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Noradrenergic innervation of the rat spinal cord caudal to a complete spinal cord transection: Effects of olfactory ensheathing glia

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ABSTRACT

Transplantation of olfactory bulb-derived olfactory ensheathing glia (OEG) combined with step training improves hindlimb locomotion in adult rats with a complete spinal cord transection. Spinal cord injury studies use the presence of noradrenergic (NA) axons caudal to the injury site as evidence of axonal regeneration and we previously found more NA axons just caudal to the transection in OEG- than media-injected spinal rats. We therefore hypothesized that OEG transplantation promotes descending coeruleospinal regeneration that contributes to the recovery of hindlimb locomotion. Now we report that NA axons are present throughout the caudal stump of both media- and OEG-injected spinal rats and they enter the spinal cord from the periphery via dorsal and ventral roots and along large penetrating blood vessels. These results indicate that the presence of NA fibers in the caudal spinal cord is not a reliable indicator of coeruleospinal regeneration. We then asked if NA axons appose cholinergic neurons associated with motor functions, i.e., central canal cluster and partition cells (active during fictive locomotion) and somatic motor neurons (SMNs). We found more NA varicosities adjacent to central canal cluster cells, partition cells, and SMNs in the lumbar enlargement of OEG- than media-injected rats. As non-synaptic release of NA is common in the spinal cord, more associations between NA varicosities and motor-associated cholinergic neurons in the lumbar spinal cord may contribute to the improved treadmill stepping observed in OEG-injected spinal rats. This effect could be mediated through direct association with SMNs and/or indirectly via cholinergic interneurons.

Introduction

The noradrenergic (NA) system can activate locomotor pattern generation in the absence of supraspinal innervation. For example, administration of noradrenaline, its precursor dopamine, and its α1- and α2-receptor agonists such as clonidine and methoxamine induce and/or modulate hindlimb locomotion in acute complete spinal transected (i.e., spinal) adult cats (Barbeau et al., 1993; Chau et al., 1998; Giroux et al., 1998), chronically transected adult spinal rodents (Guertin, 2004), and in severely injured humans (Barbeau and Norman, 2003). Administration of both noradrenaline and its agonists also elicit and maintain fictive locomotion in the neonatal spinal cord *in vitro* (Kiehn et al., 1999; Sqalli-Houssaini and Cazalets, 2000). Furthermore, transplantation of embryonic locus coeruleus tissue into the spinal cord caudal to the lesion, i.e., caudal stump, in adult spinal rats reinnervates previous targets (Gimenez y Ribotta et al., 1996) and leads to improved hindlimb stepping (Yakovleff et al., 1989; Gimenez y Ribotta et al., 1998a; Gimenez y Ribotta et al., 1998b) and recovery of withdrawal reflexes (Moorman et al., 1990). NA α1- and α2-receptors are expressed broadly throughout the gray mater in an intact spinal cord and are up-regulated in the lumbar segments after a complete spinal cord transection (Roudet et al., 1994; Roudet et al., 1996). These observations indicate that the spinal cord caudal to the lesion remains responsive to noradrenaline after the loss of coeruleospinal innervation and, with proper stimulation, can contribute to locomotor activity of spinal animals.

When OEG transplantation is combined with long-term treadmill stepping in adult spinal rats, we found that step training alone did not improve stepping, while OEG transplantation alone improved plantar step performance. When OEG transplantation and step training were combined, however, locomotor ability improved over time and step frequency and trajectory were not significantly different from sham rats (Kubasak et al., 2008). The mechanisms by which OEG transplantation and treadmill training contribute to this recovery remain unclear. Based on previous pharmacological evidence, one possibility is that OEG promote regeneration of coeruleospinal axons that then contribute to locomotor recovery. After a complete spinal cord transection, several studies interpreted the presence of NA axons in the caudal stump as evidence of spinal cord regeneration (Chen et al., 1996; Ramon-Cueto et al., 2000; Lopez-Vales et al., 2006b).

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Kubasak et al. (2008), however, reported that dopamine β-hydroxylase (DBH; a marker for noradrenergic axons)-positive axons penetrate the glial scar and enter the spinal cord caudal to the transection in both media- and OEG-injected rats. The density of DBH-positive axons immediately caudal to the lesion was higher in OEG- than media-injected rats, suggesting that regenerating DBH-positive axons may cross the glial scar and contribute to the improved hindlimb stepping seen in OEG-injected rats.

Alternatively, OEG may promote the reorganization of spinal circuits within the caudal stump that leads to the recovery of hindlimb locomotion. For example, OEG transplantation influences the frequency of interactions between serotonergic axons and motor-associated cholinergic neurons in the caudal stump (Takeoka et al., 2009), and DBH-positive axons may undergo a similar reorganization. Two groups of spinal cholinergic interneurons, the central canal cluster and partition cells (Barber et al., 1984; Phelps et al., 1984), project directly to ipsilateral and contralateral somatic motor neurons (SMNs; Houser et al., 1983; Barber et al., 1984; Phelps et al., 1984) and influence their excitability (Miles et al., 2007), and are active during fictive locomotion (Carr et al., 1995; Huang et al., 2000). As noradrenaline modulates the output of SMNs (Harvey et al., 2006) and utilizes non-synaptic as well as synaptic transmission (Beaudet and Descarries, 1978; Ridet et al., 1993; Hentall et al., 2003), axonal varicosities located within a few microns to a millimeter from motor-associated cholinergic neurons could potentially modify their activity and contribute to hindlimb stepping ability.

In this study we examined the caudal stump of a cohort of spinal rats that demonstrated differential functional recovery between media- and OEG-injected spinal rats (Kubasak et al., 2008). We sought to determine (1) if there are more DBH-axons distributed throughout the caudal stump in OEG- than media-injected spinal rats; (2) the source of DBH-positive axons in the caudal stump of adult spinal rats; and (3) if OEG transplantation influences the density of DBH-positive axons apposing motor-associated cholinergic neurons.

Materials and methods

Animal procedures

All procedures followed the NIH guidelines and were approved by the Chancellor's Animal Research Committee at UCLA. Olfactory bulb-derived OEG were dissected and immunopurified as reported in Kubasak et al. (2008). Briefly, we dissected OEG from olfactory bulbs of 8- to 10-week-old Wistar Hannover rats (Harlan Laboratories, Indianapolis IN), used the p75 nerve growth factor receptor antibody (NGFR, 1:5, Chandler et al., 1984) to immunopurify OEG from the primary culture on days 7–8, and harvested cells after 17–19 days *in vitro*. Female Wistar Hannover rats (Harlan Laboratories, Indianapolis, IN), 10–12 weeks old, were anesthetized deeply with 2% isoflurane and received a complete spinal cord transection at ~T9. Media with or without 400,000 OEG were injected 1 mm rostral and 1 mm caudal to the transection. At 1 month post-injury 50% of the rats in both the media- and OEG-injected groups began intensive manual hindlimb step training for 20 min a day, 5 days/week for 6 months (more than 50 h/rat over a 6-month period; Kubasak et al., 2008). In the present study we analyzed the entire caudal stump (i.e., from below the transection to the sacral levels) of 12 rats chosen for our previous analyses of the transection site. Three rats in each of four experimental groups (media-untrained, media-trained, OEG-untrained, and OEG-trained) represented a range of stepping abilities at 7 months (see Table 1 in Kubasak et al., 2008). Two female adult sham rats were tested for stepping kinematics (Fig. 2 in Kubasak et al., 2008) and their spinal cords were studied as positive immunohistochemical controls. To identify the association of NA axons with platelet endothelial cell adhesion molecule (PECAM)-labeled blood vessels, an additional adult OEG-injected spinal rat was perfused at both 1 and 7 months post-transection. We analyzed an additional 6 media- and 6 OEG-injected rats with another blood vessel marker, *lycopersicon esulentum* (tomato) lectin and observed similar relationships between the blood vessels and NA axons.

Tissue preparation

Rats were anesthetized deeply with ketamine (0.9 μl/g) and Anased (0.5 μl/g), perfused with 4% paraformaldehyde, and post-fixed overnight for analyses of NA axons or for 4 h for blood vessel analyses. Spinal cords were dissected, cryoprotected, frozen on dry ice, and stored at −80 °C. We sectioned all spinal cords sagittally at 25 μm and mounted the sections, so that each of the 16 slides contained every 16th section. Alternate slides were double-labeled for DBH and choline acetyltransferase (ChAT; current study). The other 50% of the sections were used to examine the distribution of serotonin and ChAT in the caudal stump (Takeoka et al., 2009).

Immunocytochemical procedures

To identify DBH-labeled axons, we used a mouse anti-rat DBH (1:1,000, Chemicon, Temecula, CA) raised against purified bovine DBH that recognizes both the soluble (70 kDa) and membrane bound (75 kDa) forms of the enzyme. For ChAT immunolabeling, a polyclonal anti-ChAT antiserum (AB144P, 1:500, Chemicon, Temecula, CA) raised against human placental ChAT was used. For tyrosine hydroxylase (TH) immunolabeling, both monoclonal (TH-16, 1:1,000, Sigma-Aldrich, St Louis, MO) and rabbit polyclonal (ab6211, 1:8,000, Abcam Inc, Cambridge, MA) antibodies were used. To most effectively identify blood vessels, we used purified mouse anti-rat CD31 (PECAM-1; 1:150, BD Biosciences, San Jose, CA).

To localize NA axons, sections were initially washed with 0.1M Tris buffer containing 1.4% NaCl and 0.1% bovine serum albumin (TBS) followed by a 30 min pre-soak in 0.3% H_2O_2 and 0.1% NaN_3. After a 1% Triton pre-soak for 15 min, sections were incubated in 1.5% normal horse serum with 0.3% Triton and an additional blocking step to reduce non-specific avidin/biotin binding (Vector Laboratories; Burlingame, CA). Sections were incubated in mouse anti-rat DBH antibody (1:1,000) overnight at room temperature. The following day, sections were rinsed in TBS, anti-mouse biotinylated IgG (1:200, Elite Standard kit, Vector laboratories) for 1 h, followed by avidin–biotin complex (1:100) for 1 h. Tissues were rinsed in 0.1 M acetate buffer for 10 min before being developed with 0.06% 3,3-diaminobenzidine (DAB) with Nickel glucose oxidase, producing a black immunoproduct.

For ChAT immunolabeling we followed the protocol reported in Takeoka et al. (2009). We rinsed the sections followed by the normal serum and avidin–biotin blocking steps before incubating with the goat anti-ChAT antiserum (1:500) overnight at room temperature. Sections were incubated with Vector rabbit anti-goat IgG (1:200, Elite Goat kit, Vector Laboratories, Burlingame, CA) and ABC (1:100) for 1 h each for signal amplification. Sections were developed in 0.02 M imidazole-DAB producing an amber brown product. The TH and PECAM protocols were identical to that reported for ChAT. The tissues were processed with sections from intact spinal cords as positive controls.

Quantification

DBH-positive axons were quantified using methods identical to those reported by Takeoka et al. (2009) and similar to those of Fouad et al. (2005) and Kubasak et al. (2008). We counted all DBH-immunopositive axons longer than 25 μm in alternate sections, i.e., 50% of the lower thoracic to sacral spinal cord. For arborized processes, we first traced and counted the longest single fiber and then other isolated immunopositive fibers. We measured the tissue