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RESEARCH ARTICLE

Microencapsulation improves inhibitory effects of transplanted olfactory ensheathing cells on pain after sciatic nerve injury

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Abstract

Olfactory bulb tissue transplantation inhibits P2X2/3 receptor-mediated neuropathic pain. However, the olfactory bulb has a complex cellular composition, and the mechanism underlying the action of purified transplanted olfactory ensheathing cells (OECs) remains unclear. In the present study, we microencapsulated OECs in alginic acid, and transplanted free and microencapsulated OECs into the region surrounding the injured sciatic nerve in rat models of chronic constriction injury. We assessed mechanical nociception in the rat models 7 and 14 days after surgery by measuring paw withdrawal threshold, and examined P2X2/3 receptor expression in $L_{4–5}$ dorsal root ganglia using immunohistochemistry. Rats that received free and microencapsulated OEC transplants showed greater withdrawal thresholds than untreated model rats, and weaker P2X2/3 receptor immunoreactivity in dorsal root ganglia. At 14 days, paw withdrawal threshold was much higher in the microencapsulated OEC-treated animals. Our results confirm that microencapsulated OEC transplantation suppresses P2X2/3 receptor expression in $L_{4–5}$ dorsal root ganglia in rat models of neuropathic pain and reduces allodynia, and also suggest that transplantation of microencapsulated OECs is more effective than transplantation of free OECs for the treatment of neuropathic pain.

Key Words: *nerve regeneration; peripheral nerve injury; sciatic nerve; microencapsulation; olfactory ensheathing cells; P2X2/3 receptor; neuropathic pain; dorsal root ganglion; sciatic chronic constriction injury; cell transplantation; NSFC grant; neural regeneration*

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Introduction

The mammalian olfactory nerve is one of the few components of the adult nervous system able to regenerate. Its regeneration capacity is attributable to the action of olfactory ensheathing cells (OECs), glial cells in the olfactory system that grow only after birth and continue to differentiate in adulthood (Tello et al., 2014). OECs are distributed in both the central and peripheral nervous systems and have a life span of 4–12 weeks. OECs inhibit gliosis and scar formation, promote myelination, and nourish nerves by secreting various neurotrophic factors that contribute to axonal sprouting and regeneration. These neurotrophic factors provide a suitable microenvironment for axon growth and migration. OECs are considered to have the strongest myelination capacity of all glial cells, and can regenerate throughout their lifespan. Therefore, they are ideal candidates for use in neuronal repair after injury. To date, remarkable effects of OEC transplantation have been obtained in studies investigating the treatment of central nervous system diseases. Shyu et al. (2008) used OEC transplantation in mouse models of cerebral ischemia, stroke, Alzheimer's disease and muscular atrophy. Furthermore, a number of studies have demonstrated the beneficial effects of OEC transplantation in the treatment of pain. Chen et al. (2010) confirmed that OEC transplantation has a therapeutic effect on intractable neuralgia in 17 patients with spinal cord injury, and Wu et al. (2011) reported that delayed OEC transplantation relieves spinal ganglion-induced nociception. However, the mechanism underlying the

therapeutic effects of OEC transplantation in pain remains unclear. We previously showed that olfactory bulb transplantation relieved neuropathic pain by inhibiting P2X3 receptor expression in the spinal cord of rats with chronic constriction injury (CCI); however, the cellular makeup of the olfactory bulb is complex.

Adenosine triphosphate acts on P2X receptors. P2X2/3 subtypes participate in the transmission of algesia and nociception information by primary sensory neurons (Zamboulis et al., 2013a, b; Dal Ben et al., 2015). In clinical practice, there is at present no specific treatment for neuropathic pain, and transplantation of olfactory ensheathing tissue or cells to promote neural repair is attracting increasing attention in this field.

Immune rejection is a key factor influencing the outcome of tissue and cell transplantation. Microencapsulation is a widely used immunoisolation technology that can overcome this complication. The basic principle of microencapsulation is to embed cells or tissue in a specific coating using chemical methods (Cameron et al., 2014). The microcapsule membrane allows nutrients, oxygen and other small molecules to pass through freely. However, the passage of macromolecules, such as immunoglobulins, is blocked, thus avoiding immune rejection (Han et al., 2014). A previous study from our group confirmed that transplantation of microencapsulated (MC) rabbit olfactory bulb cell suspension increased the expression of neurofilament 200, but suppressed that of glial fibrillary acidic protein in the injured spinal cord, and contributed to the recovery of spinal cord function (Liu et al., 2009). We also previously showed that olfactory bulb tissue transplantation reduced P2X2/3 receptor-mediated neuropathic pain (Liu et al., 2014). To date, no studies have investigated the effects of MC-OEC transplantation on neuropathic pain.

In the present study, we transplanted MC-OECs into the region surrounding the injured nerve in models of sciatic CCI, and assessed nociception by measuring mechanical paw withdrawal threshold. We also detected P2X2/3 receptor expression in rat $L_{4–5}$ dorsal root ganglion using immunohistochemical staining, to provide experimental data for OEC transplantation as a treatment for neuropathic pain.

Materials and Methods

Experimental animals

A total of 81 clean Sprague-Dawley rats of both sexes, aged 5–6 weeks and weighing 150–180 g, were provided by the Department of Laboratory Animal Science, Nanchang University, China. All protocols were approved by the Animal Care and Ethics Committee, Medical School, Nanchang University, China.

Culture and identification of OECs

One adult Sprague-Dawley rat was intraperitoneally anesthetized with 1% sodium pentobarbital 120 mg/kg, immersed in povidone iodine for 5 minutes, and decapitated. The skin on the head was removed to reveal the skull, which was then rinsed with PBS, and the posterior cranial fossa was opened to expose the olfactory bulbs. Both whole olfactory bulbs were removed, washed twice with PBS containing 100 U/L penicillin and 100 U/L streptomycin at 4°C, and then cut into pieces using ophthalmic scissors. Samples were digested with 0.25% trypsin at 37°C for 10 minutes until flocculent. The digestion was terminated by adding Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) (Boster, Wuhan, China) containing 15% fetal bovine serum (Transgen, Beijing, China). Tissue fluid was screened with a 200-mesh sieve and centrifuged at 710 × *g* for 5 minutes at room temperature. The supernatant was removed and samples were diluted with DMEM/F12. Cells at 1×10^9/L were seeded in a non-coated culture flask. Using a modification of Nash's differential adhesion method (Nash et al., 2001), suspended cells were placed in a new non-coated culture flask after 18–20 hours of culture. Thirty-six hours later, the suspended cells were transferred to a culture flask coated with poly-L-lysine (Solarbio, Beijing, China). The medium was replaced once every 3–4 days. Cell growth was observed and photographed intermittently. After 10 days of culture, cells were fixed in 4% paraformaldehyde (pH 7.4) at room temperature for 30 minutes, and blocked with 5% normal goat serum (Boster) at room temperature for 30 minutes. Cells were incubated with p75 (1:500; Affinity, Montgomery, TX, USA) at room temperature for 2 hours, with Cy3-labeled goat anti-rabbit IgG (1:100; Boster) at room temperature for 1 hour (no light) and DAPI (Boster) for 5 minutes (no light) using fluorescence microscopy (Nash et al., 2001).

Preparation of MC-OECs

The suspension was quantified at 1×10^9/L under an inverted microscope. Trypan blue staining was used to identify cell survival rate > 95%. The cell suspension was mixed 1:1 with 1.5% alginic acid solution (Sigma, St Louis, MO, USA). The mixture was sprayed into 20-mM barium chloride solution using a dual-chamber sprayer made in-house. Samples were mixed gently, without stirring, and precipitated. The supernatant was discarded. After two washes with physiological saline, microcapsules were suspended in just enough saline to cover the sedimentary microcapsules (Cameron et al., 2014).

Establishment of CCI models and experimental groups

The remaining 80 rats were equally and randomly assigned to four groups: CCI, CCI + OEC, CCI + MC-OEC, and sham. Rats in the CCI groups were injected intraperitoneally (i.p.) with 1% sodium pentobarbital (40 mg/kg). Under aseptic conditions, the main trunk of the sciatic nerve was exposed in the upper third of the rat thigh. No. 4 chromic catgut suture was tied loosely around four regions spaced 1 mm apart. The ligature did not affect the blood supply of the epineurium. In the sham group, the sciatic nerve was isolated but not constricted. Wounds were then sutured layer by layer and the rats allowed to recover from anesthesia. Reduced pain threshold and walking impairments confirmed the success of the model (Zhu et al., 2014). In the CCI + OEC and CCI + MC-OEC groups, 1×10^5 OECs or MC-OECs, respectively, per rat were transplanted into the region

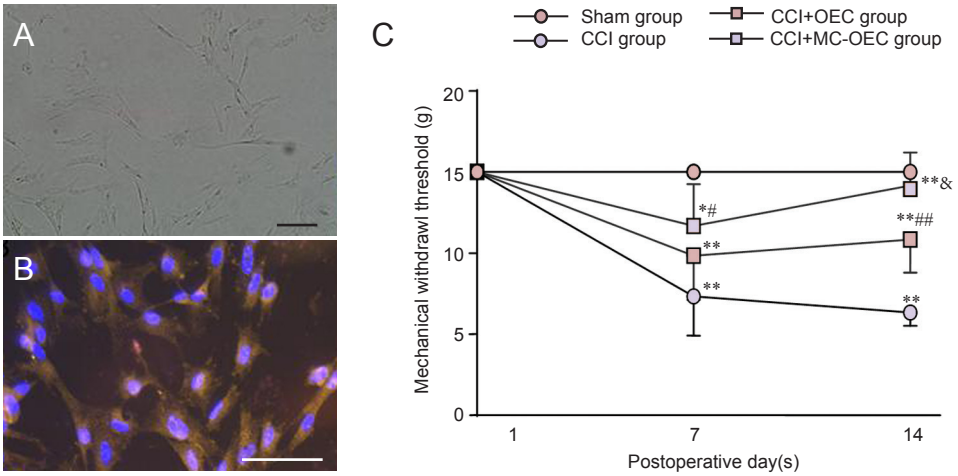

Figure 1 Morphology of OECs in culture and mechanical paw withdrawal threshold in rats with neuropathic pain.
(A) Inverted microscope image of cells at 10 days in culture; cells were bipolar, spindle-like, or had multiple processes. Scale bar: 100 μm. (B) Fluorescence microscope image of cells at 10 days in culture; blue, nuclei (DAPI); red, p75 (OEC marker). Scale bar: 100 μm. (C) Mechanical paw withdrawal threshold was higher in the CCI + OEC and CCI + MC-OEC groups than in the CCI group. At 14 days, withdrawal threshold showed greatest improvement in the CCI + MC-OEC group. *$P < 0.05$, **$P < 0.01$, *vs.* sham group at the same time point; #$P < 0.05$, ##$P < 0.01$, *vs.* CCI group at the same time point; &$P < 0.05$, *vs.* CCI + OEC group at the same time point (one-way analysis of variance and the least significant difference *post hoc* test). Data are expressed as the mean ± SD; $n = 6$ rats per group. CCI: Chronic constriction injury; MC: microencapsulated; OEC: olfactory ensheathing cell; DAPI: 4′,6-diamidino-2-phenylindole.

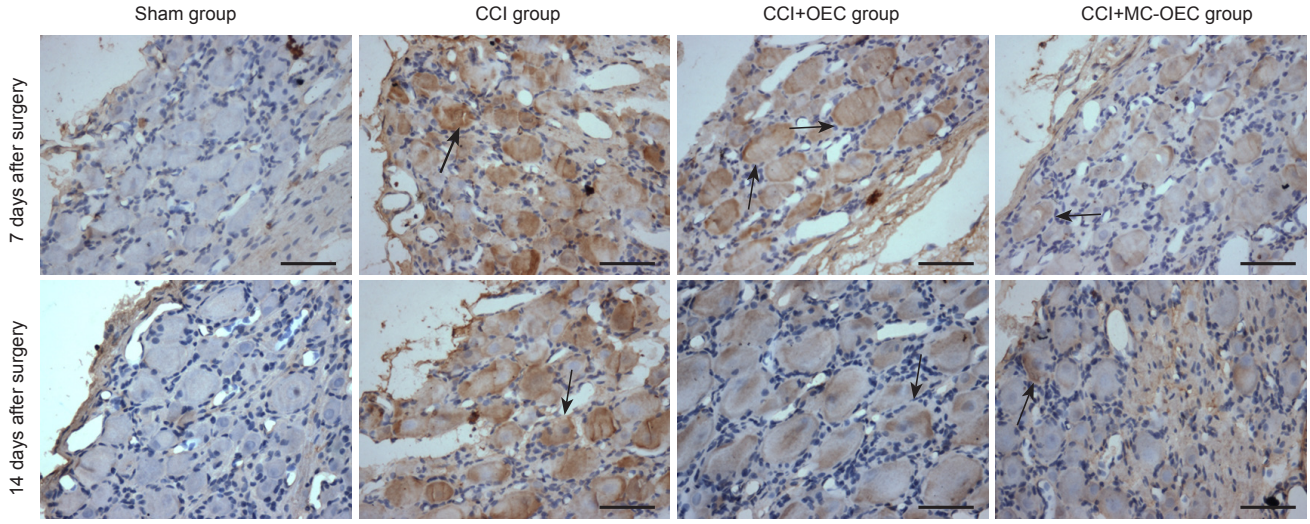

Figure 2 P2X2 receptor immunoreactivity in rat L_{4-5} dorsal root ganglia.
Immunoreactive products of the P2X2 receptor (yellow-brown, arrows), observed mainly in the cytoplasm, 7 and 14 days after surgery. CCI group had the strongest staining, followed by CCI + OEC, CCI + MC-OEC, and sham groups. Scale bars: 50 μm. CCI: Chronic constriction injury; MC: microencapsulated; OEC: olfactory ensheathing cell.

surrounding the injured nerve in the CCI models. On days 7 and 14, five rats were selected for immunohistochemical staining of P2X2 and P2X3.

Determination of mechanical withdrawal threshold in rats
The rats were placed in a transparent Plexiglas box (22 × 12 × 22 cm^3) before surgery (day 0), and 7 and 14 days after surgery. A wire mesh (1 × 1 cm^2) in the bottom of the box allowed assessment of mechanical withdrawal threshold using Von Frey filaments (Aesthesio, Danmic, CA, USA). Rats were acclimated to the box for 20 minutes before testing. The minimum bending force was 0.008 g. Successive filaments were applied 10 times each until the withdrawal threshold reached 50% (*i.e.*, paw withdrawal reflex observed five or more times for one filament) (Wang et al., 2014). Experiments were repeated in triplicate and the mean of the three values was obtained. The inter-stimulus interval was at least 15 seconds to allow stimulus-induced responses, such as foot-licking and leg-flicking, to disappear completely (Lin et al., 2014).

P2X2/3 receptor immunohistochemistry in L_{4-5} dorsal root ganglion
Rats were deeply anesthetized with 1% sodium pentobarbital (40 mg/kg, i.p.) and perfused through the ascending

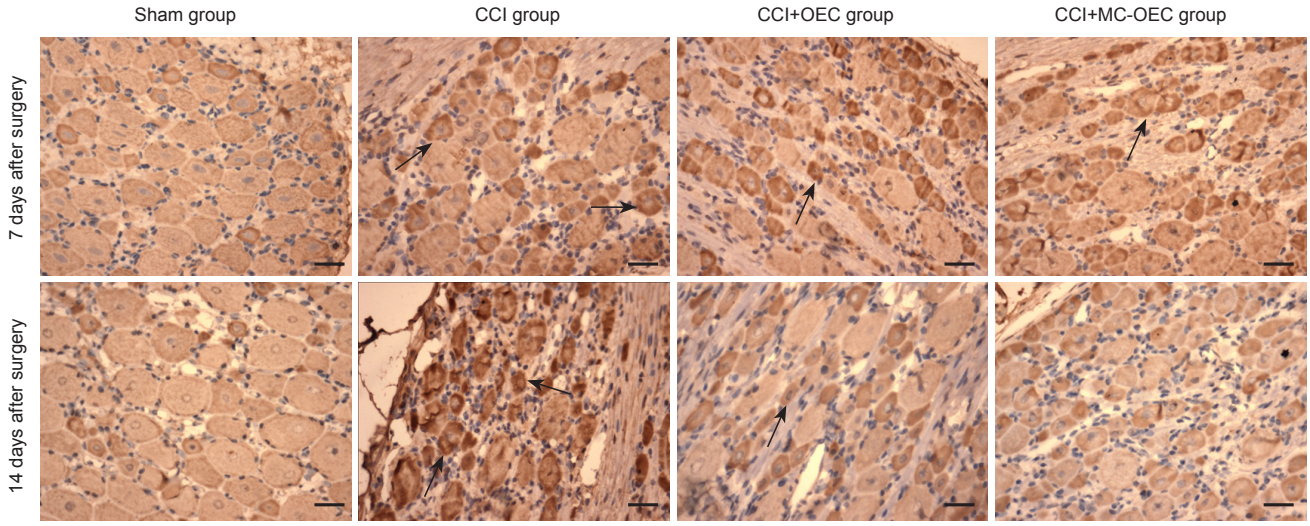

Figure 3 P2X3 receptor immunoreactivity in rat L_{4-5} dorsal root ganglia.
Immunoreactive products of the P2X3 receptor (yellow-brown, arrows), observed mainly in the cytoplasm, 7 and 14 days after surgery. CCI group had the strongest staining, followed by CCI + OEC, CCI + MC-OEC, and sham groups. Scale bars: 50 μm. CCI: Chronic constriction injury; MC: microencapsulated; OEC: olfactory ensheathing cell.

Table 1 Percentage and mean optical density of P2X2/3 receptor-immunoreactive cells in rat L_{4-5} dorsal root ganglia at 7 and 14 days (d) after surgery

	Percentage of immunoreactive cells (%)				Mean optical density of immunoreactive cells			
	P2X2		P2X3		P2X2		P2X3	
Group	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d
Sham	3.58±1.38	3.46±0.81	5.25±1.20	5.52±1.28	0.13±0.02	0.16±0.02	0.15±0.03	0.14±0.01
CCI	30.05±7.30*	40.46±10.07*	30.55±10.31*	50.27±9.99*	0.45±0.02*	0.51±0.04*	0.38±0.03*	0.43±0.03*
CCI+OEC	20.52±3.26*	15.56±2.04$^{*\#}$	20.61±6.73$^{*\#}$	17.48±2.03*	0.28±0.02$^{*\#}$	0.31±0.01$^{*\#}$	0.28±0.02$^{*\#}$	0.25±0.02$^{*\#}$
CCI+MC-OEC	8.72±0.96$^{*\#\&}$	5.58±2.00$^{*\#\&}$	10.55±2.21$^{*\#\&}$	8.36±1.02$^{*\#\&}$	0.17±0.02$^{*\#\&}$	0.18±0.02$^{\#\&}$	0.20±0.02$^{*\#\&}$	0.18±0.01$^{*\#\&}$

CCI group showed greatest P2X2/3 receptor expression, followed by CCI + OEC, CCI + MC-OEC, and sham groups. *$P < 0.05$, *vs.* sham group at the same time point; #$P < 0.05$, *vs.* CCI group at the same time point; &$P < 0.05$, *vs.* CCI + OEC group at the same time point (one-way analysis of variance and the least significant difference *post hoc* test). Data are expressed as the mean ± SD; $n = 5$ rats per group. CCI: Chronic constriction injury; MC: microencapsulated; OECs: olfactory ensheathing cells.

aorta with physiological saline followed by 4% paraformaldehyde at 4°C (pH 7.4). L_{4-5} dorsal root ganglion was obtained and embedded in paraffin blocks, which were cut into sections 4-μm thick. Paraffin sections were heated at 85°C for 20 minutes, dewaxed, and hydrated. Antigen retrieval was performed by boiling for 15 minutes. Sections were treated with 3% H_2O_2 at room temperature for 10 minutes, blocked with 10% normal goat serum (Boster) at room temperature for 15 minutes, incubated with guinea pig anti-P2X2 antibody (1:100; Millipore, Boston, MA, USA) and rabbit anti-P2X3 antibody (1:800; Millipore) in a wet box at 4°C overnight. Sections were then incubated with horseradish peroxidase-labeled goat anti-guinea pig IgG (1:100; Boster) or horseradish peroxidase-labeled goat anti-rabbit IgG (1:100; Boster) at 37°C for 50 minutes, visualized with 3,3′-diaminobenzidine, stained with hematoxylin, dehydrated, permeabilized and mounted. As a negative control test, the same procedure was performed but PBS was used instead of primary antibody. Three sections were obtained from each animal, and three non-overlapping fields were selected at random in each section using a light microscope (Olympus, Tokyo, Japan). The mean optical density and the percentage of P2X2/3 receptor-immunoreactive cells in L_{4-5} dorsal root ganglion were measured using Image-Pro Plus 6.0 software (Bio-Rad, Hercules, CA, USA). The percentage of immunoreactive cells (%) was calculated as follows: number of immunoreactive cells/total number of cells × 100%.

Statistical analysis

Data are expressed as the mean ± SD, and were analyzed using SPSS 19.0 software (SPSS, Chicago, IL, USA). Intergroup differences were explored using one-way analysis of variance and the least significant difference *post hoc* tests. $P < 0.05$ was considered statistically significant.

Results

Morphology and purity of OECs in culture

OECs cultured using Nash's differential adhesion method grew well. Under the inverted microscope, cells cultured for 10 days appeared bipolar, spindle-like or had multiple processes

(**Figure 1A**). Immunohistochemical staining revealed that the purity of p75-positive cells was above 90% (**Figure 1B**).

Effects of MC-OEC transplantation on mechanical withdrawal threshold in rats with neuropathic pain

On day 7, mechanical withdrawal threshold was lower in the CCI group than in the sham group ($P < 0.01$), but higher in the CCI + OEC and CCI + MC-OEC groups than in the CCI group ($P < 0.01$). A similar trend was observed on day 14. Furthermore, mechanical withdrawal threshold was significantly higher in the CCI + MC-OEC group than in the CCI + OEC group ($P < 0.05$; **Figure 1C**).

Effects of MC-OEC transplantation on P2X2/3 receptor expression in $L_{4–5}$ dorsal root ganglia of rats with neuropathic pain

Seven and 14 days postoperatively, immunoreactive products of P2X2/3 receptors were observable as yellow-brown spots distributed mainly in the cytoplasm. The percentage and mean optical density of P2X2/3 receptor-immunoreactive cells in rat $L_{4–5}$ dorsal root ganglia were lower in the sham group, and significantly higher in the CCI group ($P < 0.01$) at 7 and 14 days postoperatively. In the CCI + OEC group, receptor expression was lower than in the CCI group at 7 and 14 days ($P < 0.05$); the lowest expression was observed in the CCI + MC-OEC group, in which the percentage and optical density of P2X2/3-immunoreactive cells were significantly lower than those in the CCI + OEC and CCI groups ($P < 0.05$; **Figures 2**, **3**, and **Table 1**).

Discussion

We have shown that OEC transplantation reduces P2X2/3 receptor expression in $L_{4–5}$ dorsal root ganglion and increases mechanical paw withdrawal threshold in rats with neuropathic pain. Several previous studies have demonstrated that P2X receptors, in particular P2X2 and P2X3 subtypes, are involved in neuropathic pain (Jung et al., 2013; Zamboulis et al., 2013a, b). Adenosine triphosphate released by sympathetic nerve endings can activate P2X2/3 receptors in the dorsal root ganglion to contribute to neuropathic pain. In a previous study using P2X3 receptor antisense oligonucleotides, RNA interference, gene knockout technology and selective antagonists, it was shown that adenosine triphosphate and the P2X3 receptor act together to modulate the transmission of various pain signals (Prado et al., 2013). Pain is aggravated when P2X3 receptor expression is upregulated or its activity is enhanced, and downregulation or desensitization of the P2X3 receptor reduces pain (Burnstock, 2013).

Our present immunohistochemical results revealed that P2X2/3 receptor protein expression in $L_{4–5}$ dorsal root ganglia is elevated in CCI models, consistent with previous evidence. However, P2X2/3 receptor protein expression was diminished after OEC and MC-OEC transplantation. We propose the following as possible mechanisms of action: (1) OECs alter the local microenvironment by secreting various neurotrophic factors that contribute to neuronal survival and axon regeneration. At the same time, these glia promote angiogenesis in the injured area and reduce the formation of cavities within the spinal cord (Silva et al., 2014). (2) OECs ensheath nonmyelinated neurons; indeed, it was recently proposed that transplantation of these cells could be used as a treatment for demyelinating diseases (Roet and Verhaagen, 2014). (3) OECs relieve inflammation and structural damage to other cells, and promote the restoration of injured sciatic, facial, and optical nerves (Liu et al., 2014).

In the present study, noticeable improvements in behavior and immunohistochemistry were observed at 7 and 14 days in both groups that received OECs, but particularly in the CCI + MC-OEC group. We propose that this improvement was because the biocompatible high polymer microcapsules are semipermeable, blocking the passage of macromolecules such as immunoglobulins and hemocytes. Thus, an immune barrier was set up between suppressor and host cells, resulting in immune isolation. Therefore, OEC transplantation not only exerts a therapeutic effect on nerve damage, but also diminishes immunological rejection (Liu et al., 2013; Valente et al., 2013; Cao et al., 2014). Nevertheless, how MC-OECs specifically affect P2X2/3 receptor-mediated neuropathic pain warrants further investigation.

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Author contributions: *HZ, WJZ and QL provided data. HZ analyzed data, ensured the integrity of the data, wrote the paper, and performed statistical treatment. ZXL participated in study concept and design, manuscript authorization, and obtained the funding. QY, BLY, GCZ, DML, KY and HHZ provided technical or data support. ZXL and BLY served as principle investigators. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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