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Research Paper

Evidence of axon connectivity across a spinal cord transection in rats treated with epidural stimulation and motor training combined with olfactory ensheathing cell transplantation

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ABSTRACT

Olfactory ensheathing cells (OECs) are unique glia that support axon outgrowth in the olfactory system, and when used as cellular therapy after spinal cord injury, improve recovery and axon regeneration. Here we assessed the effects of combining OEC transplantation with another promising therapy, epidural electrical stimulation during a rehabilitative motor task. Sprague-Dawley rats received a mid-thoracic transection and transplantation of OECs or fibroblasts (FBs) followed by lumbar stimulation while climbing an inclined grid. We injected pseudorabies virus (PRV) into hindlimb muscles 7 months post-injury to assess connectivity across the transection. Analyses showed that the number of serotonergic (5-HT) axons that crossed the rostral scar border and the area of neurofilament-positive axons in the injury site were both greater in OEC- than FB-treated rats. We detected PRV-labeled cells rostral to the transection and remarkable evidence of 5-HT and PRV axons crossing the injury site in 1 OEC- and 1 FB-treated rat. The axons that crossed suggested either axon regeneration (OEC) or small areas of probable tissue sparing (FB). Most PRV-labeled thoracic neurons were detected in laminae VII or X, and ~25% expressed Chx10, a marker for V2a interneurons. These findings suggest potential regeneration or sparing of circuits that connect thoracic interneurons to lumbar somatic motor neurons. Despite evidence of axonal connectivity, no behavioral changes were detected in this small-scale study. Together these data suggest that when supplemented with epidural stimulation and climbing, OEC transplantation can increase axonal growth across the injury site and may promote recovery of propriospinal circuitry.

1. Introduction

A severe spinal cord injury (SCI) results in the loss of motor and sensory function below the level of the lesion. Several experimental treatments show therapeutic promise both individually and when combined with complimentary interventions. Olfactory ensheathing cells (OECs), for example, can facilitate moderate locomotor recovery when transplanted into the injured rat spinal cord (Li et al., 1997; Ramón-Cueto et al., 1998; Ramón-Cueto et al., 2000; Plant et al., 2003; López-Vales et al., 2007; Takeoka et al., 2011; Watzlawick et al., 2016) and autologous olfactory bulb-derived OEC transplantation combined with a peripheral nerve graft was associated with functional recovery in a clinically complete SCI patient (Tabakow et al., 2014). OEC-induced changes at the injury site that may promote recovery include reduction of tissue damage, modification of the astrocytic glial scar, and formation of cellular tracts that associate with astrocytes and regenerating axons (Lakatos et al., 2003; Li et al., 2005; Khankan et al., 2016). Consistent with these *in vivo* findings, *in vitro* studies reported that olfactory bulb-derived OECs secrete growth-promoting factors (Woodhall et al., 2001; Lipson et al., 2003), interact favorably with astrocytes (Lakatos et al., 2000; Fairless et al., 2005), and increase neurite outgrowth and sprouting (Chung et al., 2004; Runyan and Phelps, 2009; Khankan et al., 2015).

Lumbosacral epidural stimulation is another promising therapy that

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can restore locomotor and autonomic function in both rodents and humans with SCI (Gerasimenko et al., 2007; Harkema et al., 2011; Gad et al., 2013, 2015; Rejc et al., 2015). The combination of epidural stimulation with treadmill training generates consistent, weight-bearing locomotion in completely transected rats as early as two weeks after injury (Ichiyama et al., 2005; Lavrov et al., 2006; Gerasimenko et al., 2007; Lavrov et al., 2008). As neural activity is essential for neuronal survival and axon outgrowth during development (Mennerick and Zorumski, 2000), evidence suggests that stimulation-induced activity also enhances neuronal survival, axon growth, and synaptogenesis after injury (Al-Majed et al., 2000; Mondello et al., 2014). Additionally, long-term intraspinal microstimulation of the cervical spinal cord induced significant improvements in forelimb reaching function after a contusion injury (Kasten et al., 2013). These data suggest that spinal cord stimulation can have lasting effects on the locomotor circuitry, and when combined with activity-specific training, may promote long-term functional recovery.

While epidural stimulation with training alone promotes some recovery without axon regeneration, in this study we asked if OEC transplantation, combined with long-term epidural stimulation and climbing, would have additive effects on measures of spared tissue, astroglial bridge formation, and axon regeneration. Because many cell types may confer therapeutic benefits when transplanted after SCI compared to a media or saline-injected control (Tetzlaff et al., 2011), we chose to use dermal fibroblast (FB) transplants as cellular controls. To evaluate axon regeneration in the injury site we used novel anatomical assessments for a severe spinal cord transection model such as the transsynaptic, retrograde tracer pseudorabies virus (PRV) to evaluate synaptic connectivity between the rostral and caudal stumps, and a 3-D visualization of descending motor-associated serotonergic axons in the injury site. We then characterized PRV-labeled interneurons located rostral to the injury site to identify populations of thoracic interneurons that reformed synaptic connections with caudal hindlimb circuits.

2. Materials and methods

2.1. Animals

The Chancellor's Animal Research Committee at UCLA approved all experiments. Rats were housed under standard conditions with free access to food and water. Olfactory bulb-derived OECs and skin FBs were obtained from 8 to 10 week old female enhanced green fluorescent protein (eGFP)-expressing Sprague-Dawley rats (Perry et al., 1999). One eGFP-expressing rat was used to generate the OECs or FBs transplanted into each spinal rat. An overdose of ketamine-xylazine was used for euthanasia before the extraction of olfactory bulbs and abdominal skin biopsies. Ten female Sprague-Dawley rats (Charles River, 10–12 weeks old) received complete mid-thoracic spinal cord transections and were maintained for 6–7 months.

2.2. Olfactory bulb-derived OEC and FB cultures

Methods to prepare OEC primary and immunopurified cultures were similar to those of Ramón-Cueto et al. (2000) and identical to the description in Khankan et al. (2016). OECs were dissected from the first two layers of eGFP-expressing rat olfactory bulbs. Following cell dissociation, OECs were maintained *in vitro* for 5 days and immunopurified using anti-p75-nerve growth factor receptor antibody (1:5; clone 192). Purified OECs were cultured for an additional 7 days before transplantation. Rats were shaved and skin biopsies from the abdominal wall were dissociated into FB cultures as described (Takashima, 2001; Khankan et al., 2016). FBs were passaged 1–2 times before transplantation (12–14 days *in vitro*).

2.3. Implantation of stimulating electrodes and intramuscular recording electrodes

Rats were deeply anesthetized with 1–2.5% isoflurane gas during all surgeries. A midline longitudinal incision was made in the scalp and two stainless steel screws were inserted through pilot holes so that the tip of the screws contacted the cortical dura mater. Teflon-coated wire electrodes (AS632, Cooner Wire) were attached to a head-plug connector (Amphenol) and the head-plug was secured to the skull as described in Iyer et al. (2010). Next, partial laminectomies were performed at spinal levels L2 and S1 and the epidural stimulating electrodes were fixed to the dorsal dura mater and attached to the head-plug (Ichiyama et al., 2005). Surgical EMG implantation methods were similar to those in Roy et al. (1992) and Gad et al. (2013). The skin and fascial incisions exposed the bellies of the soleus and tibialis anterior (TA) muscles bilaterally. Two wires from the head-plug connector were routed underneath the skin from the cranium to each muscle. The electrodes were positioned in the mid-belly of the muscle and secured with two sutures. Proper electrode positioning was verified by muscle contractions elicited by stimulation and then a ground wire was placed subcutaneously in the dorsum of the rat. The skin was sutured and disinfected with betadine, pain medication and fluids were administered, and the rats recovered in an incubator.

2.4. Spinal cord injury and cell transplantation

In a second surgery 3 weeks later, a skin incision was made at vertebral levels T6 to L1, the paravertebral muscles were retracted, and partial laminectomies were performed at T7 and T8. The dorsal dura was incised longitudinally and then laterally at both ends to expose the spinal cord. The spinal cord was completely transected near spinal level T7/T8 with micro-scissors, leaving the ventral and lateral dura mostly intact. The rostral and caudal stumps were separated, lifted, and the intact dura was scraped with a glass probe and small cotton balls to sever any spared axons.

Before transplantation, OECs or FBs were rinsed, centrifuged, and re-suspended at a concentration of 100,000 cells per 1 μl in serum-free DMEM (Khankan et al., 2016). Immediately after the transection, ~400,000 total OECs (n = 5) or FBs (n = 5) were injected stereotactically into the spinal cord 1 mm rostral and caudal to the transection as in Ramón-Cueto et al. (2000). The spinous processes of the T7-T13 vertebra were stabilized with a stainless steel bar to protect the transection site. Bladder expressions were performed 3×/day for the first month, and 2×/day at 12-hour intervals thereafter. Rats were inspected daily for weight loss or dehydration and their urine was tested weekly with Multistix 10 SG reagent strips (Siemens). Investigators were blinded to the cell treatment groups from the time of transplantation until completion of the study.

2.5. Epidural stimulation, climb training, and behavioral analyses

Before surgery, rats were trained to climb a 1-inch grid positioned at 60 and 90-degree angles from the horizontal plane (Ramón-Cueto et al., 2000; Ziegler et al., 2011). The inclined grid was connected to a 36-inch tower attached to a low-friction Plexiglas platform covered on 3 sides with dark plastic. Rats received a food reward when they completed the climb and lifted themselves onto the slippery platform. Climb training was conducted for 20 minutes/day, 3×/week, for 6 months, starting 1 month after spinal cord transection. Rats received 40 Hz epidural stimulation during each climbing session at a voltage of 95% of threshold, which was calculated daily by determining the minimum stimulation to elicit a palpable muscle contraction. Climbing tests were videotaped and EMG activity in the soleus and TA muscles was recorded pre-injury, and with and without epidural stimulation at 3, 5, and 7 months post-injury.

Two independent observers reviewed the videos of the climbing