BRAIN RESEARCH 1426 (2011) 54–72

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Olfactory and respiratory lamina propria transplantation after spinal cord transection in rats: Effects on functional recovery and axonal regeneration

Lígia Aline Centenaro[a,b], *Mariane da Cunha Jaeger*[b], *Jocemar Ilha*[a,b], *Marcelo Alves de Souza*[c], *Pedro Ivo Kalil-Gaspar*[b], *Núbia Broetto Cunha*[a,b], *Simone Marcuzzo*[a,b], *Matilde Achaval*[a,b,*]

[a]*Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500, CEP: 90050-170, Porto Alegre, RS, Brazil*

[b]*Laboratório de Histofisiologia Comparada, Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500, CEP: 90050-170, Porto Alegre, RS, Brazil*

[c]*Laboratório de Neuroendocrinologia do Comportamento, Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500, CEP: 90050-170, Porto Alegre, RS, Brazil*

ARTICLE INFO

Article history:
Accepted 23 September 2011
Available online 1 October 2011

Keywords:
Spinal cord injury
Olfactory lamina propria
Respiratory lamina propria
Olfactory ensheathing cell
Delayed transplantation
Hindlimb motor function

ABSTRACT

Spinal cord injury (SCI) has very poor clinical prospects, resulting in irreversible loss of function below the injury site. Although applied in clinical trials, olfactory ensheathing cells transplantation (OEC) derived from lamina propria (OLP) is still a controversial repair strategy. The present study explored the efficacy of OLP or respiratory lamina propria (RLP) transplantation and the optimum period after SCI for application of this potential therapy. Adult male rats were submitted to spinal cord transection and underwent acute, 2-week or 4-week post-injury transplantation with pieces of OLP (containing OECs) or RLP (without OECs). After grafting, animals with OLP and RLP showed discrete and similar hindlimb motor improvement, with comparable spinal cord tissue sparing and sprouting in the lesion area. Acute transplantation of OLP and RLP seems to foster limited supraspinal axonal regeneration as shown by the presence of neurons stained by retrograde tracing in the brainstem nuclei. A larger number of 5-HT positive fibers were found in the cranial stump of the OLP and RLP groups compared to the lesion and caudal regions. Calcitonin gene-related peptide fibers were present in considerable numbers at the SCI site in both types of transplantation. Our results failed to verify differences between acute, 2-week and 4-week delayed transplantation of OLP and RLP, suggesting that the limited functional and axon reparative effects observed could not be exclusively related to OECs. A greater understanding of the effects of these tissue grafts is necessary to strengthen the rationale for application of this treatment in humans.

* *Corresponding author at:* Laboratório de Histofisiologia Comparada, Instituto de Ciências da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500, CEP: 90050-170, Porto Alegre, RS, Brazil. Tel.: +55 51 33083624; fax: +55 51 33083092.
E-mail address: achaval@ufrgs.br (M. Achaval).

Abbreviations: 2WDC, 2-Week Delayed Control; 2WDT, 2-Week Delayed Treated; 4WDC, 4-Week Delayed Control; 4WDT, 4-Week Delayed Treated; AC, Acute Control; AT, Acute Treated; BBB, Basso, Beattie and Bresnahan Scale; FG, Fluorogold; OEC, Olfactory Ensheathing Cells; OLP, Olfactory Lamina Propria; RLP, Respiratory Lamina Propria; SCI, Spinal Cord Injury

0006-8993/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2011.09.054

1. Introduction

Spinal cord injury (SCI) results in loss of central control of motor, sensorial, and autonomic functions below the site of injury (van den Berg et al., 2010). Despite the application of neuroprotective treatments, such as methylprednisolone or interleukin-10, the clinical prospects for spinal cord lesions are currently very poor (Fitch and Silver, 2008; Takami et al., 2002b). Functional disabilities occur due to local neuronal death and loss of ascending and descending axons in the spinal cord, either by direct trauma or secondary damage (Hausmann, 2003; Ramer et al., 2005).The hostile environment produced by glial scarring, the presence of inhibitory molecules associated with oligodendrocyte myelin and inadequate neurotrophin supply are responsible for impaired regeneration of severed axons after SCI (Franssen et al., 2007).

In attempts to provide a cellular milieu appropriate for axonal regrowth and the restoration of lost neural circuits, several primary cell types have been used in transplantations into the SCI site (Kwon and Tetzlaff, 2001; Tetzlaff et al., 2011). The olfactory system has attracted considerable interest as a promising source of cells for transplantation after SCI, because of its capacity for lifelong regeneration (Lindsay et al., 2010). The main focus of attention in the olfactory tissue has been a unique type of glia, known as the olfactory ensheathing cells (OECs) (Doucette, 1991; Raisman, 2001; Ramón-Cueto and Muñoz-Quiles, 2011). These cells reside within the two main regions of the olfactory axis: peripherally, in the lamina propria and centrally, along the nerve fiber layer of the olfactory bulb (OB) (Au and Roskams, 2003). The OECs are responsible for maintaining an environment which favors neurite outgrowth and the creation of new functional synapses in the central nervous system (Au and Roskams, 2003; Franssen et al., 2007).

Due to their supposed axon regenerative properties, OECs have been extensively studied in animal models of SCI. Although some research has shown locomotor and axonal regeneration improvements, a consensus on the efficacy of this cellular transplantation and mode of action has yet to be reached (Barnett and Riddell, 2007; Boyd et al., 2004; Franssen et al., 2008; Kubasak et al., 2008; Raisman and Li, 2007; Ramón-Cueto and Avila, 1998; Ramón-Cueto et al., 1998, 2000; Tetzlaff et al., 2011). The source of OECs for transplantation into injured spinal cord is also subject of debate (Richter et al., 2005). However, the use of olfactory lamina propria (OLP) grafts, which is a more accessible source of OECs in humans, could enable a safer approach for autologous transplantation (Bianco et al., 2004; Féron et al., 1998; Franklin, 2002).

The devastating prognosis associated with the social and economic impacts, has led to increased efforts to find therapies that provide functional recovery for people who undergo severe SCI (Blight, 2002; van den Berg et al., 2010). According to previous studies, the use of OLP transplantation is a promising, though controversial, repair strategy (Lu et al., 2001, 2002; Steward et al., 2006). In the present study we hypothesized that the OECs present in OLP grafts could create a favorable glial environment that would favor neurite and axonal outgrowth after thoracic spinal cord transection in rats. Thus, OLP transplantation could produce higher levels of hindlimb motor recovery when compared to respiratory lamina propria (RLP), which is a graft devoid of OECs. Additionally, we tested the efficacy of OLP transplantation in three different therapeutic windows (acutely, 2 weeks and 4 weeks post-injury), since another key aspect in the translation of this therapy to clinical practice is their potential to produce axonal regeneration even when transplantation is delayed after SCI.

2. Results

2.1. *Hindlimb motor function*

Fig. 1 illustrates average Basso, Beattie, and Bresnahan scale scores (BBB) before and across the post-injury survival interval for the experimental groups. Prior to the injury (naive test), no differences were observed in the average of inter-group BBB scores and the animals showed normal locomotor activity (scored as 21). By contrast, at 5 days post-injury (test 1) there was a complete flaccid paralysis of both hindlimbs movements in most animals and BBB scores were 0.21±0.09 for the acute control group (AC), 0.23±0.16 for the acute treated group (AT), 0.18±0.09 for the 2-week delayed control group (2WDC), 0.21±0.09 for the 2-week delayed treated group (2WDT), 0.16±0.09 for the 4-week control group (4WDC), 0.41±0.37 for the 4-week treated group (4WDT) (mean±SEM). Instead of the slight recovery of motor skills observed from 20 days after SCI to the end of this study, there were no differences between the average BBB scores obtained at any time point comparing acute, 2-week or 4-week OLP transplanted groups with their respective RLP control groups (one-way repeated measures ANOVA; acute groups $F_{(1,20)}=0.13$, $p>0.05$; 2-week delayed groups $F_{(1,22)}=1.66$, $p>0.05$; 4-week delayed groups $F_{(1,22)}=0.11$, $p>0.05$). In the last functional test, the BBB scores were 3.5±0.9 for the AC group; 2.7±0.5 for the AT group; 2.6±0.4 for the 2WDC group; 3.0±0.6 for the 2WDT group; 2.6±0.6 for the 4WDC group and 2.0±0.4 for the 4WDT group. No differences were found when data from the last functional test were compared between all the studied groups (one-way ANOVA $F_{(5,65)}=0.57$, $p>0.05$).

2.2. *Spinal tissue sparing and sprouting*

Analysis of the glial fibrillary acidic protein (GFAP) immunoreactive sections revealed a variation in the morphology of the lesion sites among the experimental groups: some rats displayed transparent cavities that separated their spinal cord stumps, while others contained smaller cavities. The preserved tissue area, determined by the presence of healthy looking cells and GFAP immunoreactivity, was measured to quantify the repair effects produced by OLP or RLP transplantation. Although no significant differences were found between the groups (one-way ANOVA $F_{(5,21)}=0.75$, $p>0.05$), the AT and 4WDT groups presented higher levels of spinal tissue sparing (488.7±101.1; 613.2±77.1, respectively) when compared to their respective controls, the AC and 4WDC groups (303.1±77.3; 414.8±96.4, respectively). The 2-week delayed transplantation of both lamina propria grafts seems to promote similar spinal tissue sparing levels (450.9±123.2; 478.6±120.9 respectively) (Fig. 2A).

The presence of sprouting axons was indicated by growth associated protein-43 (GAP-43) immunoreactivity at the SCI site of the groups (AC—0.1±0.0; AT—0.2±0.0; 2WDC—0.1±0.0; 2WDT—0.1±0.0; 4WDC—0.1±0.0; 4WDT—0.2±0.0). The

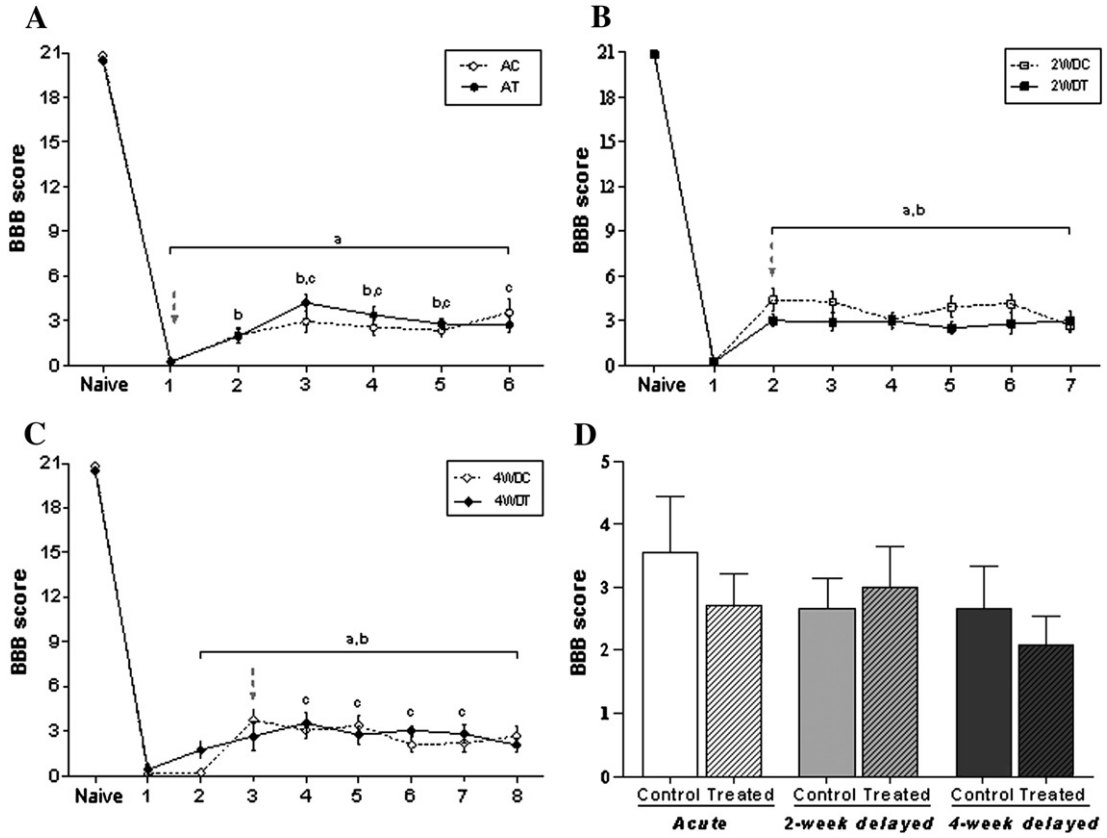


Fig. 1 – (A) BBB scores before (naive) and postoperatively at days 5, 20, 35, 50, 65, 80 (1–6 tests) after spinal cord transection and acute OLP or RLP transplantation. a—$p<0.001$ for AC and AT groups compared to naive; b—$p<0.05$ for AC group compared to test 1; c—$p<0.05$ for AT compared to test 1. (B) BBB scores before (naive) and postoperatively at days 5, 20, 35, 50, 65, 80, 95 (1–7 tests) after spinal cord transection and 2-week delayed OLP or RLP transplantation. a—$p<0.001$ for 2WDC and 2WDT groups compared to naive; b—$p<0.01$ for 2WDC and $p<0.05$ for 2WDT compared to test 1. (C) BBB scores before (naive) and postoperatively at days 5, 20, 35, 50, 65, 80, 95, 110 (1–8 tests) after spinal cord transection and 4-week delayed OLP or RLP transplantation. a—$p<0.001$ for 4WDC and 4WDT groups compared to naive; b—$p<0.05$ for 4WDC compared to test 1; c—$p<0.05$ for 4WDC group compared to test 1. (D) BBB final scores of each experimental group after spinal cord transection and 42 days after OLP or RLP transplantation at different times. No differences were found when all experimental groups were compared ($p>0.05$). Arrows indicate the time at which the experimental groups underwent OLP or RLP transplantation. Abbreviations: 2WDC—2-Week Delayed Control; 2WDT—2-Week Delayed Treated; 4WDC—4-Week Delayed Control; 4WDT—4-Week Delayed Treated; AC—Acute Control; AT—Acute Treated; BBB—Basso, Beattie, and Bresnahan scale; OLP—Olfactory Lamina Propria; RLP—Respiratory Lamina Propria.

optical densitometry for this protein showed no differences when comparing acute, 2-week or 4-week OLP transplanted groups with their respective RLP controls (one-way ANOVA $F_{(5,25)}=0.64$, $p>0.05$) (Figs. 2B, C, D).

2.3. *Retrograde tracer labeled cells*

Few fluorogold (FG) positive neurons were found in the brain areas of the animals that received OLP and RLP transplantation (Table 1). The observed labeled cell bodies were comparable in size and appeared in clusters, as previously described by Vavrek et al. (2007).

The primary and secondary motor cortices (M1/M2) did not exhibit labeled neurons in any groups. Only one animal, from the AC group, was found to have FG-positive neurons in the primary somatosensory cortex (S1). In the brainstem, animals from the AC group showed labeled neurons in the spinal vestibular nucleus (SpVe), lateral vestibular nucleus (LVe), caudal and rostral pontine reticular nuclei (PnO/PnC) and animals from the AT group exhibited FG-positive neurons in the dorsal and ventral medullary reticular fields (MdD/MdV), raphe nuclei (Ra), SpVe, LVe and PnO/PnC nuclei. In the 2-week delayed groups, FG-labeled neurons were observed in the LVe nuclei of the 2WDC group and in the PnO/PnC of the 2WDT group. The 4WDC group exhibited few FG-positive neurons in the LVe nuclei, while no labeled neurons were observed in any studied areas of the 4WDT group (Table 1).

2.4. *Serotonin (5-HT) and calcitonin gene-related peptide (CGRP) pattern fibers in caudal, rostral and lesion areas*

Animals transplanted with both types of lamina propria had most evident 5-HT immunostained fibers in the rostral stump of longitudinal spinal cord sections (AC—0.9±0.2; AT

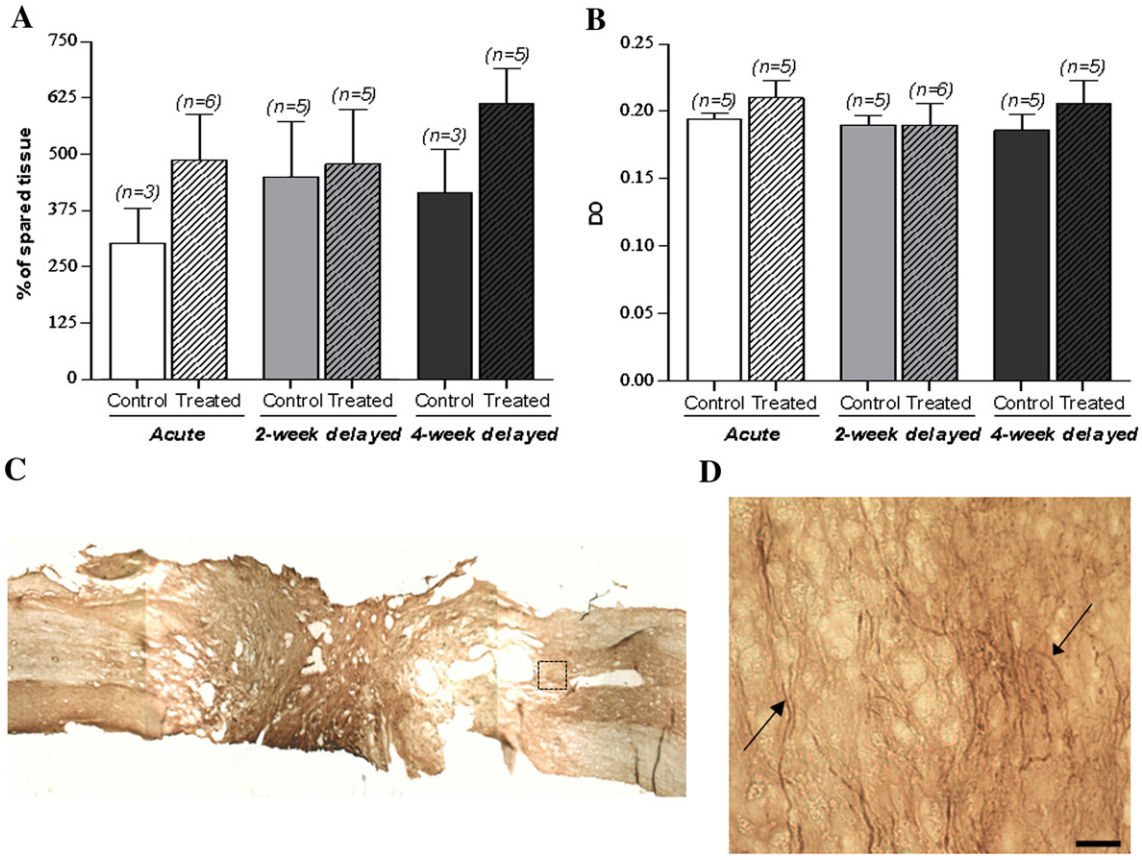


Fig. 2 – (A) Spinal cord spared tissue immunostained with GFAP in animals submitted to spinal cord transection and olfactory or respiratory lamina propria transplantation. No differences were found between groups ($p > 0.05$). (B) GAP-43 immunoreactivity in the lesion region of injured rats transplanted with both types of lamina propria. No differences were found between groups ($p > 0.05$). (C) Representative longitudinal spinal cord section immunostained by GAP-43, showing the lesion site. (D) Higher magnification of the highlighted box, showing GAP-43 positive sprouting axons (arrows). Magnification—100×, scale bar—100 μm. Abbreviations: GAP-43—Growth Associated Protein-43; GFAP—Glial Fibrillary Acidic Protein.

—1.0±0.5; 2WDC—0.5±0.3; 2WDT—0.4±0.0; 4WDC—0.7±0.2; 4WDT—0.6±0.0). A GFAP negative region delineated the injured area in the spinal cord and, in most animals, 5-HT fibers did not extend beyond the vicinity of the lesion border (AC—0.00±0.0; AT—0.01±0.0; 2WDC—0.00±0.0; 2WDT—0.01±0.0; 4WDC—0.03±0.0; 4WDT—0.02±0.0). Moreover, in the majority of slices analyzed, no 5-HT labeled axons were found in the caudal stump (AC—0.00±0.0; AT—0.00±0.0; 2WDC—0.01±0.0; 2WDT—0.01±0.0; 4WDC—0.00±0.0; 4WDT—0.00±0.0) (Figs. 3, 4). No differences were detected in the 5-HT immunoreactivity of the rostral, lesion and caudal regions of spinal cord when all groups were compared (Kruskal–Wallis $p=0.37$; $p=0.73$; $p=0.34$, respectively) (Fig. 6).

As expected, ascending sensory fibers immunostained by CGRP were detected in the caudal stump of the experimental groups (AC—1.27±0.3; AT—1.08±0.3; 2WDC—1.42±0.6; 2WDT—1.42±0.8; 4WDC—0.87±0.2; 4WDT—1.10±0.2). There were considerable levels of CGRP fibers in the lesion region (AC—1.71±0.4; AT—1.37±0.3; 2WDC—0.88±0.2; 2WDT—1.19±0.1; 4WDC—0.85±0.2; 4WDT—1.75±0.5), showing that both OLP and RLP transplantation stimulated the growth/sprouting of CGRP fibers in animals submitted to SCI. In the rostral stump, CGRP immunoreactive fibers were also detected in all groups, but particularly in the AT and 2WDC groups (AC—1.56±0.9; AT—3.58±2.1; 2WDC—4.10±3.0; 2WDT—1.40±0.6; 4WDC—1.79±0.9; 4WDT—1.29±0.5) (Figs. 3, 5). No differences in the rostral, lesion and caudal regions of the spinal cord were observed comparing the groups (Kruskal–Wallis $p=0.97$; $p=0.25$; $p=0.90$, respectively) (Fig. 6).

3. Discussion

The hindlimb motor recovery and axonal growth-promoting effects of OECs, OLP and olfactory mucosa have been studied in different models of spinal cord lesions (see Table 2). In the present study, we have investigated and compared the restorative efficiency of OLP and RLP transplants, in three different therapeutic windows (acutely, 2-week and 4-week delayed), after a complete thoracic spinal cord transection in adult rats. By the twelfth week after transplantation, animals with OLP or RLP showed a discrete and similar hindlimb motor improvement. All transplants produced comparable results for spinal cord tissue sparing and sprouting, evaluated using GFAP and GAP-43

Table 1 – Number and location of fluorogold retrogradely stained cells in the individual rats ($n=3$)

	MdV/ MdD	Ra	SpVe	LVe	LC	PnC/ PnO	M1/ M2	S1
Acute control	0	0	6	25	0	16	0	0
	0	0	0	7	0	0	0	32
	0	0	0	0	0	0	0	0
Acute treated	15	0	3	16	0	4	0	0
	4	5	12	19	0	7	0	0
	0	0	0	0	0	12	0	0
2 weeks delayed control	0	0	0	3	0	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	6	0	0	0	0
2 weeks delayed treated	0	0	0	0	0	0	0	0
	0	0	0	0	0	31	0	0
	0	0	0	0	0	0	0	0
4 weeks delayed control	0	0	0	0	0	0	0	0
	0	0	0	6	0	0	0	0
	0	0	0	0	0	0	0	0
4 weeks delayed treated	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0

Note the absence of labeling cells in *LC* and M1/M2. MdV/MdD—Ventral and Dorsal Medullary Reticular Fields; PnO/PnC—Caudal and Rostral Pontine Reticular Nuclei; Ra—Raphe Nuclei; SpVe—Spinal Vesribular Nucleus; LVe—Lateral Vestibular Nucleus, LC—*Locus Coeruleus*; M1/M2—Primary and Secondary Motor Cortex; S1—Primary Somatosensory Cortex.

immunohistochemistry. Acute transplantation of OLP and RLP seems to foster some limited supraspinal axonal regeneration, as indicated by the presence of cells stained by retrograde tracing in brainstem nuclei. However, retrogradely labeled cells in cortical areas were only observed following acute RLP transplantation. A larger number of 5-HT positive fibers were found in the cranial stump of the OLP and RLP groups compared to the lesion and caudal regions analyzed. CGRP fibers were present in considerable number at the SCI site in both transplantation types.

Although the mechanisms underlying the regenerative properties of OECs in the SCI site are not completely elucidated, reduction of glial scarring (Lu et al., 2006), facilitation of axon re-entry into the host–graft interface (Li et al., 2005), reduction of proteoglycan expression (García-Alías et al., 2004), angiogenesis (Richter et al., 2005), remyelination (Sasaki et al., 2006) and growth-factors release (Lipson et al., 2003) are considered the main benefits of this cell transplantation (Tetzlaff et al., 2011). We were able to detect the presence of OECs in the lamina propria before and after grafting in the transection site, but the limitations of our study were the lack of the OECs quantification and the inability to investigate the possible migratory properties of these cells after transplantation. Nevertheless, some aspects of OECs behavior after transplantation have been previously documented. In an olfactory nerve injury, OECs were seen to remain at the lesion site forming a conduit that can guide regenerating nerve axons, analogously to Schwann cells after a peripheral nerve injury (Li et al., 2005; Williams et al., 2004). After a cervical spinal cord injury model, Lu et al. (2006) failed to demonstrate any unique migratory properties of OECs, concluding that these cells probably spread due to pressure at the injection site,

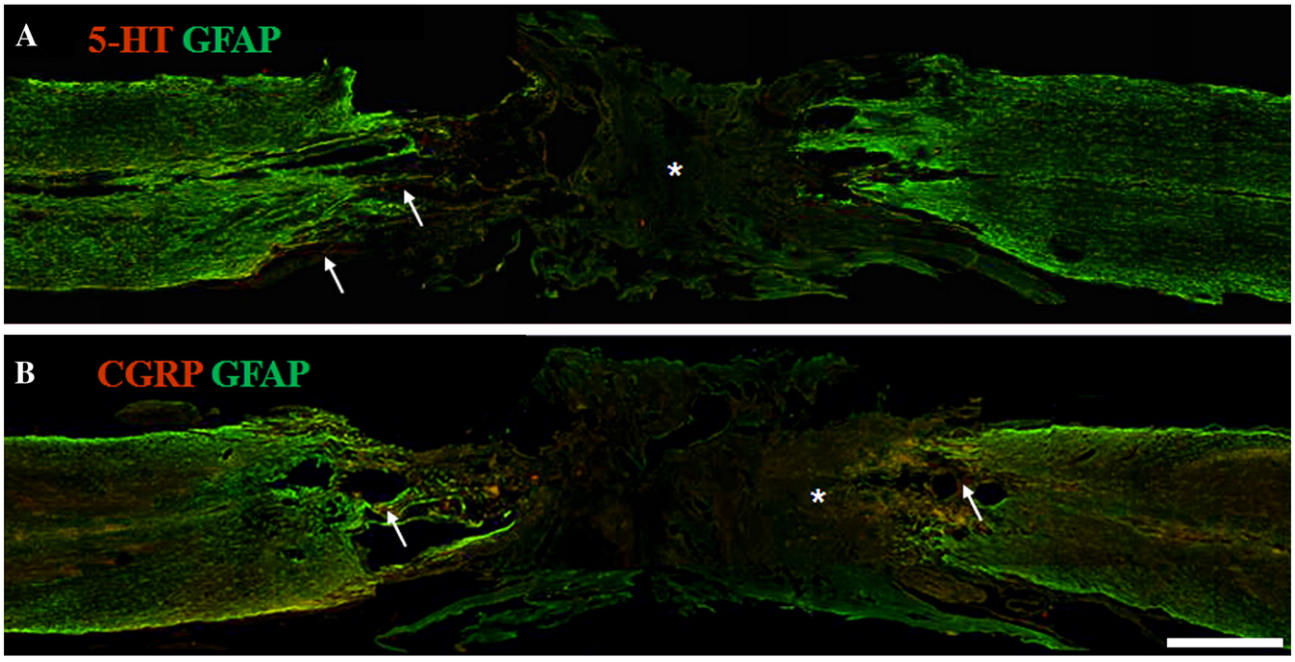


Fig. 3 – 5-HT and CGRP fiber regeneration in a representative animal with moderate cavitation at the lesion site, approximately 18 weeks after spinal cord transection. (A) There are 5-HT fibers surrounding the margin of the transection site in the rostral stump (arrows), but the majority of these sprouts do not continue in the GFAP-negative lesion area (asterisk). 5-HT fibers are not seen in the caudal stump. (B) CGRP fibers are found surrounding the border of the transection site in both stumps (arrows) and penetrating the GFAP-negative lesion area (asterisk). Rostral is to the left. Photomontages were made from digital images with magnification of 100×, 2.2 μm of optical stack thickness and 11 confocal planes. Pixel size—638.92 × 638.92 μm. Scale bar—500 μm. Abbreviations: 5-HT—Serotonin; CGRP—Calcitonin gene-related peptide; GFAP—Glial Fibrillary Acidic Protein.

without active migration. On the other hand, Richter et al. (2005) showed a superior migratory ability of OECs derived from lamina propria when compared to OECs derived from OB after crush of spinal cord dorsolateral funiculus at the C3–C4 level. Thus, the migratory capacity of these cells after transplantation into different injury sites is still controversial.

OLP transplants have the dual advantage of providing a physical bridge that could mechanically favor axonal sprouting as well as being a reservoir of OECs (Lu et al., 2002). RLP provides the same bridging function and shares many of the cell types with OLP (olfactory nerve bundles, trigeminal nerve fibers, Schwann cells, endothelium, interstitial fibroblasts and tissue resident immune cells) (Mackay-Sim and St John, 2011). These shared cells present in RLP may have been responsible for the hindlimb motor improvement and the CGRP regeneration observed at the lesion site (Lindsay et al., 2010). On the other hand, the restoration of a cell continuum alone within the spinal cord may have largely contributed to the results found with both transplant types. According to this latter hypothesis, animals in which 4 mm were removed from spinal cord and with a matrigel only-bridge showed BBB scores comparable to those observed in the RLP groups. In the animals transplanted with matrigel, myelinated axons were exhibited in the injury site, with 5-HT positive fibers crossing the lesion and penetrating the caudal stump (Fouad et al., 2005). In another similar study, alginate-based capillary gels were inserted after transection of the dorsal column at the C3 level. Similarly, a robust growth of coerulospinal projections and GAP-43 positive fibers was shown within the hydrogel (Prang et al., 2006). However, animals submitted to spinal cord transection and injections of culture medium only (without any bridge at the lesion), also obtained BBB scores that were very close to those observed with our OLP/RLP grafts. Many GAP-43-immunoreactive axons were found in the stumps of these culture-medium-injected group and some CGRP-positive axons invaded the lesion epicenter (López-Vales et al., 2006). In the present study, a lesion-only control group was not included in order to avoid the use of a large number of animals. Moreover, animals without any type of transplantation would not develop the immune responses present in the other groups submitted to heterologous tissue transplantation. More studies are required to verify whether comparable outcomes reported in this study could be found in either untreated or matrigel-only bridge groups, in order to elucidate the possible positive effects exerted by cells other than OECs present in the RLP after spinal cord transection.

Previous studies have emphasized the importance of an appropriate post-injury period for repair after SCI (Schiwy et al., 2009; Takami et al., 2002a). Most experimental studies only performed OECs or tissue transplants acutely (Guest et al., 2008; Kubasak et al., 2008; Lu et al., 2001; Ramón-Cueto and Avila, 1998; Ramón-Cueto et al., 2000). However, transplantation of purified OECs or lamina propria after SCI in humans implies delayed grafting (Tetzlaff et al., 2011). The release of cytotoxic metabolites derived from hemorrhage and/or inflammation could be prevented by more acute interventions, but this procedure could be harmful to the transplanted cells at an intermediate stage (Deumens et al., 2006; Martin et al., 1996). Lu et al. (2001, 2002) showed a great improvement in hindlimb motor function, spinal reflex and enhanced regeneration of raphespinal fibers with OLP transplantation immediately or 4 weeks after spinal cord transection. On the other hand, Steward et al. (2006) failed to find evidence of functional recovery and showed only limited regeneration of raphespinal axons after spinal cord transection and 4-week delayed OLP transplantation. In our study, functional recovery, tissue sparing and axon sprouting/regeneration outcomes were comparable between animals with OLP or RLP grafts, uninfluenced by the different transplantation times (acute, 2 weeks or 4 weeks post-injury). Raphespinal axons rarely extended beyond the lesion border, but CGRP fibers were evident in the center of the lesion after both types of transplants. Thus, the optimal time-window for cellular or tissue transplantation continues to be ill-defined, but this parameter does not seem to limit the effects obtained from the grafts. In addition, as CGRP axon regeneration may be related to nociception transmission, interventions that favor axonal regeneration after SCI must be controlled to ensure that appropriate rather than inappropriate connections are restored (Richter et al., 2005).

Despite current controversy in animal studies, clinical trials using cultured OECs from lamina propria or olfactory mucosa grafts have been made in chronically injured humans (Chhabra et al., 2009; Féron et al., 2005; Lima et al., 2006, 2010; Mackay-Sim et al., 2008). There is still divergence regarding the functional results and, moreover, the procedures used in some of these studies were not administered according to formal clinical trial protocols (Mackay-Sim and St John, 2011).

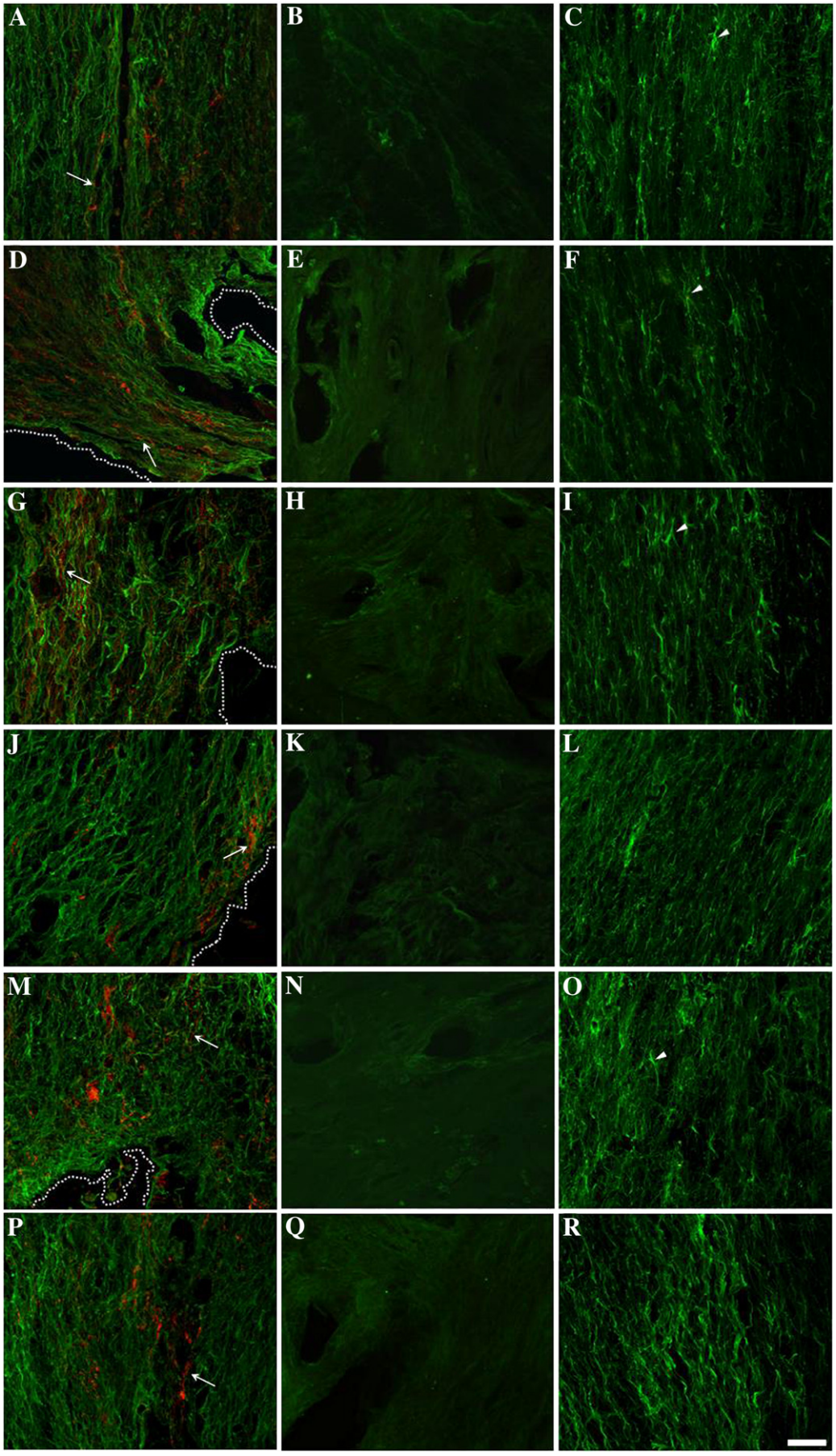
3.1. *Conclusions*

Acute, 2-week or 4-week delayed OLP and RLP transplantation produced a discrete functional recovery over time and comparable CGRP fiber sprouting in the lesion site, but failed to produce regeneration of raphespinal descendent fibers. OECs are only one cell type found in olfactory mucosa, which is a tissue of considerable cellular complexity. This is particularly relevant when clinical trials involving the transplantation of these tissue samples in a complex injury such as the damaged central nervous system are conducted (Lindsay et al., 2010). A better understanding of the effects of OLP and RLP transplantation in SCI animal models is necessary in order to strengthen the rationale for the application of this treatment in humans. Additionally, cells transplants combined with other therapies, such as the administration of MAG, OMP and NOGO-A inhibitors, growth-factors, and/or treadmill step training may increase the possible beneficial results after spinal cord injury.

4. Experimental procedure

4.1. *General*

Experimental procedures were approved by the Research Ethics Committee of the Universidade Federal do Rio Grande do Sul (No. 2007892). The animals were cared for in accordance with Arouca Brazilian law (11794/2008) and the National Institute of Health's Guidelines for Care and Use of Laboratory Animals (publication No. 85-23, revised 1985). The


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R

animal handling recommendations of the Brazilian Society for Neurosciences and the International Brain Research Organization were also followed.

A total of 108 male *Wistar* rats (local breeding colony), 280–380 g in body weight, and 13 weeks old were used in this study. Groups of two or three animals were maintained in standard plexigas boxes (46×24×15 cm), under 12:12 h light/dark cycle, in a temperature controlled environment (20±2 °C) with food and water *ad libitum*. The animals were tested during the light phase of the photo cycle.

Initially, animals were separated in experimental animals (n=72) and lamina propria donors (n=36). Experimental animals (n=72) were again randomly divided into six groups: (1) *AC*—rats submitted to RLP transplantation, immediately after spinal cord transection (n=11); (2) *AT*—rats submitted to OLP transplantation, immediately after spinal cord transection (n=12); (3) *2WDC*—rats submitted to RLP transplantation, two weeks after spinal cord transection (n=12); (4) *2WDT*—rats submitted to OLP transplantation, two weeks after spinal cord transection (n=12); (5) *4WDC*—rats submitted to RLP transplantation, four weeks after spinal cord transection (n=12); (6) *4WDT*—rats submitted to OLP transplantation, four weeks after spinal cord transection (n=12). All efforts were made to minimize the number of animals studied and their suffering. Thus, similarly to a previous studies, a lesion-only group (i.e., without any type of transplantation) was not included (Lu et al., 2001, 2002; Steward et al., 2006).

4.2. *Spinal cord transection*

For spinal cord transection procedure, animals were anesthetized using pentobarbital (40 mg/kg, i.p., Cristália, São Paulo—SP, Brazil) and maintained on a heating pad. The hair overlying the area of interest was shaved and the skin was cleaned. A midline incision in the thoracic area was made and muscle/connective tissues were dissected to expose the T8-T9 vertebrae. After a laminectomy, the spinal cord was transected at two levels using microscissors (approximately 2–3 mm apart). The segment between these incisions was removed, leaving a gap (Fig. 7A, left). To ensure completeness of the lesion, the spinal cord stumps were lifted, placed back into the vertebral canal and a curved needle was passed through the lateral extension of vertebral canal at lesion center (Ilha et al., 2011; Ramón-Cueto et al., 2000). A piece of hemostatic sponge (Technew, São Paulo—SP, Brazil) was placed on the transection site, and then muscles, connective tissue and skin were sutured. The animals were gently warmed until recovery.

4.3. *Post-operative care*

Animals received the analgesic Dimorph (morphine sulfate, s.c., 0.08–0.16 mg/kg, Cristália, São Paulo—SP, Brazil) twice a day, during the first 4 days post-injury. Rats were also treated prophylactically with Baytril (Enrofloxacin, s.c., 2.5 mg/kg, Bayer, São Paulo—SP, Brazil) to prevent urinary tract infections for 14 days. Bladders were manually expressed twice a day until it was no longer distended and palpable, indicating that the animal had developed an automatic bladder voidance reflex (15–20 days). Animals were daily monitored for infections and general health throughout the post-injury survival period. Animals did not exhibit autophagia during the experimental period.

4.4. *Dissection and lamina propria preparation*

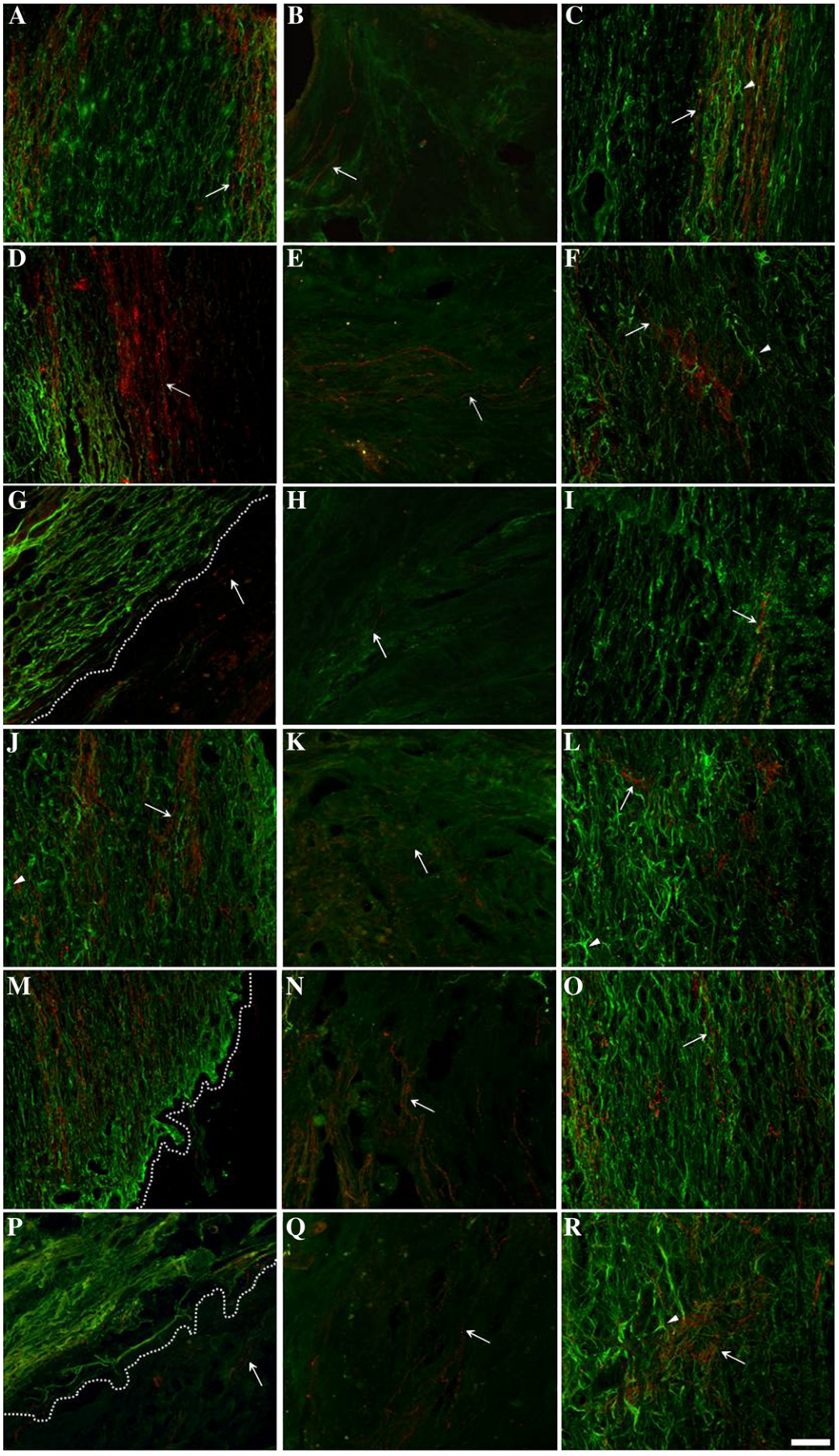
OLP and RLP were dissected according to the method described by Steward et al. (2006). Donors male *Wistar* rats (n=36), 280–380 g in body weight and 13 weeks old, were decapitated. The head was bisected just off the midline in such a way as to allow visualization of the nasal septum and OB. The nasal septum was removed using microscissors and placed in a Petri dish containing Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F12 tissue culture media (DMEN/F12, Sigma-Aldrich, USA).

Olfactory mucosa bilaterally lines the posterior part of the nasal septum and its lamina propria contains OECs. Fig. 8A shows a coronal section of the olfactory mucosa, with the olfactory epithelium and OECs in lamina propria. These fusiform glial cells were identified by their immunoreactivity for p75 neurotrophin receptor (rabbit anti-p75NTR, 1:300, Sigma-Aldrich, USA, N3908), S-100 (rabbit anti-S-100, 1:600, Sigma-Aldrich, USA, S2644) and GFAP in low intensity (mouse anti-GFAP, 1:400, Sigma-Aldrich, USA, G3893) (Ramer et al., 2004; Ramón-Cueto and Avila, 1998).

Respiratory mucosa is thinner than olfactory mucosa and bilaterally covers the dorso-anterior part of the nasal septum. As shown in Fig. 8B, RLP is devoid of OECs. However, p75, S-100 and GFAP markers alone are not exclusive to these glial cells and the staining observed in RLP could be related to the presence of Schwann cells from the trigeminal nerve (Mackay-Sim and St John, 2011).

Using a scalpel, two similar sized pieces of olfactory or respiratory mucosa were dissected from the donor's nasal septum and immediately placed in ice-cold DMEN/F12. In the respiratory tissue dissection, the vomeronasal nerve was avoided. Olfactory and respiratory tissues were separately in-

Fig. 4 – Longitudinal spinal cord sections, double-stained for 5-HT (red) and GFAP (green) at approximately 18 weeks post-injury. AC group (A–C), AT group (D–F), 2WDC group (G–I), 2WDT group (J–L), 4WDC group (M–O) and 4WDT group (P–R). Left—rostral stump (dashed lines represents the lesion border); Center—lesion epicenter (GFAP negative); Right—caudal stump. The majority of 5-HT axons were located in rostral non-injured areas and stopped at the border of the scar. Note the absence of 5-HT axon immunoreactivity in the lesion epicenter and caudal stump. Arrows indicate 5-HT fibers. Arrowheads point to astrocytic cell body. Magnification—40×. Optical stack thickness—0.69 μm. Pixel size—397×397 μm. Scale bar—50 μm. Abbreviations: 2WDC—2-Week Delayed Control; 2WDT—2-Week Delayed Treated; 4WDC—4-Week Delayed Control; 4WDT—4-Week Delayed Treated; 5-HT—Serotonin; AC—Acute Control; AT—Acute Treated; GFAP—Glial Fibrillary Acidic Protein.


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R

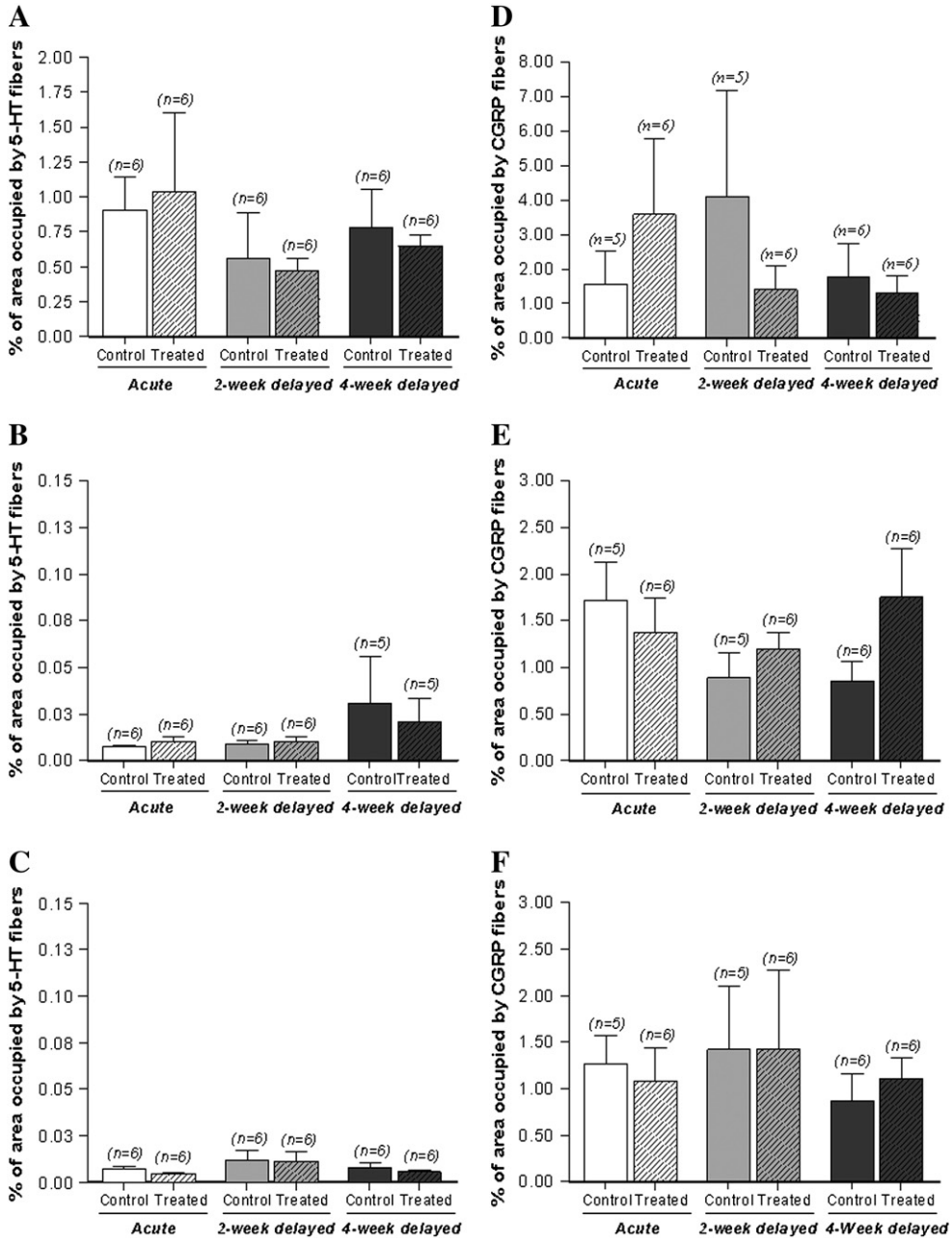


Fig. 6 – Quantitative data of 5-HT (A–C) and CGRP (D–E) axon profiles in rostral (above), lesion (center) and caudal (below) regions of spinal cord at approximately 18 weeks post-injury. There were no statistical differences between groups transplanted with olfactory or respiratory lamina propria in the studied fibers ($p > 0.05$). Abbreviations: 5-HT—Serotonin; CGRP—Calcitonin gene-related peptide.

cubated in 2.4 units/mL dispase II solution (Sigma-Aldrich, Germany, D4693) at 37 °C. After enzymatic digestion, both types of lamina propria samples were carefully separated from the epithelium using a micro-spatula under a dissection microscope and then cut into small pieces (approximately 3–4 mm^2 for grafting). Then, the tissue was rinsed with Hank's

Fig. 5 – Longitudinal spinal cord sections, double-stained for CGRP (red) and GFAP (green) at approximately 18 weeks post-injury. AC group (A–C), AT group (D–F), 2WDC group (G–I), 2WDT group (J–L), 4WDC group (M–O) and 4WDT group (P–R). Left—rostral stump (dashed lines represents the lesion border); Center—lesion epicenter (GFAP negative); Right—caudal stump. Note the presence of CGRP axon immunoreactivity in the lesion epicenter, rostral and caudal stumps. Arrows indicate CGRP fibers. Arrowheads point to astrocytic cell body. Magnification—40×. Optical stack thickness—0.69 μm. Pixel size—397 × 397 μm. Scale bar—50 μm. Abbreviations: 2WDC—2-Week Delayed Control; 2WDT—2-Week Delayed Treated; 4WDC—4-Week Delayed Control; 4WDT—4-Week Delayed Treated; AC—Acute Control; AT—Acute Treated; CGRP—Calcitonin gene-related peptide; GFAP—Glial Fibrillary Acidic Protein.

Buffered Salt Solution (HBSS, Sigma-Aldrich, Brazil) and placed in iced DMEM/F12 until transplantation into the host.

4.5. *Transplantation of OLP and RLP*

The acute animal groups were transplanted immediately after spinal cord transection with RLP (AC group) or OLP (AT group). The other animal groups received RLP and OLP grafts 2 weeks post-SCI (2WDC and 2WDT groups, respectively) and 4 weeks post-SCI (4WDC and 4WDT groups, respectively). For this procedure, rats were re-anesthetized (as described above) and the original incision was re-opened. Scar tissue was removed and the gap between the rostral and caudal stumps was filled with pieces of respiratory (2WDC and 4WDC groups) or olfactory (2WDT and 4WDT groups) lamina propria (Fig. 7A, right). A piece of hemostatic sponge (Hemospon, Technew, São Paulo—SP, Brazil) was placed over the transplantation site to ensure blood homeostasis. Again, muscle and skin layers were sutured and post-operatory care was maintained as previously described.

Approximately 18 weeks after spinal cord injury, the viability of grafted tissue was demonstrated by the presence of fusiform-shaped OECs immunoreactive for p75NTR, S-100 and GFAP at the site of spinal cord transection (Fig. 8C). RLP control grafts continued to be devoid of OECs, as confirmed by the lack of cells expressing the three markers used in the lesion area (Fig. 8D).

4.6. *Behavioral assessment*

Hindlimb motor function was assessed using the BBB locomotor rating (Basso et al., 1996). This scale is qualitative, widely used and designated to assess the functional recovery of hindlimbs after lesions in thoracic spinal cord. The score of this scale ranges from 0 (no hindlimb movement) to 21 (normal movement of the hindlimbs). In this study, BBB assessment was accomplished preoperatively (naive) and postoperatively after the SCI (at days 5, 20, 35, 50, 65, 80 post-injury for the AC and AT groups; at days 5, 20, 35, 50, 65, 80, 95 post-injury for the 2WDC and 2WDT groups; and at days 5, 20, 35, 50, 65, 80, 95, 110 post-injury for the 4WDC and 4WDT groups). For each test, rats were placed in an open-field (60×30×40 cm) for 5 min. The test session was recorded with a video camera (Sony Handycam DCR-SR88, São Paulo—SP, Brazil) to allow later analysis by a blinded observer. The scores of the left and right hindlimbs were averaged and taken as the BBB score of each animal.

4.7. *Retrograde tracer injection*

At the end of behavioral analysis, rats were anesthetized as described above. An incision was made at the T12 vertebrae level to expose the spinal cord below the SCI site. After a laminectomy, FG retrograde tracer (2% dextran tetramethylenerhodamine, Biotium Inc., Hayward—CA, USA) was injected using a stereotaxic apparatus (Insight, Ribeirão Preto—SP, Brazil) coupled to a 1 μL Hamilton syringe (Hamilton Company, Reno—NV, USA). Three injections of FG (0.05 μL, 1 min duration each) were made at midline (0.5, 0.8 and 1.5 mm deep) and 1 mm laterally (0.5, 0.8 and 1.2 mm deep) in each side of this spinal cord level (Steward et al., 2006). Post-operatory care was done as previously described.

4.8. *Tissue preparation and immunohistochemistry*

One week after the retrograde tracing injections, rats received an overdose of pentobarbital (100 mg/kg body weight, i.p., Cristália, São Paulo—SP, Brazil) and were transcardially perfused with saline solution and buffered 4% paraformaldehyde (pH 7.4) using a peristaltic pump (30 mL/min, Milan Equipamentos Científicos, Colombo—PR, Brazil). Brain, brainstem and thoracic spinal cord with approximately 2-cm long (including the lesion site) were removed, post-fixed in the same fixative solution and cryoprotected in 15% and 30% sucrose in phosphate buffer saline (PBS). Prior to embedding in Tissue-Tek, spinal cord samples were photographed with a digital camera (Sony Cyber-Shot DSC-S950, São Paulo—SP, Brazil) on a dark background to provide morphological visualization of the injury site (Fig. 7B). After this, samples were quickly frozen in isopentane (Merck, Germany) cooled in liquid nitrogen and stored at −80 °C.

Primary somatosensory cortex, primary and secondary motor cortex and the entire brainstem were serially sliced (200 μm thick, 150 μm apart) using a cryostat (CM1850, Leica, São Paulo—SP, Brazil) to allow retrograde tracer visualization. These sections were mounted on gelatin-coated glass slides, covered with aqueous mounting medium (FluorSave, Calbiochem, Darmstadt, Germany) and coverslips. The entire spinal cord samples were longitudinally cut (25 μm), in a series of 5 slides per animal with 7–8 sections per slide. Two slides per animal were used to perform immunohistochemistry by the peroxidase method (Sternberger, 1979). Initially, sections were washed in PBS, followed by a 30 min period with 3% hydrogen peroxide (H_2O_2). After several washes in PBS, sections were pre-incubated in 1% albumin solution with 0.4% triton X-100 (PBS-Tx). Then, slices were incubated for 48 h at 4 °C in either GFAP (rabbit anti-GFAP, 1:200, DAKO Denmark A/S, Denmark, Z0334) or GAP-43 antibodies (mouse anti-GAP-43, 1:500, Santa Cruz Biotechnology Inc., USA, SC33705). Sections were rinsed in PBS-Tx and re-incubated in goat anti-rabbit IgG (1:100, Sigma-Aldrich, USA, R2004) or goat anti-mouse IgG (1:100, Sigma-Aldrich, USA, M8642) for 2 h. Following PBS washes, slices were placed in peroxidase anti-peroxidase (1:500, Sigma-Aldrich, USA, P1291) for 1 h and 30 min. The immunohistochemical reaction was developed by incubating the slices in a medium containing 0.06% 3,3 diaminobenzidine (DAB, Sigma-Aldrich, USA, D5637) and then in the same solution containing 1 μM of 3% H_2O_2 per mL of DAB medium for 10 min each. Finally, slices were rinsed with PBS, dehydrated with ethanol, cleared with xylene and covered with Permount and coverslips. Control sections were prepared by omitting the primary antibody and replacing it with PBS.

In double staining protocols, fibre tracts were stained using the following antibodies: rabbit anti-serotonin (1:5000, Sigma-Aldrich, USA, S5545) for serotonergic axons in the spinal cord coming from raphe nuclei; and rabbit anti-CGRP (1:1500, courtesy of Dr. Rodrigo, Instituto Cajal, Spain) as a marker for ascending sensory neurons. Fibrous scar borders were defined using immunoreactivity to GFAP (mouse anti-GFAP, 1:400, Sigma-Aldrich, USA, G3893). The protocol consisted of

Table 2 – Studies performed with several types of olfactory ensheathing cells transplantation in different spinal cord injury models.

Reference and injury model	Species/strain	Graft properties	Transplantation time	Behavioral results	Histological results	Survival
Takami et al. (2002a) –Moderate contusion at T9 level	Adult female Fisher rats	–OB OECs from adult female Fischer rats –SCs from adult female Fischer rats –Combination of OECs/SCs	–7 days post—injury	–No improvements of BBB score in OEC group –Increase in BBB scores at 8–11 weeks post-injury in SCs only group (10.8 to 11.8)	–Less cavitation and more sparing in grafted groups –Less intense GFAP and CSPG staining in OEC—only grafts versus SCs –Higher number of propriospinal and brainstem axons reached long distances beyond the grafted area with SCs and SC/OEC grafts but not with OEC only –Corticospinal fibers terminate closer to the lesion epicenter in grafted animals.	–12 weeks
Barakat et al. (2005) –Moderate contusion at T9 level	Adult female Fischer rats	–OB OECs from adult female Fischer rats –SCs from adult female Fisher rats	–8 weeks post-injury	–SCs but not OECs resulted in increased BBB scores (Control: 8.5; SC group: 10.2; OEC group: 8.5) –SCs but not OECs resulted in small improvements in base of support and hindpaw rotation	–8–11 weeks after transplantation, SCs survive better than OECs –NF positive axons where observed in OEC transplantation –SC transplants, but not OEC, contained significantly more CGRP and 5-HT positive axons –CGRP fibers arise primarily from DRGs adjacent to the lesion –SCs did not promote corticospinal fibers growth	–19 weeks
Pearse et al. (2007) –Moderate contusion at T8 level	Adult females Fischer rats	–OB OECs from adult female Fischer rats –SCs from adult Fischer rats –FBs from adult Fischer rats	–7 days post-injury	–At 9 weeks, only SC+OEC injection group increased BBB scores (12.3) vs. FBs transplant (9.8) and injury controls (10.7) –No transplant reduced gridwalk errors or reduced base of support and stride length	–Cell survival decreased to a low level by 3 weeks post-transplantation, especially when injected at transection site –Migration of OEC—only was not observed –At later times, significant host SCs infiltration was shown –No sensory or supraspinal axon growth into transplants –Host axons were associated with or ensheathed by transplanted glia –Numerous myelinated axons were found within regions of grafted SCs but no OECs	3 days or 3, 9 and 28 weeks
Richter et al. (2005) –Crush of dorsolateral funiculus at C3–C4 level	Adult male Sprague Dawley rats	–OB OECs of P5 mice expressing eGFP –LP OECs of P5 mice expressing eGFP	–Immediately post injury	–Not reported	–OEC survival was higher when transplanted in both stumps –Both OEC types reduced lesion and cavity formation, increased angiogenesis, endogenous Schwann cell infiltration and axonal sprouting –LP–OECs increased outgrowth of axonal subpopulations but also increased autotomy	–1 or 28 days

(continued on next page)

Table 2 *(continued)*

Reference and injury model	Species/strain	Graft properties	Transplantation time	Behavioral results	Histological results	Survival
Lu et al. (2006) –C4 spinal cord dorsal column wire knife lesion	Adult female Fischer rats	–OECs from olfactory mucosa of postnatal day 5 Fischer rats –Bone stromal cells of adult female Fischer rats FBs of adult female Fischer rats	–Immediately post-injury	–Not reported	–OECs failed to support bridging of corticospinal axons –The "bridging" tract of OECs formed within 1 h of cell injection, increasing the possibility that cells passively spread –NF positive axons penetrate into the lesion site dependently or independently from regions with OEC tracts SCs infiltrated in OEC grafts of lesion cavity and associate with penetrating axons –OECs proliferated in injection sites, cell tracts and lesion sites, indication that these cells can also accumulate through proliferation	–1, 3, 12 or 24 h –3, 7, 9 days PI – 4 weeks
Ramón-Cueto et al. (1998) –Complete transection at T9 level (4 mm gap)	Adult female Fischer rats	–OECs from adult Fischer rats and SCs cells from adult Fischer rats within a channel with Matrigel	–Immediately post-injury	–Not reported	–Numerous NF, GAP-43, CGRP, and 5-HT positive fibers traversed both interfaces of the cord with the channel filled with SCs and OECs stumps injection –5-HT axons extended long distances along the connective tissue outside of the channels to the caudal spinal cord –Ascending propriospinal axons were seen caudally to the graft –OECs integrated and migrated through host and graft tissue	–6 weeks
López-Vales et al. (2007) –Complete transection at T8 level	Adult female Sprague Dawley rats	–OB OECs from P22-P23 Sprague Dawley rats	–Immediately post-injury –30 min post-injury –7 days post-injury	–OECs reduced hindlimb hyperreflexia and increased BBB scores (Control: 0–2; Acute OEC group: 4,4; Delayed OEC group: 3,7) –OECs transplants recovered MEP	–OECs produced axonal regeneration of raphespinal, coerulospinal and corticospinal axons within the caudial stump –Expression of GFAP and NG2 was reduced in perilesional cord segments in transplanted animals	–36 weeks
López-Vales et al. (2007) –Complete transection at T8 level	Adult female Sprague Dawley rats	–OB OECs from P22-P23 Sprague Dawley rats	–45 days post-injury	–OECs increased BBB scores (Control: 0.92; OEC group: 2.5) –Limited recovery of MEP with OEC vs. no recovery in control	–No significant amounts of corticospinal and 5-HT positive axon growth through the lesion site and into the caudal spinal cord from transplanted animals –Some 5-HT axons extended long distances through the gray matter –Delayed transplantation of OECs failed to reduce astrogliosis	–20 weeks
Lu et al. (2001) –Complete transection at T10 level (1–2 mm gap)	Adult female Sprague Dawley rats	–OLP from adult Sprague Dawley rats –LP OECs from adult Sprague Dawley rats	–Immediately post-injury	–Both OEC types increased BBB scores (Control: 0–2; OEC group: 6–8) –OECs recovered spinal reflex circutry (assessed using the	–Nerve fibers passed through the transection site in OLP transplanted animals –5-HT positive fibers were found distal to the transection site –Retrograde labeling of brainstem raphe and gigantocellularis neurons were observed,	–10 weeks

				rate-sensitive depression of the H-reflex)	indicating regeneration of descending pathways in OLP transplanted animals	
Lu et al. (2001) –Complete transection at T10 level	Adult female Sprague Dawley rats	–OLP from adult female Sprague Dawley rats –RLP from adult female Sprague Dawley rats	–4 weeks post-injury	–OLP significantly increased BBB scores (OLP group: 4.3; RLP group: 1.0)	–5-HT positive axons were observed caudal to the transection site in OLP transplanted animals	–10 weeks for histology and 14 weeks for behavior
Steward et al. (2006) –Complete transection at T10 level	Adult female Sprague Dawley rats	–OLP from adult female Sprague Dawley rats –RLP from adult female Sprague Dawley rats	–4 weeks post-injury	–No significant differences in BBB scores between groups at any time point –No differences in bladder retention of urine	–FG injection caudal to the lesion did not reveal evidence of regeneration of descending axons across transection site –Few 5-HT positive axons extended in both lamina propria transplants; –Few 5-HT positive axons were also found caudal to the injury in 2 animals that received OLP and in one animal that received RLP	–10 weeks
Present study –Complete transection at T8–T9 level (1–2 mm gap)	Adult male Wistar rats	–OLP from adult male Wistar rats –RLP from adult male Wistar rats	–Immediately post-injury –2 weeks post-injury –4 weeks post-injury	–No significant differences in BBB scores between groups at 42 days post-transplantation (AC: 3.5; AT: 2.7; 2WDC: 2.6; 2WDT: 3.0 4WDC: 2.6; 4WDT: 2.0)	–All transplants produced comparable results for spinal cord tissue sparing and sprouting evaluated using GFAP and GAP-43 staining –Acute transplantation of OLP and RLP seems to foster some limited supraspinal axonal regeneration as observed with FG tracing –A higher number of 5-HT positive fibers was found in the cranial stump of OLP and RLP groups compared to the lesion and caudal regions; CGRP fibers were present in considerable number at the SCI site in both types of transplantation	–Approximately 18 weeks

Abbreviations: 2WDC-2-Week Delayed Control; 2WDT—2-Week Delayed Treated; 4WDC—4-Week Delayed Control; 4WDT—4-Week Delayed Treated; 5-HT—Serotonin; AC—Acute Control; AT—Acute Treated; BBB—Basso, Beattie, and Bresnahan Scale; CGRP—Calcitonin Gene-Related Peptide; CSPG—Chondroitin Sulfate Protooglycan; DRGs—Dorsal Root Ganglia; eGFP—Green Fluorescent Protein-expressing; FBs—Fibroblasts; FG—Fluorogold; GAP-43—Growth Associated Protein-43; GFAP—Glial Fibrillary Acidic Protein; LP OECs—Olfactory Ensheathing Cells from Lamina Propria; MEP—Motor Evoked Potentials; NF—Neurofilament; OB OECs—Olfactory Ensheathing Cells from Olfactory Bulb; OEC—Olfactory Ensheating Cells; OLP—Olfactory Lamina Propria; RLP—Respiratory Lamina Propria; SCs—Schwann Cells; WGA–HRP—Agglutinin–Horseradish Peroxidase.

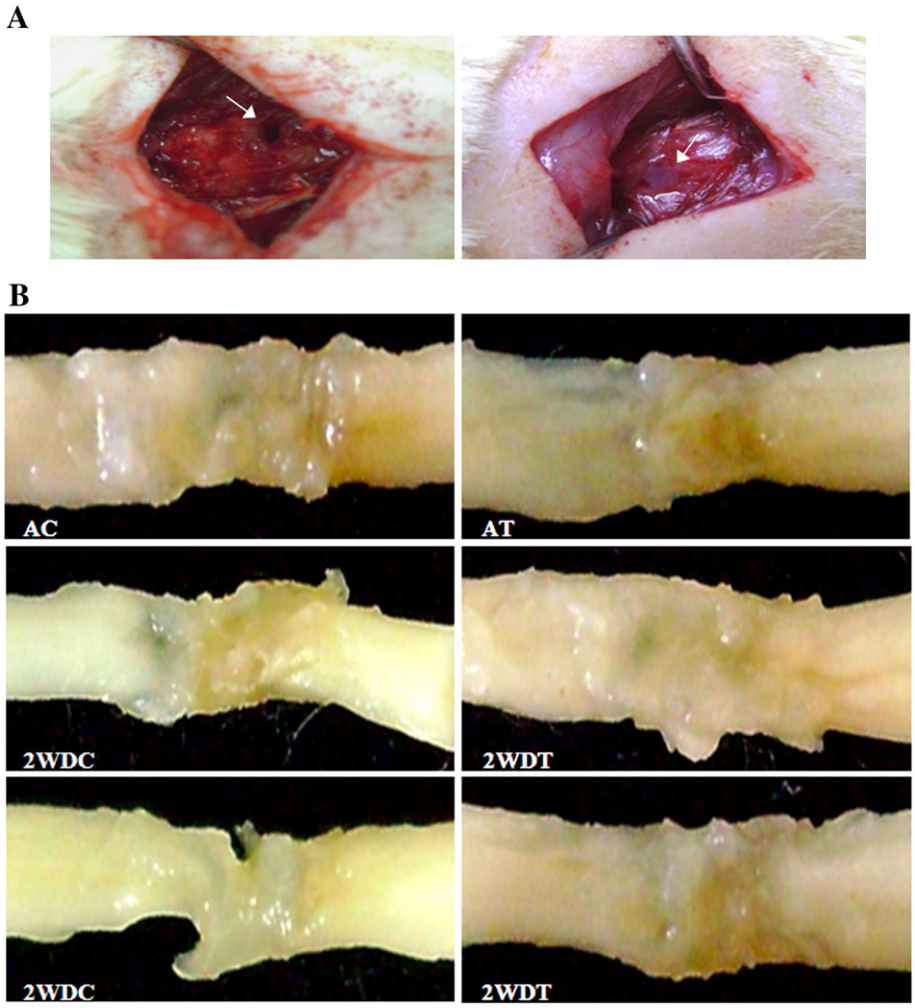


Fig. 7 – (A) Illustration of the cavity formed as a result of the spinal cord injury procedure (left, arrow) and appearance of the transplanted olfactory or respiratory lamina propria in the lesion gap (right, arrow). (B) Macroscopic view of the spinal cords from different animal groups. Note that the transection site is filled with transplanted tissue and these transplants bridge a gap between the rostral (left) and caudal segments.

washing the sections with PBS, followed by permeabilization with 0.25% PBS-Tx. After this, sections were blocked in 1% albumin for 30 min. Incubation with the first antibodies was carried out in 1% albumin in PBS-Tx at 4 °C for 48 h. Following PBS washes, sections were incubated in secondary antibodies anti-mouse Alexa 488 (1:500, Molecular Probes, Invitrogen, USA, A10680) and anti-rabbit Alexa 555 (1:500, Molecular Probes, Invitrogen, USA, A21428). The slides were covered with aqueous mounting medium (FluorSave, Calbiochem, Darmstadt, Germany) and coverslips.

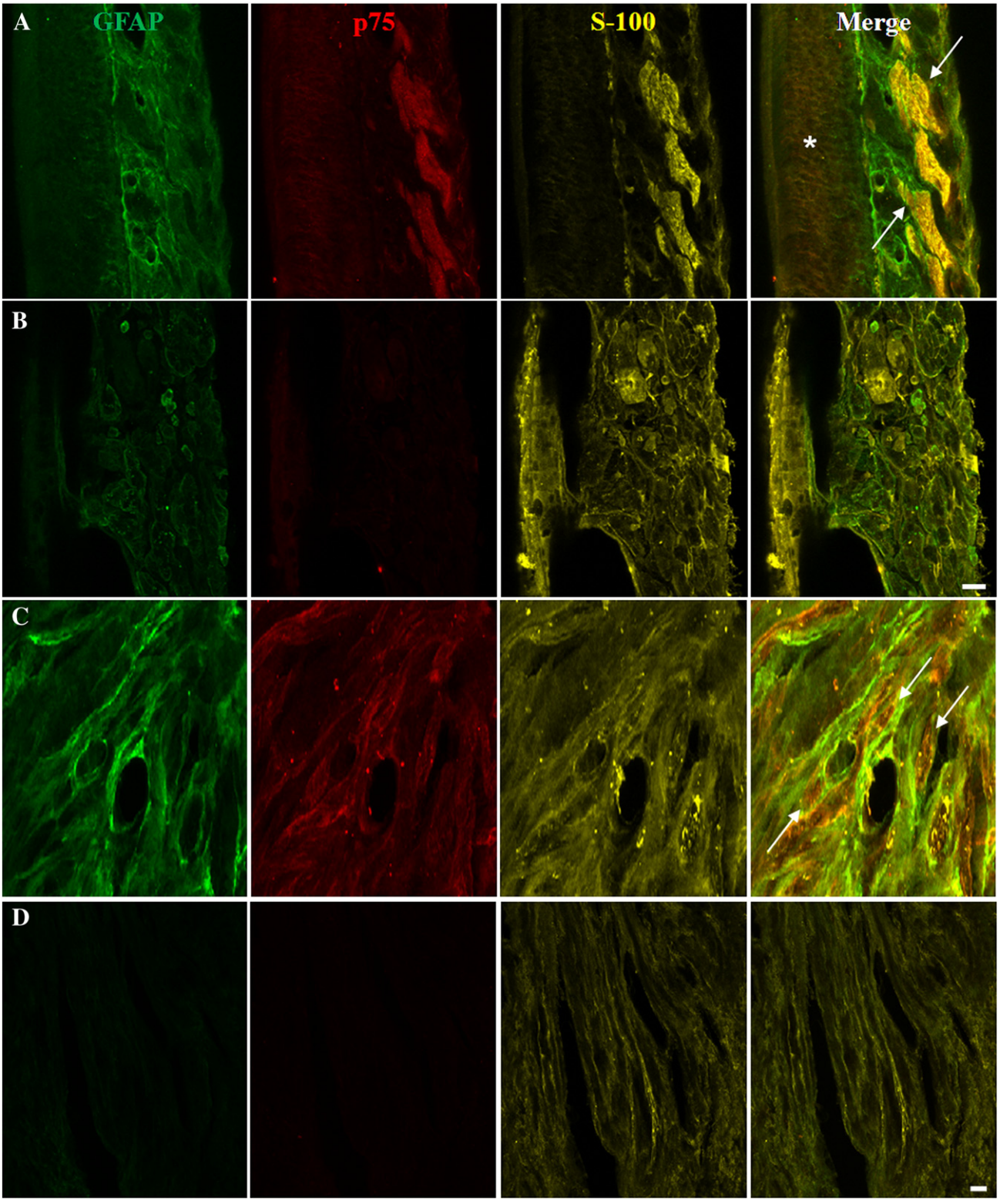
4.9. *Image analysis*

4.9.1. *Retrograde tracing assessment*

Sections from the entire brainstem and sensorimotor cortex (n=3 per group) were visualized in serial stack images

Fig. 8 – (A) Olfactory and (B) respiratory mucosa dissected from nasal septum of adult rat, immunostained by GFAP (first column), p75 (second column) and S-100 (third column). Olfactory ensheathing cells were identified by immunoreactivity for the three markers used (arrows). Respiratory lamina propria is devoid of olfactory ensheathing cells. Asterisks indicate olfactory epithelium. Magnification—600× (water objective). Pixel size—265×265 μm (A—0.57 μm optical stack thickness and 44 confocal planes; B—1.0 μm optical stack thickness and 21 confocal planes). Scale bar—20 μm. (C) Olfactory and (D) respiratory lamina propria at the site of spinal cord transection, immunostained by GFAP (first column), p75 (second column) and S-100 (third column). Olfactory ensheathing cells maintained their fusiform aspect and continued to express the same three markers observed before transplantation (arrows). Respiratory lamina propria remains devoid of olfactory ensheathing cells. Magnification—400×. Pixel size—497×497 μm (C—0.76 μm optical stack thickness and 21 confocal planes; D—0.1 μm optical stack thickness and 14 confocal planes). Scale bar—20 μm. Abbreviations: GFAP—Glial Fibrillary Acidic Protein, p75—p75 Neurotrophin Receptor.

(11.96 μm thick, 16–17 serial stack images per slice) obtained with an Olympus confocal FV-1000. Plan-Apochromat 10× objective lens were used (Numerical aperture - NA 0.30) and the pinhole was set in automatic mode. The *FG signal* (blue) was visualized with a wide band ultraviolet excitation filter (Excitation—331 nm, Emission—418 nm). Images were made using a photomultiplier detector and all pictures were analyzed with Image J Software 1.42q (Wayne Rasband, National Institutes of Health, USA). The total number of FG labeled neurons in the propriospinal and selected supraspinal regions, i.e., MdV/MdD (5 slices per animal on average), PnO/PnC (7 slices per animal on average), Ra (including the raphe pallidus, raphe obscurus, raphe magnus—20 slices per animal on average), SpVe (7 slices per animal on average), LVe (6



slices per animal on average), *locus coeruleus* (LC—4 slices per animal on average), M1/M2 (25 slices per animal on average) and S1 (20 slices per animal on average) was counted bilaterally by a blinded observer (Iannotti et al., 2004; Xu et al., 1995). Axons from these nuclei project to the thoracolumbar spinal cord and play important roles in locomotor function (Holstege and Kuypers, 1987; Iannotti et al., 2004; Kim et al., 2002). The location and number of FG-stained cellular bodies were determined from each section using an overlaid grid and a stereotaxic atlas (Paxinos and Watson, 1998).

4.9.2. *Spinal tissue sparing and GAP-43 optical densitometry*

Images of diaminobenzidine-stained spinal cord sections (20×) were taken using a Nikon Microscope Optiphot-2 (Japan) coupled to a CMOS camera (518 CU, Micrometrics) and analyzed with Image J Software 1.42q. Subsequently, digital RGB (24-bit) images with resolution of 254×254 DPI were converted to grayscale (8-bit) and corrected for unequal illumination (shading correction). All lightning conditions and magnifications were held constant.

To evaluate spinal tissue sparing, pictures of GFAP-immunostained spinal cord sections were captured with the lesion-part in the center. Samples with no continuity between rostral and caudal stumps were discarded from this analysis. After standardized background corrections, black-and-white 8-bit images were thresholded and tissue area fractions measured in each section. Since not all sections of the whole spinal could be used for analysis, volume and total area values of spinal cord tissue sparing could not be obtained. On average, 5 images were analyzed from each rat and a mean of spared tissue was calculated for experimental group (6 animals each group) (adapted from Kubasak et al., 2008).

Images of GAP-43 immunohistochemistry were also obtained from the injured part of the spinal cord. After standardized background corrections, a mask of each spinal cord section image was created using an auto-threshold tool from Image J, hence avoiding vacuolization and interrupted tissue integrity. Thereafter, optical densities (OD) of the images were measured from whole injury regions within the area of interest, i.e., the mask itself. OD was calculated using the following formula:

$$OD = -\log[(INT(x,y)-BL)]/(INC-BL)]$$

Where "OD" is the optical density; "INT (x,y)" or intensity is the intensity at pixel (x,y), "BL" or black is the intensity generated when no light goes through the material and "INC" is the intensity of the incidental light.

Around 6–16 images were analyzed from each rat and 6 animals were analyzed per group.

4.9.3. *Axon profile quantification*

5-HT and CGRP fiber populations were also identified using a Nikon Microscope Optiphot-2 (Japan) with a green excitation filter for the Alexa 555 signal (G-2A, Excitation—510/560). Double-labeling with GFAP antibody was used to delineate the fibrous scar borders and the signal for Alexa 488 was detected using a blue excitation filter (B-2A, Excitation—450/490). Pictures with resolution of 254×254 DPI, were taken at magnification of 200× using a CMOS camera (518 CU, Micrometrics) and analyzed with Image J Software 1.42q. The total area occupied by 5-HT or CGRP axons was determined separately in the rostral, lesion and caudal regions, throughout the width of the tissue sections. To assess 5-HT fibers, pictures were taken of the rostral stump (in the region with abundant visible astrocytes), the central part of the lesion (approximately) and near the scar border of the caudal stump. Analogously, images of CGRP fibers were taken of the caudal stump (in the region with abundant visible astrocytes), in the central part of the lesion (approximately) and near the scar border of the rostral stump. All images (on average, 19 pictures per spinal cord region in each animal, 6 animals per group) were turned into binary (black and white) and a constant threshold value was used to measure the total percentage area (%) occupied by axon fibers.

4.10. *Statistical analysis*

Data were expressed as means±SEM. Open field locomotor scores were analyzed between groups using analysis of variance (ANOVA) with *time* as the repeated measure. When there were statistically significant F values ($p \leq 0.05$), Bonferroni's *post hoc* tests were conducted by comparing OLP transplantation with the corresponding RLP group. Regarding assessment of spinal tissue sparing and regional optical densitometry, all groups were analyzed using one-way ANOVA followed by Bonferroni's *post hoc* test. The Kruskal–Wallis test was used for axon profile data (5-HT or CGRP). Values were run on SPSS 11.5 (Statistical Package for the Social Sciences, Inc., USA).

Acknowledgments

The authors would like to thank Lorena F. Orlandini for animal care, Henrique B. Biehl and Carlos E. L. Santos for their assistance with confocal microscopy (Centro de Microscopia Eletrônica da UFRGS). Technical assistance was provided by Silvia Barbosa and Antônio Severino. We also thank Ana Paula Horn and Lauren Valentim for their helpful information in immunofluorescence. This study was supported by grants from CNPq and CAPES. There was no conflict of interests.

REFERENCES

Au, E., Roskams, A.J., 2003. Olfactory ensheathing cells of the lamina propria in vivo and in vitro. Glia 41, 224–236.

Barakat, D.J., Gaglani, S.M., Neravetla, S.R., Sanchez, A.R., Andrade, C.M., Pressman, Y., Puzis, R., Garg, M.S., Bunge, M.B., Pearse, D.D., 2005. Survival, integration, and axon growth support of glia transplanted into the chronically contused spinal cord. Cell Transplant. 14, 225–240.

Barnett, S.C., Riddell, J.S., 2007. Olfactory ensheathing cell transplantation as a strategy for spinal cord repair—what can it achieve? Nat. Clin. Pract. Neurol. 3, 152–161.

Basso, D.M., Beattie, M.S., Bresnahan, J.C., 1996. Graded histological and locomotor outcomes after spinal cord contusion using the

NYU weight-drop device versus transection. Exp. Neurol. 139, 244–256.
Bianco, J.I., Perry, C., Harkin, D.G., Mackay-Sim, A., Féron, F., 2004. Neurotrophin 3 promotes purification and proliferation of olfactory ensheathing cells from human nose. Glia 45, 111–123.
Blight, A.R., 2002. Miracles and molecules—progress in spinal cord repair. Nat. Neurosci. 5, 1051–1054.
Boyd, J.G., Lee, J., Skihar, V., Doucette, R., Kawaja, M.D., 2004. LacZ-expressing olfactory ensheathing cells do not associate with myelinated axons after implantation into the compressed spinal cord. Proc. Natl. Acad. Sci. U. S. A. 101, 2162–2166.
Chhabra, H.S., Lima, C., Sachdeva, S., Mittal, A., Nigam, V., Chaturvedi, D., Arora, M., Aggarwal, A., Kapur, R., Khan, T.A., 2009. Autologous olfactory [corrected] mucosal transplant in chronic spinal cord injury: an Indian Pilot Study. Spinal Cord 47, 887–895.
Deumens, R., Koopmans, G.C., Honig, W.M., Maquet, V., Jerome, R., Steinbusch, H.W., Joosten, E.A., 2006. Limitations in transplantation of astroglia-biomatrix bridges to stimulate corticospinal axon regrowth across large spinal lesion gaps. Neurosci. Lett. 400, 208–212.
Doucette, R., 1991. PNS–CNS transitional zone of the first cranial nerve. J. Comp. Neurol. 312, 451–466.
Féron, F., Perry, C., Cochrane, J., Licina, P., Nowitzke, A., Urquhart, S., Geraghty, T., Mackay-Sim, A., 2005. Autologous olfactory ensheathing cell transplantation in human spinal cord injury. Brain 128, 2951–2960.
Féron, F., Perry, C., McGrath, J.J., Mackay-Sim, A., 1998. New techniques for biopsy and culture of human olfactory epithelial neurons. Arch. Otolaryngol. Head Neck Surg. 124, 861–866.
Fitch, M.T., Silver, J., 2008. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp. Neurol. 209, 294–301.
Fouad, K., Schnell, L., Bunge, M.B., Schwab, M.E., Liebscher, T., Pearse, D.D., 2005. Combining Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord. J. Neurosci. 25, 1169–1178.
Franklin, R.J., 2002. Obtaining olfactory ensheathing cells from extra-cranial sources a step closer to clinical transplant-mediated repair of the CNS? Brain 125, 2–3.
Franssen, E.H., de Bree, F.M., Essing, A.H., Ramón-Cueto, A., Verhaagen, J., 2008. Comparative gene expression profiling of olfactory ensheathing glia and Schwann cells indicates distinct tissue repair characteristics of olfactory ensheathing glia. Glia 56, 1285–1298.
Franssen, E.H., de Bree, F.M., Verhaagen, J., 2007. Olfactory ensheathing glia: their contribution to primary olfactory nervous system regeneration and their regenerative potential following transplantation into the injured spinal cord. Brain Res. Rev. 56, 236–258.
García-Alías, G., López-Vales, R., Forés, J., Navarro, X., Verdú, E., 2004. Acute transplantation of olfactory ensheathing cells or Schwann cells promotes recovery after spinal cord injury in the rat. J. Neurosci. Res. 75, 632–641.
Guest, J.D., Herrera, L., Margitich, I., Oliveria, M., Marcillo, A., Casas, C.E., 2008. Xenografts of expanded primate olfactory ensheathing glia support transient behavioral recovery that is independent of serotonergic or corticospinal axonal regeneration in nude rats following spinal cord transection. Exp. Neurol. 212, 261–274.
Hausmann, O.N., 2003. Post-traumatic inflammation following spinal cord injury. Spinal Cord 41, 369–378.
Holstege, J.C., Kuypers, H.G., 1987. Brainstem projections to spinal motoneurons: an update. Neuroscience 23, 809–821.
Iannotti, C., Ping Zhang, Y., Shields, C.B., Han, Y., Burke, D.A., Xu, X.M., 2004. A neuroprotective role of glial cell line-derived neurotrophic factor following moderate spinal cord contusion injury. Exp. Neurol. 189, 317–332.
Ilha, J., Centenaro, L.A., Broetto Cunha, N., de Souza, D.F., Jaeger, M., do Nascimento, P.S., Kolling, J., Ben, J., Marcuzzo, S., Wyse, A.T., Gottfried, C., Achaval, M., 2011. The beneficial effects of treadmill step training on activity-dependent synaptic and cellular plasticity markers after complete spinal cord injury. Neurochem. Res. 36, 1046–1055.
Kim, E.S., Kim, G.M., Lu, X., Hsu, C.Y., Xu, X.M., 2002. Neural circuitry of the adult rat central nervous system after spinal cord injury: a study using fast blue and the Bartha strain of pseudorabies virus. J. Neurotrauma 19, 787–800.
Kubasak, M.D., Jindrich, D.L., Zhong, H., Takeoka, A., McFarland, K.C., Muñoz-Quiles, C., Roy, R.R., Edgerton, V.R., Ramón-Cueto, A., Phelps, P.E., 2008. OEG implantation and step training enhance hindlimb-stepping ability in adult spinal transected rats. Brain 131, 264–276.
Kwon, B.K., Tetzlaff, W., 2001. Spinal cord regeneration: from gene to transplants. Spine (Phila Pa 1976) 26, S13–S22.
Li, Y., Li, D., Raisman, G., 2005. Interaction of olfactory ensheathing cells with astrocytes may be the key to repair of tract injuries in the spinal cord: the 'pathway hypothesis'. J. Neurocytol. 34, 343–351.
Lima, C., Escada, P., Pratas-Vital, J., Branco, C., Arcangeli, C.A., Lazzeri, G., Maia, C.A., Capucho, C., Hasse-Ferreira, A., Peduzzi, J.D., 2010. Olfactory mucosal autografts and rehabilitation for chronic traumatic spinal cord injury. Neurorehabil. Neural Repair 24, 10–22.
Lima, C., Pratas-Vital, J., Escada, P., Hasse-Ferreira, A., Capucho, C., Peduzzi, J.D., 2006. Olfactory mucosa autografts in human spinal cord injury: a pilot clinical study. J. Spinal Cord Med. 29, 191–203.
Lindsay, S.L., Riddell, J.S., Barnett, S.C., 2010. Olfactory mucosa for transplant-mediated repair: a complex tissue for a complex injury? Glia 58, 125–134.
Lipson, A.C., Widenfalk, J., Lindqvist, E., Ebendal, T., Olson, L., 2003. Neurotrophic properties of olfactory ensheathing glia. Exp. Neurol. 180, 167–171.
López-Vales, R., Forés, J., Navarro, X., Verdú, E., 2007. Chronic transplantation of olfactory ensheathing cells promotes partial recovery after complete spinal cord transection in the rat. Glia 55, 303–311.
López-Vales, R., Forés, J., Verdú, E., Navarro, X., 2006. Acute and delayed transplantation of olfactory ensheathing cells promote partial recovery after complete transection of the spinal cord. Neurobiol. Dis. 21, 57–68.
Lu, J., Féron, F., Ho, S.M., Mackay-Sim, A., Waite, P.M., 2001. Transplantation of nasal olfactory tissue promotes partial recovery in paraplegic adult rats. Brain Res. 889, 344–357.
Lu, J., Féron, F., Mackay-Sim, A., Waite, P.M., 2002. Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord. Brain 125, 14–21.
Lu, P., Yang, H., Culbertson, M., Graham, L., Roskams, A.J., Tuszynski, M.H., 2006. Olfactory ensheathing cells do not exhibit unique migratory or axonal growth-promoting properties after spinal cord injury. J. Neurosci. 26, 11120–11130.
Mackay-Sim, A., Féron, F., Cochrane, J., Bassingthwaighte, L., Bayliss, C., Davies, W., Fronek, P., Gray, C., Kerr, G., Licina, P., Nowitzke, A., Perry, C., Silburn, P.A., Urquhart, S., Geraghty, T., 2008. Autologous olfactory ensheathing cell transplantation in human paraplegia: a 3-year clinical trial. Brain 131, 2376–2386.
Mackay-Sim, A., St John, J.A., 2011. Olfactory ensheathing cells from the nose: clinical application in human spinal cord injuries. Exp. Neurol. 229, 174–180.
Martin, D., Robe, P., Franzen, R., Delree, P., Schoenen, J., Stevenaert, A., Moonen, G., 1996. Effects of Schwann cell transplantation in a contusion model of rat spinal cord injury. J. Neurosci. Res. 45, 588–597.
Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates, fourth ed. Academic Press, San Diego.

Pearse, D.D., Sanchez, A.R., Pereira, F.C., Andrade, C.M., Puzis, R., Pressman, Y., Golden, K., Kitay, B.M., Blits, B., Wood, P.M., Bunge, M.B., 2007. Transplantation of Schwann cells and/or olfactory ensheathing glia into the contused spinal cord: survival, migration, axon association, and functional recovery. Glia 55, 976–1000.

Prang, P., Müller, R., Eljaouhari, A., Heckmann, K., Kunz, W., Weber, T., Faber, C., Vroemen, M., Bogdahn, U., Weidner, N., 2006. The promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels. Biomaterials 27, 3560–3569.

Raisman, G., 2001. Olfactory ensheathing cells—another miracle cure for spinal cord injury? Nat. Rev. Neurosci. 2, 369–375.

Raisman, G., Li, Y., 2007. Repair of neural pathways by olfactory ensheathing cells. Nat. Rev. Neurosci. 8, 312–319.

Ramer, L.M., Ramer, M.S., Steeves, J.D., 2005. Setting the stage for functional repair of spinal cord injuries: a cast of thousands. Spinal Cord 43, 134–161.

Ramer, L.M., Richter, M.W., Roskams, A.J., Tetzlaff, W., Ramer, M.S., 2004. Peripherally-derived olfactory ensheathing cells do not promote primary afferent regeneration following dorsal root injury. Glia 47, 189–206.

Ramón-Cueto, A., Avila, J., 1998. Olfactory ensheathing glia: properties and function. Brain Res. Bull. 46, 175–187.

Ramón-Cueto, A., Cordero, M.I., Santos-Benito, F.F., Avila, J., 2000. Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. Neuron 25, 425–435.

Ramón-Cueto, A., Muñoz-Quiles, C., 2011. Clinical application of adult olfactory bulb ensheathing glia for nervous system repair. Exp. Neurol. 229, 181–194.

Ramón-Cueto, A., Plant, G.W., Avila, J., Bunge, M.B., 1998. Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. J. Neurosci. 18, 3803–3815.

Richter, M.W., Fletcher, P.A., Liu, J., Tetzlaff, W., Roskams, A.J., 2005. Lamina propria and olfactory bulb ensheathing cells exhibit differential integration and migration and promote differential axon sprouting in the lesioned spinal cord. J. Neurosci. 25, 10700–10711.

Sasaki, M., Black, J.A., Lankford, K.L., Tokuno, H.A., Waxman, S.G., Kocsis, J.D., 2006. Molecular reconstruction of nodes of Ranvier after remyelination by transplanted olfactory ensheathing cells in the demyelinated spinal cord. J. Neurosci. 26, 1803–1812.

Schiwy, N., Brazda, N., Muller, H.W., 2009. Enhanced regenerative axon growth of multiple fibre populations in traumatic spinal cord injury following scar-suppressing treatment. Eur. J. Neurosci. 30, 1544–1553.

Sternberger, L.A., 1979. Immunocytochemistry, second ed. John Wiley & Sons, New York.

Steward, O., Sharp, K., Selvan, G., Hadden, A., Hofstadter, M., Au, E., Roskams, J., 2006. A re-assessment of the consequences of delayed transplantation of olfactory lamina propria following complete spinal cord transection in rats. Exp. Neurol. 198, 483–499.

Takami, T., Oudega, M., Bates, M.L., Wood, P.M., Kleitman, N., Bunge, M.B., 2002a. Schwann cell but not olfactory ensheathing glia transplants improve hindlimb locomotor performance in the moderately contused adult rat thoracic spinal cord. J. Neurosci. 22, 6670–6681.

Takami, T., Oudega, M., Bethea, J.R., Wood, P.M., Kleitman, N., Bunge, M.B., 2002b. Methylprednisolone and interleukin-10 reduce gray matter damage in the contused Fischer rat thoracic spinal cord but do not improve functional outcome. J. Neurotrauma 19, 653–666.

Tetzlaff, W., Okon, E.B., Karimi-Abdolrezaee, S., Hill, C.E., Sparling, J.S., Plemel, J.R., Plunet, W.T., Tsai, E.C., Baptiste, D., Smithson, L.J., Kawaja, M.D., Fehlings, M.G., Kwon, B.K., 2011. A systematic review of cellular transplantation therapies for spinal cord injury. J. Neurotrauma 28, 1611–1682.

van den Berg, M.E., Castellote, J.M., de Pedro-Cuesta, J., Mahillo-Fernandez, I., 2010. Survival after spinal cord injury: a systematic review. J. Neurotrauma 27, 1517–1528.

Vavrek, R., Pearse, D.D., Fouad, K., 2007. Neuronal populations capable of regeneration following a combined treatment in rats with spinal cord transection. J. Neurotrauma 24, 1667–1673.

Williams, S.K., Franklin, R.J., Barnett, S.C., 2004. Response of olfactory ensheathing cells to the degeneration and regeneration of the peripheral olfactory system and the involvement of the neuregulins. J. Comp. Neurol. 470, 50–62.

Xu, X.M., Guenard, V., Kleitman, N., Bunge, M.B., 1995. Axonal regeneration into Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord. J. Comp. Neurol. 351, 145–160.