el receptor p75 NTR, que puede actuar como un factor quimiotáctico para la guía de los axones sensoriales (Gong et al., 1994), y un conjunto de receptores de factores solubles quimioatrayentes secretados por el bulbo olfatorio, imprescindibles para la migración y crecimiento de la glía y de los axones sensoriales del epitelio nasal hacia el bulbo olfatorio (Liu et al., 1995). Además, la GE también expresa enzimas relacionados con el metabolismo anabólico de la L-Serina, la cual da soporte para las neuronas (Yamasaki et al., 2001).

Por medio de estudios inmunohistoquímicos y de biología molecular, se ha constatado que las células de la GE son productoras de una amplia variedad de factores tróficos. No sólo los sintetizan y los secretan, sinó que además expresan muchos de sus respectivos receptores, atribuyéndoles una función paracrina y autocrina, así como la posibilidad de unión y presentación de factores tróficos a células vecinas (Chuah y West, 2002). En este sentido, está demostrado que la glía envolvente secreta FGF1 y FGF2 (Chuah y Teague, 1999), PDGF-B (Kott et al., 1994), BDNF, GDNF, Neurturin y GGF2 (Woodhall et al., 2001), NT4/5 (Boruch et al., 2001), varias isoformas del gen de las neurogulinas (NRG-1) (Thompson et al., 2000, Woodhall et al., 2000), y expresan en sus membranas celulares los receptores FGFr1 y perlecan (Chuah y Teague, 1999, Hsu et al., 2001), p75 NGFr, TRK B, GFR-1, GFR-2, el receptor de la neurturina (MTN) (Woodhall et al., 2001) y ErbB-2, ErbB-3 (Salehi-Ashtiani y Farbman, 1996).

No obstante, ni los procesos enzimáticos, ni los factores directamente responsables de promover la regeneración axonal están completamente esclarecidos. Diversos trabajos han demostrado la necesidad por parte de las células de la GE de presentar una elevada concentración intracelular de iones calcio para promover la extensión de neuritas de las raíces dorsales (Sonigra et al., 1999, Hayat et al., 2003). La importancia del calcio en los procesos de crecimiento y plasticidad axonal están ampliamente

fundamentados (Gomez y Spitzer, 1999), y los autores sugieren que, posiblemente la GE posea canales de calcio no dependientes de voltaje, los cuales juegan un papel fundamental en los procesos de promoción de la regeneración (Hayat et al., 2003)

Una de las propiedades más interesantes que la GE posee es la capacidad de migrar del lugar donde ha sido transplantada a otras zonas del SNC y la de integrarse en el parénquima medular lesionado (Boruch et al., 2002, Ramón-Cueto et al., 1998, Li et al., 1998). Estos dos factores, que por ejemplo no comparten las células de Schwann, son imprescindibles para su uso como terapia reparadora, ya que ofrece la seguridad de que las células transplantadas no serán rechazadas ni por tanto eliminadas del parénquima medular y además podrán ejercer su función no sólo en el lugar de transplante sino en zonas alejadas del mismo.

2.2 Transplante de glía envolvente en diferentes modelos de lesión del SNC

Las propiedades exclusivas de la glía envolvente la convierte en una candidata muy atractiva para ser transplantada en zonas del SNC lesionadas, con el propósito de mimetizar las condiciones generadas en el bulbo olfatorio y crear un ambiente favorable para el crecimiento axonal. El primer transplante de GE se realizó en un modelo de avulsión de las raíces dorsales de roedores. Los investigadores encontraron que, a diferencia de los animales no trasnplantados, los axones sensoriales aferentes de los animales con transplante glial, atravesaban barrera astrocítica que conforma la zona de entrada de las raíces dorsales a la médula espinal o "Dorsal Root Entry Zone" (Ramón-Cueto y Nieto-Sampedro, 1994). Electrofisiológicamente también se demostró que los axones sensoriales regenerados realizaban sinapsis funcionales con las motoneuronas diana, de tal forma que restablecían la circuitería refleja dañada por la lesión. Además, por medio de estudios inmunohistoquímicos se evidenció cómo las células transplantadas seguían a los axones regenerativos en su camino hacia el interior del parénquima medular (Navarro et al., 1999). Recientemente, Pascual y colaboradores (2002) han demostrado, utilizando el mismo paradigma experimental, que los animales con el transplante de GE recuperan los reflejos de micción perdidos tras la lesión. Concluyendo que la regeneración axonal inducida por la GE desemboca en el restablecimiento de la función (Pascual et al., 2002).

El transplante de GE también se ha realizado en lesiones parciales y selectivas de la médula espinal. Raisman y colaboradores (1997, 1998) realizaron la lesión selectiva del tracto corticoespinal, y observaron en los animales con transplante celular, cómo los axones corticoespinales regeneraban a través del injerto celular y se extendian caudalmente varios milímetros de la lesión (Li et al., 1997). También evidenciaron que la regeneración del tracto corticoespinal iba acompañada de la recuperación de la función ejercida por las neuronas de dicho tracto, y los animales recuperaban la eficiencia en el test de "food reaching" (Li et al., 1998). Ruitenberg y colaboradores (2003) realizaron la lesión del

tracto rubroespinal, y al transplantar GE modificada genéticamente para expresar BDNF y NT-3, observaron que los axones rubroespinales atravesaban la lesión y que los animales recuperaban la función (Ruitenberg et al., 2003). En lesiones más severas, el transplante celular también ha producido mejoras en los animales. Así, en el modelo de sección completa se ha evidenciado la mejor habilidad locomotora de los animales con transplante de GE, y por medio de estudios histológicos se ha observado la regeneración de axones supraespinales (Ramón-Cueto et al., 1998, Ramón-Cueto et al., 2000). En otra serie de experimentos, Lu y colaboradores (2001, 2002) transplantaron mucosa olfatoria en la medula espinal seccionada y evaluaron si el transplante agudo después de la lesión o el diferido (7 días después) influía en la respuesta de los animales. En ambos trabajos observaron que tanto el transplante agudo como el diferido potenciaban la regeneración de axones supraespinales, y los animales mejoraban el control de la marcha. El papel ejercido por el transplante de GE también se ha estudiado en otras funciones que no fueran la de locomoción. En este sentido Li (2003) realizó una hemisección cervical en los animales y evaluó la recuperación del control de la respiración y de la escalada. Los resultados obtenidos indican que los animales con transplante de GE mejoraron en ambas funciones espinales, evidenciando que la GE permite el restablecimiento funcional de varios sistemas independientes. Asismismo, el efecto del transplante de GE también se ha evaluado en el sistema nervioso periférico. Su transplante en cámaras neurales de silicona permitió la regeneración de axones periféricos a través de un "gap" limitante de 15 mm en el nervio ciático de ratas (Verdú et al., 1999). Guntinas-Lichius y colaboradores (2001) observaron que el transplante de GE en una rama del nervio facial promovía la reinervación muscular por medio de la formación masiva de colaterales y la subsiguiente inervación inespecífica. Estos autores sugieren que la alta colateralización axonal está posiblemente inducida por la amplia secreción de factores tróficos.

La capacidad que la GE posee para envolver y mielinizar los axones sensoriales de una forma similar a cómo lo hacen las células de Schwann abre la posibilidad de utilizar estas células como terapia remielinizadora en enfermedades desmielinizantes como la esclerosis múltiple. Experimentos "in vitro" ya mostraban este papel remielinizador (Devon y Doucette, 1992). Franklin y colaboradores (1996) observaron que al transplantar GE en médulas espinales tratadas con bromuro de etidio, los axones desmielinizados por la lesión eran remielinizados por la glía envolvente transplantada. Estudios posteriores han demostrado que los axones mielinizados con glía envolvente son funcionales y conducen los potenciales de acción, con unas características similares a cómo lo hacen las células de Schwann (Imaizumi et al., 1998).

No obstante, aunque en la mayoría de resultados publicados muestran que los animales con transplante de GE presentan una mejor respuesta que los animales de los grupos control, recientemente

han aparecido varios artículos que ponen en tela de juicio las acciones beneficiosas ejercidas por la GE. En uno de estos trabajos se observó que las GE transplantadas en lesiones en el tronco del encéfalo no promovieron la regeneración axonal. Los autores describen cómo las células transplantadas migraron hacia el soma neuronal, y que posiblemente por este motivo no dieron soporte para la regeneración axonal (Guñido-Cabrera et al., 2000). Recientemente, en un modelo de contusión de la médula espinal, Takami y colaboradores (2002) han observado que el transplante de células de Schwann pero no de GE promueve la recuperación parcial de la habilidad locomotora. Aunque en ambos grupos con transplante celular el área de parénquima medular preservado fue mayor que la observada en los animales control, sólo los animales que recibieron el transplante de células de Schwann mejoraron en la prueba locomotora del campo abierto (Takami et al., 2002). En esta misma línea, Resnick y colaboradores (2003) han encontrado que el transplante de GE en roedores sujetos a una contusión leve no ha influenciado en la respuesta locomotora de los animales, y éstos han presentado los mismos déficits que los animales no tratados.

Estos resultados controvertidos ponen de relieve la necesidad de realizar nuevos estudios tanto *in vitro* como *in vivo* para poder demostrar la relación directa e inequívoca entre la glía envolvente y la neuroprotección y regeneración medular. No obstante, antes de refutar las posibles ventajas de las GE, hay que tener en cuenta dos consideraciones intrínsecas al modelo de transplante y de las propias GE, que pueden influir en los resultados obtenidos. En primer lugar, cada vez van apareciendo más trabajos en los que se evidencia la heterogeneidad celular de la GE. La expresión de marcadores celulares así como la secreción de factores tróficos es específica para cada subpoblación celular, y no sólo entre las diferente zonas del sistema olfatorio (epitelio, nervio y bulbo olfatorio), sinó también dentro de cada uno de estos segmentos (Miragall et al., 1994, Pixley 1992, Au et al., 2003). Por tanto, según el proceso de purificación celular utilizado se aislarán un grupo concreto de GE, cuyas propiedades serán posiblemente diferentes a las del resto, y en último término condicionarán los efectos que ejercerán. El criterio de purificación y selección de la GE no es unánime entre los investigadores, y en consecuencia, existen distintos protocolos para ello. Uno de estos procedimientos consiste en eliminar a las células contaminantes del cultivo primario. Para ello se añaden substancias que matan a las células en división, mayoritariamente fibroblastos, y mantienen las células de la GE intactas (Chuah y Au, 1993). Otros protocolos, en cambio, seleccionan la población de células de la GE de las demás células, ya sea por técnicas de inmunopurificación, por ejemplo seleccionar las células p75 positivas (Ramón-Cueto et al., 1998, Gudiño-Cabrera y Nieto-Sampedro, 1995), o por medio de técnicas de citometría de flujo aislar las células que expresan un marcador concreto, como O4 (Barnett et al., 1993). En cambio, otros investigadores transplantan directamente las células cultivadas, sin hacer ningún proceso de selección

(Li et al., 1998), o a partir de las propiedades de adhesión de las células al cultivo (Nash et al., 2003). Recientemente, se ha descrito que el transplante mixto de GE con células de las meninges que envuelven el bulbo olfatorio permite una remielinización más extensa de axones medulares previamente desmielinizados (Lakatos et al., 2003).

En segundo lugar, tampoco hay un criterio unánime en lo que se refiere a las propiedades del cultivo de GE, tanto en lo que se refiere a la naturaleza de los medios y de los reactivos del cultivo ni a los tiempos para la expansión y purificación celular. Esta posible fuente de variabilidad ha quedado patente en un trabajo en el que se evidencia que la GE cultivada durante más de 15 días entra en un estado de senescencia y disminuyen la expresión de p75 NTR (Rubio et al., 2002). Estos datos ponen de relieve la importancia de las características del cultivo para la biología y las propiedades de las células a transplantar.

En cambio, existen otras variables intrínsecas al experimento que no afectan al resultado final. Por ejemplo, en la presente tesis se ha demostrado que el método de transplante de las células no altera las funciones ejercidas por éstas. Así, el transplante de GE, ya sea por medio de inyecciones de aire comprimido o por medio de una inyección manual continua, promueve la preservación tisular y la recuperación de las habilidades locomotoras de los animales.

2.3 El transplante de glía envolvente promueve la preservación del parénquima medular y el restablecimiento funcional de la médula espinal

En los trabajos descritos anteriormente, la glía envolvente ha sido transplantada en modelos de lesión orientados ha evaluar la regeneración o remielinización inducida en los axones centrales o periféricos. El modelo de lesión fotoquímica utilizado en la presente tesis, ofrece el abordaje de una variable de distinta naturaleza. Al realizar una lesión incompleta no permite evaluar la regeneración axonal, ya que no es posible identificar la causa que provoca la recuperación de la función del animal. Ésta podría ser debida a la preservación, emisión de ramas colaterales o regeneración de los axones centrales. En cambio, la lesión fotoquímica nos ha permitido evaluar el posible papel neuroprotector ejercido por el transplante celular, ya que al producir lesiones que desatan las cascadas de lesión secundaria, se afecta a la estructura y función medular. Si el transplante glial, o cualquier otra terapia experimental consigue reducir o mitigar los efectos de la lesión secundaria, el parénquima medular estará más preservado y posiblemente el animal poseerá mayor control sensoriomotor. Otros estudios también han utilizado modelos de contusión en los que de forma similar, se ha generado una cavidad que ha afectado heterogéneamente a los distintos tractos espinales y en los que no se ha evaluado la regeneración sino la neuroprotección (Takami et al., 2002, Plant et al., 2002).

La promoción de la regeneración axonal así como la neuroprotección ejercida en el parénquima medular por el transplante de GE puede ser debido a que estas células gliales sean capaces de modular la reactividad de los astrocitos y de las células de la microglía endógena subyacente a toda lesión medular, o que sean capaces de modular la plasticidad sináptica de las neuronas lesionadas, o a ambas cosas a la vez.

La integración en el parénquima medular lesionado y la secreción de factores tróficos, así como de otras muchas moléculas aún por descubrir, producen la modulación del ambiente glial. En los trabajos realizados tanto a corto como largo plazo, atribuimos la mayor preservación de parénquima medular en los animales transplantados a la reducción de la reactividad astrocitaria, evaluada a partir del grado de hipertrofia celular, ya sea por medio de la evaluación de la expresión de GFAP, o de la expresión de proteoglicanos. Hemos constatado que en las secciones medulares de los animales no tratados o de aquellos que recibieron un transplante de células de Schwann la expresión de estos marcadores es mucho mayor que en las de los animales con transplante de GE.

Tras una lesión del SNC, el papel desarrollado por los astrocitos es claramente bipolar, promoviendo tanto la estabilización y preservación del tejido lesionado, como promoviendo un ambiente citotóxico e inhibitorio para la supervivencia y regeneración axonal (Eddleston 1993, David y Lacroix 2003, Benveniste 1993). Las causas que conducen a un astrocito a adoptar una u otra respuesta no están esclarecidas, y posiblemente tanto las características propias del astrocito, como las propias del ambiente generado tras la lesión influirán notoriamente en su respuesta. Es por ello que la actuación directa o indirecta sobre el astrocito puede conseguir desplazar la balanza hacia un lado u otro.

En las capas más superficiales del bulbo olfatorio, los astrocitos y la GE son los responsables de crear un ambiente glial especial, favorable para el crecimiento y regeneración axonal. El hecho de que ambos tipos celulares desarrollen un papel tan destacado abre la posibilidad de que existan señales de comunicación entre ambas poblaciones gliales. Esta hipótesis podría explicar que ambos tipos de células sean las únicas del sistema nervioso que expresan la proteína nexina-1 (Reinhard et al., 1988). Estudios *in vitro* sugieren que esta proteína realiza una función quimiotrópica para el crecimiento de neuritas (Zurn et al., 1988). Además, tanto las células de la GE como los astrocitos expresan conexina 43, lo que abre la posibilidad de que haya una comunicación entre ambos tipos celulares por medio de las uniones intercelulares de tipo “GAP junctions” (Miragall et al., 1992).

Diferentes estudios muestran que la relación entre los astrocitos y la GE no sólo se da en situaciones fisiológicas, sinó que lesiones experimentales del nervio o bulbo olfatorio ponen de manifiesto esta posible comunicación. Al realizar una lesión del bulbo olfatorio, los astrocitos desatan rápidamente su reacción hipertrófica y sellan la herida producida. Pero la cicatriz formada es

temporalmente transitoria, y a los pocos días, los astrocitos vuelven a adoptar su morfología normal permitiendo el paso a los axones en crecimiento (Anders y Johnson, 1990). En un estudio posterior, estos mismos autores compararon la regeneración de las neuronas olfatorias en un grupo de animales en los que se les realizó una axotomía del nervio olfatorio a nivel de la placa cribiforme con un segundo grupo de animales a los cuales se realizó la misma lesión y luego se les transplantó, en la zona de la sección, una pieza de tejido gliótico maduro obtenido de animales a los que se les había realizado una lesión del nervio sesenta días antes del transplante (Anders y Hurlock, 1996). Los estudios histológicos con trazadores mostraron que a las tres semanas después de la sección del nervio olfatorio los axones del epitelio nasal habían cruzado la zona lesionada y habían regenerado hasta el bulbo. En cambio, en los animales con el transplante del tejido gliótico, ningún axón sensorial consiguió cruzar la zona de lesión ni reinervar el bulbo. A partir de estos resultados, los autores sugieren que la glía envolvente puede modular la formación de la cicatriz glial, haciendo que los astrocitos sean temporalmente reactivos. Hay que añadir que probablemente, una vez la cicatriz glial está formada y es madura, no hay posible modulación por parte de la glía envolvente por lo que se hace impermeable para la regeneración axonal.

Estudios *in vitro* han evidenciado la comunicación entre glía envolvente y astrocitos de otras zonas del sistema nervioso central que no son el bulbo olfatorio. En cultivos de glía envolvente del bulbo olfatorio se observó que el sobrenadante obtenido de cultivos de astrocitos corticales inducía su proliferación y supervivencia. Seguidamente se purificó una de las moléculas responsables de ejercer estos efectos en las GE y se las identificó como neuregulinas (Pollock et al., 1999). Recientemente en estudios de cocultivos de GE y astrocitos, y células de Schwann y astrocitos se observó como las células de Schwann inducían la hipertrofia y aumento de la expresión de proteoglicanos en los astrocitos. En cambio, el cultivo de astrocitos y de GE no aumentaba la reactividad glial ni la expresión de proteoglicanos en estas células y además permitía la migración de la glía envolvente (Lakatos et al., 2000).

El transplante de GE es capaz de modular la reactividad de los astrocitos medulares. Los resultados obtenidos en esta tesis, así como los obtenidos por otros autores confirman este hecho (Lakatos et al., 2000, Takami et al., 2002). Estos resultados sugieren varias conclusiones atractivas. En primer lugar indican que la glía envolvente del bulbo olfatorio es capaz de modular la reactividad de astrocitos que no forman parte del bulbo olfatorio, sino de aquellos que son de la médula espinal. Esto sugiere que la glía puede tener un peso específico más importante que el astrocito del bulbo para promover la regeneración. La segunda idea concierne a la ventana de modulación. Mientras que en nuestros experimentos el transplante de células de GE fue agudo, los transplantes realizados por

Takami y colaboradores (2002) son siete días depués de realizar la lesión. Sin embargo, en ambos casos se observa una reducción de la reactividad astrocitaria. Esto sugiere que hay una ventana terapéutica, que se inicia en el mismo momento en que ocurre la lesión, y que se mantiene como mínimo sietes días después de la misma. Durante este periodo se inicia la modulación de la reactividad astrocitaria, la cual se mantiene a lo largo del tiempo. Por último en los diferentes estudios realizados, hemos observado cómo la reactividad astrocitaria estaba modulada en la propia zona de lesión o en regiones próximas a éstas, indicando que los efectos del transplante no sólo se circunscriben a la zona de lesión sino que puede abarcar áreas más extensas del parénquima medular.

Los resultados electrofisiológicos obtenidos en los estudios a largo plazo sugieren que el transplante de GE modula la onda H, el análogo eléctrico del reflejo de estiramiento, de la musculatura distal de las extremidades posteriores. Mientras que en los animales control (inyección de DMEM) y en aquellos con transplante de células de Schwann la amplitud de esta onda aumentó después de la lesión y se mantuvo, a lo largo de todo el seguimiento, muy por encima de los valores preoperatorios, en los animales con transplante de GE la amplitud de la onda H aumentó, pero gradualmente volvió a los valores preoperatorios.

Hasta la actualidad, el único trabajo en el que se hace referencia a una modulación sináptica ejercida por el transplante de GE es el llevado a cabo por Lu y colaboradores (2001), quienes realizaron una sección de la médula espinal, y transplantaron piezas de epitelio mucoso en la zona de lesión. Los investigadores encontraron que los reflejos de estiramiento registrados en los animales con transplante eran similares a los obtenidos en animales no operados, ya que al estimular repetidamente el nervio periférico, la señal refleja registrada se abolía rápidamente, fenómeno característico de animales sanos y que no ocurría en los animales sin transplante. Estudios histológicos posteriores mostraron cómo fibras serotoninérgicas de origen supraespinales cruzaban la zona lesionada con transplante. Los autores atribuyen al control supraespinal conseguido, el restablecimiento de la actividad refleja normal en los animales (Lu et al., 2001)

En nuestro modelo de lesión parcial de la médula espinal no podemos valorar el restablecimiento del control reflejo mediado por la regeneración de axones del tronco del encéfalo o del cortex motor, ya que los axones que presentan continuidad en la médula tanto pueden ser regenerativos como que hayan sobrevivido a la lesión. Es por ello que establecemos tres posibles hipótesis para explicar los resultados obtenidos.

La primera es aquella en la que si las células de la GE ejercen un papel neuroprotector, los animales con el injerto celular presentarán una mayor preservación de fibras descendentes y ascendentes, por lo que tendrán un mayor control sobre las motoneuronas e interneuronas lumbares.

Está demostrado que la pérdida de control supraespinal sobre las aferencias Ia (Calancie et al., 1993), así como la sección de las columnas dorsales (Decima et al., 1986) o cambios en la excitabilidad de las motoneuronas (Taylor et al., 1984) desembocan en las hiperreflexia y en último término en la espasticidad muscular (Hiersemenzel et al., 2000).

En segundo lugar puede ser que si las células de la GE son capaces de migrar por el parénquima medular, y son capaces de secretar neurotrofinas y otras moléculas de señalización, cabe la posibilidad de que éstas ejerzan una modulación o remodelación de las funciones de las motoneuronas y de los circuitos espinales. Existen evidencias experimentales que sugieren que las neurotrofinas regulan la expresión de canales iónicos, influyen en la excitabilidad de la membranas, modulan la transmisión sináptica y regulan cambios en la estructura axonal y de las dendritas (Mc Allister, 1999).

En tercer lugar, hay una hipótesis que propone que la pérdida de control supraespinal inducida por una lesión medular, puede ser contrarrestada por cambios a nivel de la estructura segmentaria de la médula espinal. De tal forma que la formación de ramas colaterales de los axones sensoriales aferentes de las raíces dorsales, harían aumentar el “input” sensorial sobre las motoneuronas, potenciando el desarrollo de reflejos con el subsiguiente restablecimiento parcial de la locomoción (Helgren and Goldberg, 1993). El aumento de la onda H podría reflejar esta situación (Cope et al., 1988). El transplante de GE, a diferencia de lo que sucede en el sistema nervioso periférico, podría limitar la formación de colaterales de los axones sensoriales aferentes, situación descrita previamente por Li y colaboradores (1998), por tanto mitigar el aumento de los regflejos.

Por último se debe recalcar que, el rol neuroprotector que atribuimos a las células de la glía envolvente en el modelo de lesión fotoquímica, no se contradice con las propiedades promotoras de la regeneración o remielinizantes atribuidas a estas céluals. Cabe pensar que la GE desarrolla todas estas funciones de forma natural en el sistema olfatorio. Es característico del sistema olfatorio la continua dinámica entre los procesos de degeneración y de nacimiento de nuevas neuronas. Para que estos procesos lleguen a buen fin, y las posibles secuelas de uno no afecten al otro ni al sistema en general, debe existir una precisa concatenación de los eventos celulares. De tal forma que, una vez que las neuronas sensoriales han muerto tendrán que ser rápidamente eliminadas, tal y como sucede en el sistema nervioso periférico. Además es posible que existan mecanismos que limiten el ambiente lesivo formado por la muerte accidental de las neuronas, y que inhiban los procesos lesivos, característicos de las lesiones del SNC que conducirían al aumento de la lesión. Es posible que la glía envolvente del bulbo olfatorio en conjunción con los demás tipos celulares presentes realice esta función, por medio del soporte trófico necesario para no agravar la lesión particular y para proteger a las neuronas de los productos lesivos originados por la muerte de células vecinas.

Los resultados obtenidos en la presente tesis permiten avanzar en el estudio del transplante de glía envolvente del bulbo olfatorio para conseguir el ansiado restablecimiento de la función de la médula espinal lesionada. No obstante, serán necesarios futuros estudios, tanto *in vitro* como *in vivo*, que permitan elucidar los mecanismos moleculares responsables de la neuroprotección ejercida, que identifiquen las señales responsables de la comunicación entre las células de la glía envolvente y los astrocitos u otras células endógenas medulares, así como la interacción con la circuiteria propioespinal y supraespinal. También será imprescindible estudiar los cambios, si es que lo sufren, de las células de la GE al ser transplantadas, así como será necesaria la optimización de los protocolos de transplante, en lo que se refiere a población celular concreta, momento y lugar de transplante y concentración celular idónea, para obtener los resultados más óptimos.

CONCLUSIONES

1.- La lesión fotoquímica de la médula espinal, basada en la aplicación tópica de rosa de bengala y la subsiguiente irradiación con una fuente de luz fría, produce una lesión en el parénquima medular, cuyos efectos son similares a los producidos por otros modelos experimentales de lesión traumática, y cuya severidad es graduable en función del tiempo de fotoactivación. Además, se ha evidenciado la importancia de la ubicación anatómica de la lesión medular, ya que se ha constatado que los animales con lesiones en los segmentos espinales lumbares altos (L2) presentan mayor déficit motor que los animales con lesiones similares en los segmentos torácicos medios (T8).

2.- Los estudios a corto plazo (15 dpo), en los que se ha transplantado GE en una lesión fotoquímica severa de 5 minutos de irradiación, indican que las células gliales ejercen un papel neuroprotector en la médula espinal, ya que los animales con transplante presentan una mayor preservación de parénquima medular en comparación de los animales sin transplante. Una de las posibles causas efectoras de la neuroprotección es la disminución de la hipertrofia y de la expresión de proteoglicanos de los astrocitos medulares de los animales con transplante. Sugiriendo que las GE modulan la reactividad astrocitaria, y que en consecuencia, modulan el ambiente glial formado tras la lesión.

3.- Los estudios realizados a largo plazo (90 dpo) corroboran los resultados obtenidos a corto plazo, ya que tras una lesión fotoquímica leve, los animales con transplante de GE preservan más tejido medular y presentan menor reactividad glial, así como una mejor respuesta neurológica y electrofisiológica que los animales del grupo control. Además se ha evidenciado como el procedimiento de transplante, ya sea por inyección gradual con una aguja Hamilton, o bien, por inyecciones pulsátiles consecutivas con una bomba de aire, no influyen en el papel efector de las células transplantadas.

4.- La comparación a largo plazo de los efectos del transplante de GE y de SCs en una lesión fotoquímica leve indica que ambos tipos celulares ejercen un papel neuroprotector de la médula espinal, y los animales de ambos grupos presentaron una mejor respuesta de las habilidades conductuales que los del grupo control. No obstante, los animales con GE presentaron una mejor respuesta electrofisiológica que los animales con SCs y control. Asimismo, los resultados histológicos muestran que el transplante de GE reduce la hipertrofia astrocitaria y la expresión de proteoglicanos en comparación con los animales con transplante de SCs y control y mantienen la conectividad de las vías descendentes espinales.

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