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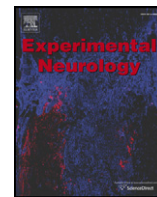
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# Noradrenergic innervation of the rat spinal cord caudal to a complete spinal cord transection: Effects of olfactory ensheathing glia

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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.

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## 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  $\alpha$ 1- and  $\alpha$ 2-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 (Yakovlev 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  $\alpha$ 1- and  $\alpha$ 2-receptors are expressed broadly throughout the gray matter 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  $\beta$ -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  $\mu$ l/g) and Anased (0.5  $\mu$ l/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  $-80^{\circ}\text{C}$ . We sectioned all spinal cords sagittally at 25  $\mu$ m 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%  $\text{H}_2\text{O}_2$  and 0.1%  $\text{NaN}_3$ . 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 immunoprecipitate.

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  $\mu$ m 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



volume processed for DBH-immunoreactivity using Openlab (Improvision Inc., Lexington, MA) software and calculated the DBH-fiber density. NA varicosities adjacent to cholinergic neurons were quantified as reported in Takeoka et al. (2009) and similar to that used by Miller and Salvatierra (1998) and Mullner et al. (2008). The quantification method is based on the assumption that NA is released both synaptically and non-synaptically and does not distinguish between these transmission modes. We counted the number of DBH-positive varicosities directly adjacent (maximum of 1–2  $\mu\text{m}$  away) to the cholinergic somata or processes at high magnification with the light microscope and normalized these counts per unit volume ( $\text{mm}^3$ ).

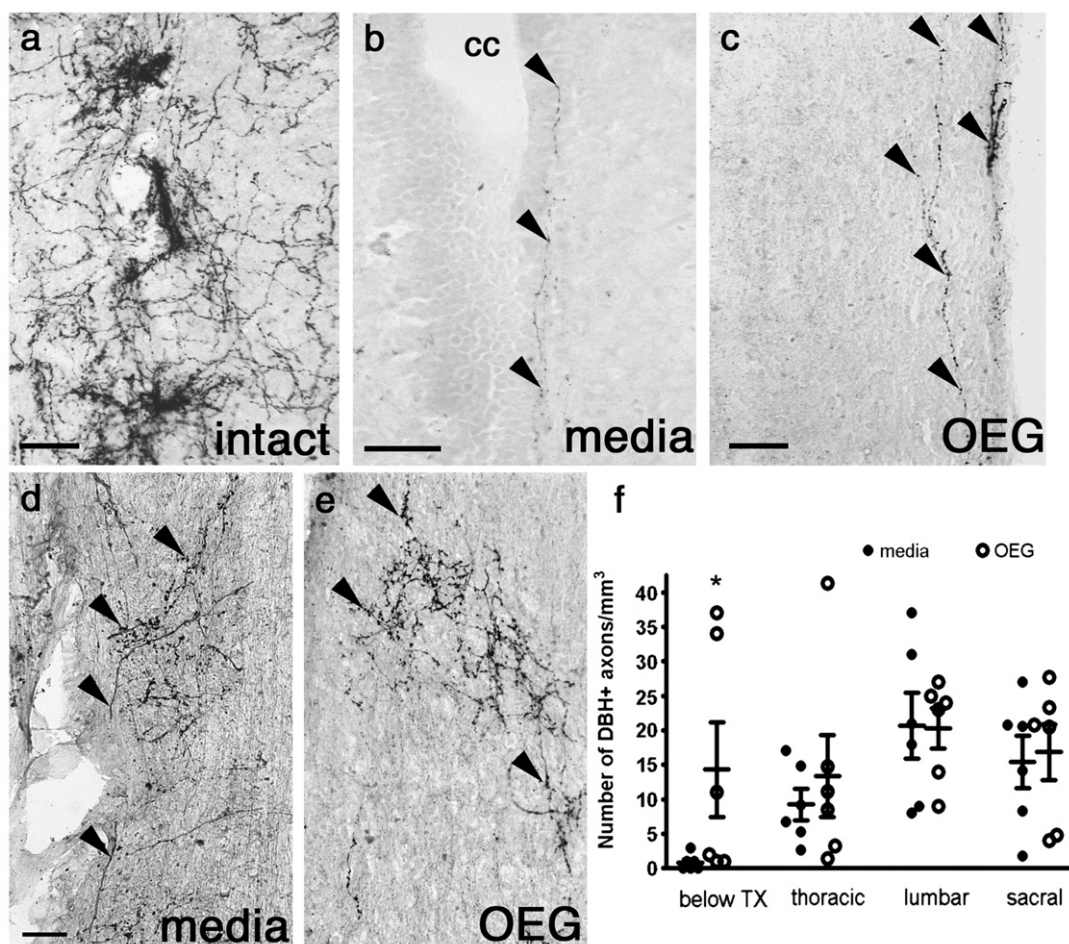
### Statistical analyses

All statistical analyses were carried out using the resampling method (Efron and Tibshirani, 1991) that computationally simulates the null hypothesis with minimal assumptions about the data distribution or estimators (i.e., the means in this study) and, therefore, is suitable for small samples. Scripts for individual analyses were written using Resampling stats in MATLAB software package (Resampling Stats, Inc, Arlington, VA; MATLAB 7.0, Mathworks, Inc, Natick, MA). Statistical significance of mean differences was set at  $P < 0.05$ .

### Results

#### Media- and OEG-injected adult spinal rats have NA axons throughout the caudal stump

DBH-positive axons densely innervate the dorsal horn, laminae VII, VIII, and X of an intact spinal cord, and concentrate along the sympathetic preganglionic neurons in lamina VII (Fig. 1a; Aramant et al., 1986; Fritschy et al., 1987; Proudfit and Clark, 1991; Rajaofetra et al., 1992a; Rajaofetra et al., 1992b). The descending coeruleospinal tract originates mainly from the ventral caudal locus coeruleus and the A5 and A7 cell groups in the pontine lateral tegmentum (Satoh et al., 1977; Moore and Bloom, 1979; Westlund et al., 1981, 1983) and degenerates within several weeks after a complete spinal cord transection (Commissiong, 1985). Surprisingly, however, we detected a small number of NA axons immediately caudal to the transection site in both media- and OEG-injected spinal rats (Kubasak et al., 2008). Although 25% of spinal rats analyzed contained no DBH-positive axons just below the lesion (Kubasak et al., 2008), we now report that all rats in the same cohort of spinal rats had DBH-positive axons in the entire caudal stump, regardless of transplantation status. These DBH-immunoreactive axons were often present within lamina X (Fig. 1b), laminae I–III (Figs. 1d, e) and most commonly coursing rostrocaudally within or along the edge of the white matter (Fig. 1c).



**Fig. 1.** Noradrenergic (NA) axons in sagittal sections of lower thoracic spinal cord processed for dopamine  $\beta$ -hydroxylase (DBH) from an intact rat (a), and media- (b, d), and OEG-injected (c, e) rats 7 months after transection. (a–c) Many DBH-labeled axons are found around presumed sympathetic preganglionic neurons in lamina VII in an intact (a) rat. Only a few DBH immunopositive axons (arrowheads) are detected in lamina X along the central canal (cc; b) or at the edge of the white matter (c) in spinal rats. (d, e) DBH-positive axons (arrowheads) arborize extensively in the dorsal horn. (f) The density of DBH-labeled fibers is greater in OEG- than media-injected rats just below the transection site (data from Kubasak et al., 2008), but not significantly different in the remainder of the caudal spinal cord. Bars in (f) represent the mean  $\pm$  SEM for 6 media- and 6 OEG-injected rats. Scale a–e = 50  $\mu\text{m}$ .



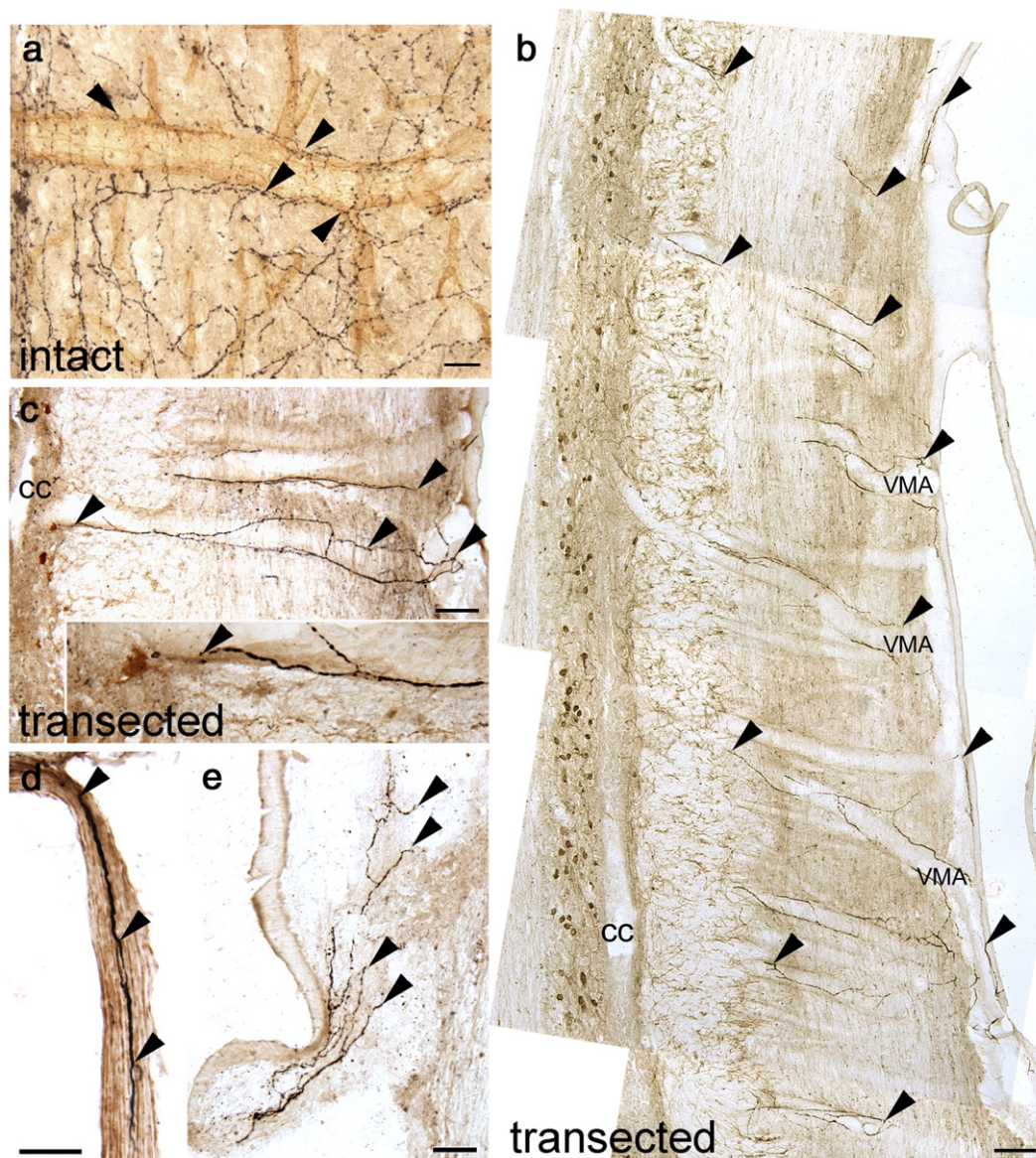
Although OEG-injected rats contained more DBH-labeled axons in the glial fibrillary acidic protein (GFAP)-positive region immediately below the transection than media-injected rats (Kubasak et al., 2008), there were no differences in axonal density between media- and OEG-injected rats within the remainder of the caudal stump (Fig. 1f; thoracic:  $P=0.34$ , lumbar:  $P=0.49$ , sacral:  $P=0.40$ ).

As the administration of noradrenaline improves the stepping performance of spinal animals, we asked if the overall density of NA axons in the caudal stump would correlate with the better hindlimb stepping performance observed in the OEG-trained rats compared to the other groups (Kubasak et al., 2008). When we compared the density of NA axons in the OEG-trained group to each of the other three groups, none were statistically different. We then asked if step training influenced the density of DBH-positive axons present in the caudal stump. Neither training groups combined (OEG- and media-trained vs. OEG- and media-untrained;  $P=0.25$ ) nor comparison of training status within the same transplantation status (OEG-trained

vs. OEG-untrained or media-trained vs. media-untrained;  $P=0.22$  and  $P=0.23$ , respectively) affected the DBH fiber density. Thus the overall density of DBH-positive axons below the lesion did not correlate with the stepping ability of spinal rats.

#### Peripheral NA axons innervate the spinal cord caudal to the transection

Amenta et al. (1987) reported that NA axons associated with the spinal cord blood vessels could originate from peripheral sympathetic neurons. Other studies demonstrated that NA axons coursed along meningeal blood vessels and entered the spinal cord or associated with blood vessels found within the ventral and dorsal roots (McNicholas et al., 1980; Karlsson and Hildebrand, 1993; Hildebrand et al., 1997). In intact rats, DBH-positive axons coursed near PECAM-positive large and small diameter blood vessels (Fig. 2a), but could not be distinguished from the abundant coeruleospinal innervation. In spinal rats, however, the association of DBH-positive axons with



**Fig. 2.** Noradrenergic (NA) axons enter the spinal cord in association with blood vessels via ventral roots and meninges regardless of transplantation status. (a) Lumbar spinal cord of an intact rat shows numerous dopamine  $\beta$ -hydroxylase (DBH)-positive axons (black; arrowheads) aligned along a penetrating blood vessel identified with platelet endothelial cell adhesion molecule (PECAM; brown). (b, c) DBH-positive axons (black; arrowheads) course along the wall of large ventralmedium arteries (VMA) and extend toward lamina X that contains ChAT-positive central canal cluster cells (amber brown; c is enlarged in inset). cc, central canal (d) A ventral root contains DBH-positive axons (arrowheads) in adult spinal rats. (e) DBH-labeled axons (arrowheads) often are detected along the meninges. Scale a = 25  $\mu$ m, Scale d, e = 50  $\mu$ m, Scale b, c = 100  $\mu$ m.



blood vessels was clearly evident along the large diameter ventral–median spinal arteries (Fig. 2b; VMA). These DBH-labeled fibers course along the endothelial wall of the VMA within the white matter and extend toward lamina X (Fig. 2c). We also found DBH-positive axons within the ventral (Fig. 2d), and less frequently the dorsal (data not shown) roots as reported in various species (Stevens et al., 1983; Hara and Kobayashi, 1987; Kummer, 1994). Most commonly, we detected branches of DBH-positive axons within the meninges (Fig. 2e). We also verified that NA axons entering the caudal spinal cord from the periphery expressed TH, the rate limiting enzyme for the synthesis of catecholamines (data not shown).

To determine the relationship between DBH-positive axons and blood vessels in the caudal stump, we identified the vessels with anti-PECAM. We often detected DBH-positive axons associated with large diameter (25 to 50  $\mu\text{m}$ ) blood vessels in the white matter, within the ventral roots (data not shown), and the gray matter (Fig. 3a). Less frequently, we found DBH-labeled axons along small blood vessels (5–15  $\mu\text{m}$  diameter) in the gray matter (Fig. 3b). DBH-positive axons readily penetrated the GFAP-negative scar formed around the lesion in both media- and OEG-injected rats (Kubasak et al., 2008). Therefore, in addition to supraspinal reinnervation, the association of sympathetic axons with blood vessels contributed to the DBH-labeled axons detected in the GFAP-negative zone at 1 (Fig. 3c) or 7 months (Fig. 3d) post-transection. Labeled axons course along PECAM-positive blood vessels within the transection site and often associate with blood vessels that penetrate the GFAP-negative zone from the periphery (data not shown). Together, our data suggest that NA axons within or below the transection site may be sympathetic axons and once these axons enter the spinal cord, they cannot be distinguished from coerulespinal regeneration.

*Intrinsic DBH-positive somata are present in the cervical spinal cord but not below the transection*

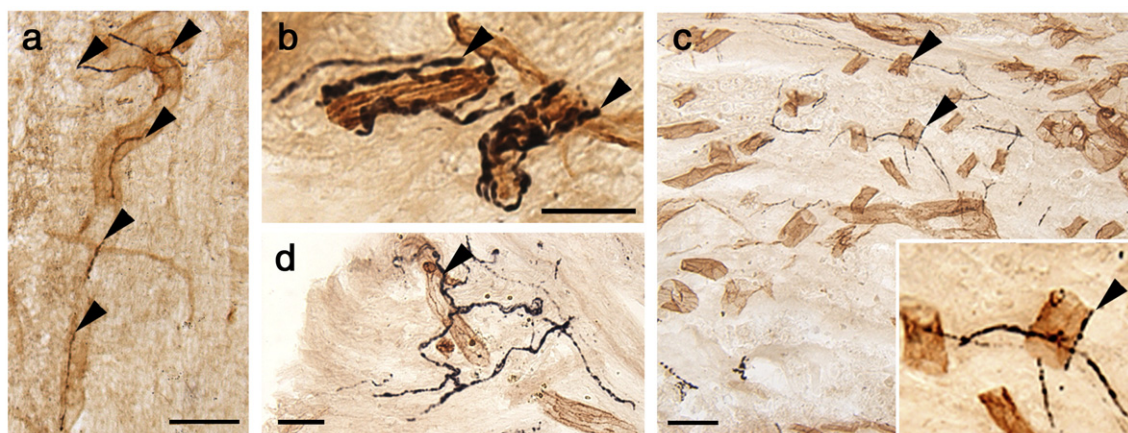
We asked if there are other sources that could contribute to NA axons in the caudal stump of spinal rats. Mouchet et al. (1986) first reported the presence of intrinsic spinal NA neurons in adult rats but found them only at cervical levels, whereas TH-positive dopaminergic interneurons were present at both the cervical and sacral levels. In contrast, Cassam et al. (1997) detected DBH-positive interneurons throughout the spinal cord of intact rats and reported that their number increased in the spinal cord caudal to a complete mid-thoracic transection. Therefore, we asked if the DBH-labeled axons in the caudal stump are derived from NA interneurons. We rarely

detected DBH-immunolabeled somata and found them only in the cervical dorsal and lateral funiculi (Fig. 4a). The DBH-positive somata were 20–35  $\mu\text{m}$  in diameter with bipolar or multipolar processes. We also found a few TH-positive somata that were 20–25  $\mu\text{m}$  in diameter in the upper cervical gray matter (Fig. 4b) that resembled those reported by Mouchet et al. (1986) and Cassam et al. (1997). We did not detect any DBH-positive interneurons between the lower thoracic and sacral segments of our spinal rats but found some TH-positive somata (0–15 somata per rat) in the sacral lateral white matter (Fig. 4c). Sacral TH-positive interneurons were typically bipolar and smaller (20–25  $\mu\text{m}$ ) than those at the cervical level, and embedded in the lateral white matter or occasionally in the lateral gray matter.

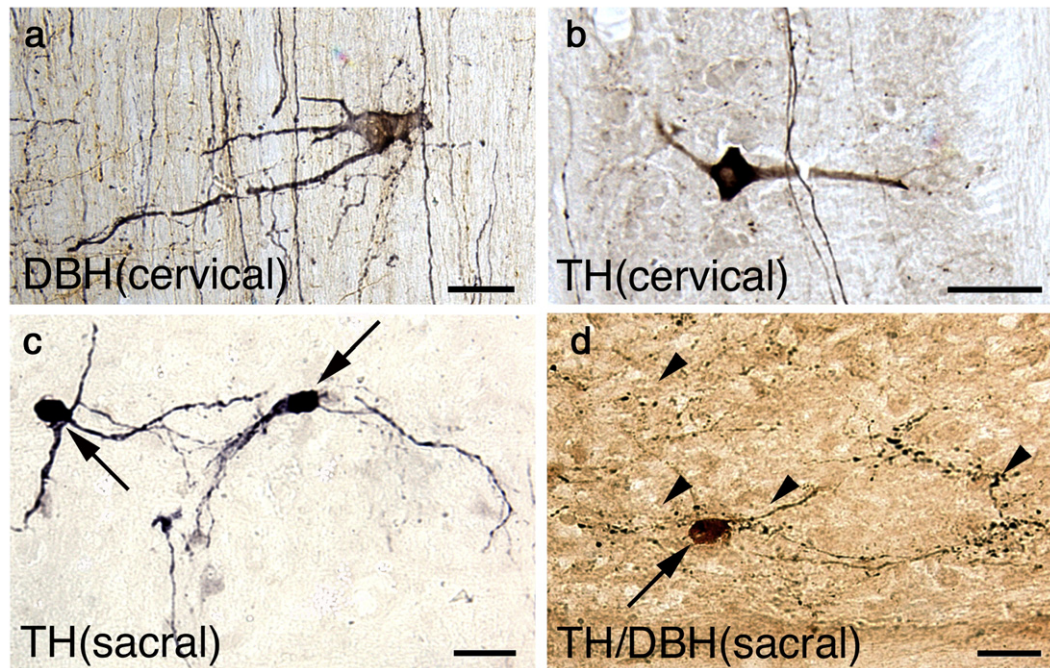
To verify that the small number of TH-labeled neurons observed in the sacral cords were dopaminergic but not NA, we performed double-immunolabeling experiments. Although we detected a number of DBH-positive axons near TH-positive somata, we found no evidence of double-labeled somata (Fig. 4d). Thus, in the present study it appears that the catecholaminergic somata in the sacral cord are not a source of DBH-positive axons below the complete transection.

*DBH-positive axons appose motor-associated cholinergic neurons in the caudal stump of spinal rats*

In intact rats, DBH-positive axons course near motor-associated cholinergic spinal cord neurons, i.e., central canal cluster cells (Fig. 5a), SMNs (Fig. 5f; Commissiong et al., 1978), and partition cells (Fig. 6a). As both media- and OEG-injected rats contain DBH-positive axons that associate with these cholinergic neurons, we asked if these appositions were more common in OEG- than media-injected rats (Figs. 5b, c). In the lower thoracic and upper lumbar segments both central canal cluster cells and centrally located sympathetic preganglionic neurons reside in dorsal lamina X (Barber et al., 1991) and are difficult to distinguish conclusively. Therefore we only evaluated the proximity of DBH-labeled varicosities to these cholinergic neurons in the lower lumbar and sacral segments. More DBH-positive varicosities apposed central canal cluster cells in lower lumbar segments of OEG- than media-injected spinal rats, but there were no differences found at the sacral levels. When the total number of appositions was combined, however, we detected significantly more DBH-positive axons apposed to central canal cluster cells in OEG- than in media-injected rats (Fig. 5d, lumbar:  $P=0.006$ , sacral:  $P=0.09$ , combined:  $P=0.003$ ). To determine if long-term treadmill step training affects the density of NA axons associated with central canal cluster cells, we compared the number of appositions relative to training status (i.e., OEG- and media-



**Fig. 3.** Noradrenergic (NA) axons labeled with dopamine  $\beta$ -hydroxylase (DBH) associate with blood vessels caudal to (a, b) and in the (c, d) injury site in adult spinal rats. (a, b) After 7 months, DBH-positive axons (black; arrowheads) in the caudal stump of spinal rats associate with large (a; 35  $\mu\text{m}$ ) and small (b; 10  $\mu\text{m}$ ) blood vessels identified with the platelet endothelial cell adhesion molecule (PECAM, brown). (c, d) DBH-positive axons (arrowheads) associate with PECAM-labeled blood vessels within the astroglial scar. Inset in c depicts the close association of NA axons along vessels. Scale a, c, d = 50  $\mu\text{m}$ , Scale b = 25  $\mu\text{m}$ .



**Fig. 4.** Noradrenergic cells are present only at cervical levels while dopaminergic somata are distributed at cervical and sacral levels. (a, b) Rare NA (a; labeled with dopamine  $\beta$ -hydroxylase, DBH) and dopaminergic (b; labeled with tyrosine hydroxylase, TH) interneurons are found in the dorsal funiculi at upper cervical levels. (c, d) TH-positive only interneurons (c; arrows) at the sacral levels have smaller somata than those found at the cervical levels. Double-labeling experiments detected a TH-positive soma (d; amber brown, arrow) in the sacral spinal cord that was DBH-negative with DBH-labeled axons (black, arrowheads) coursing nearby. Scale a, b = 50  $\mu$ m, Scale c, d = 25  $\mu$ m.

trained vs. OEG- and media-untrained). We found no training effect on DBH appositions along central canal cluster cells at either the lumbar or sacral levels or when the results from both levels are combined (lumbar:  $P=0.31$ , sacral:  $P=0.34$ , combined:  $P=0.37$ ; data not shown).

Compared to the central canal cluster cells in lamina X, fewer DBH-positive axons course near SMNs in adult spinal rats (Figs. 5g, h). Although more DBH-positive axons apposed SMNs in OEG- than media-injected rats at the lumbar levels, other segmental levels did not differ (Fig. 5e, thoracic:  $P=0.17$ , lumbar:  $P=0.014$ , sacral:  $P=0.095$ ). When the total number of DBH varicosities near SMNs is combined and averaged for all three spinal segments, however, more appositions are detected in OEG- than media-injected rats (Fig. 5e, combined:  $P=0.017$ ). In contrast, step training did not affect the number of DBH appositions along SMNs at any spinal cord level caudal to the transection or when the results were combined (thoracic:  $P=0.29$ ; lumbar:  $P=0.07$ , sacral:  $P=0.31$ , combined:  $P=0.06$ ; data not shown). C-boutons are derived from cholinergic interneurons that terminate on SMNs (Houser et al., 1983; Nagy et al., 1993). Since we previously found evidence of these cholinergic terminals on SMNs in all spinal rats (Takeoka et al., 2009), we suggest that cholinergic interneurons, i.e., partition and central canal cluster cells, can modulate the SMN excitability even after a complete spinal cord transection.

We chose to analyze the partition cells only in the lumbar enlargement where they do not intermingle with the cholinergic sympathetic and parasympathetic preganglionic neurons and where most of the rhythmogenic centers in the spinal cord reside (Grillner and Zangger, 1979; Kjaerulff and Kiehn, 1996; Bonnot and Morin, 1998; Kiehn and Kjaerulff, 1998). In intact spinal cords, DBH-positive axons appear to appose partition cells (Fig. 6a). Occasional DBH-labeled axons also course near the somata and large dendrites of partition cells found in both media- and OEG-injected rats (Figs. 6b, c). Interestingly, we found more DBH-positive varicosities apposing partition cells in OEG- than media-injected rats ( $P=0.004$ ; Fig. 6d), while step training did not influence the number of associations ( $P=0.15$ ; data not shown).

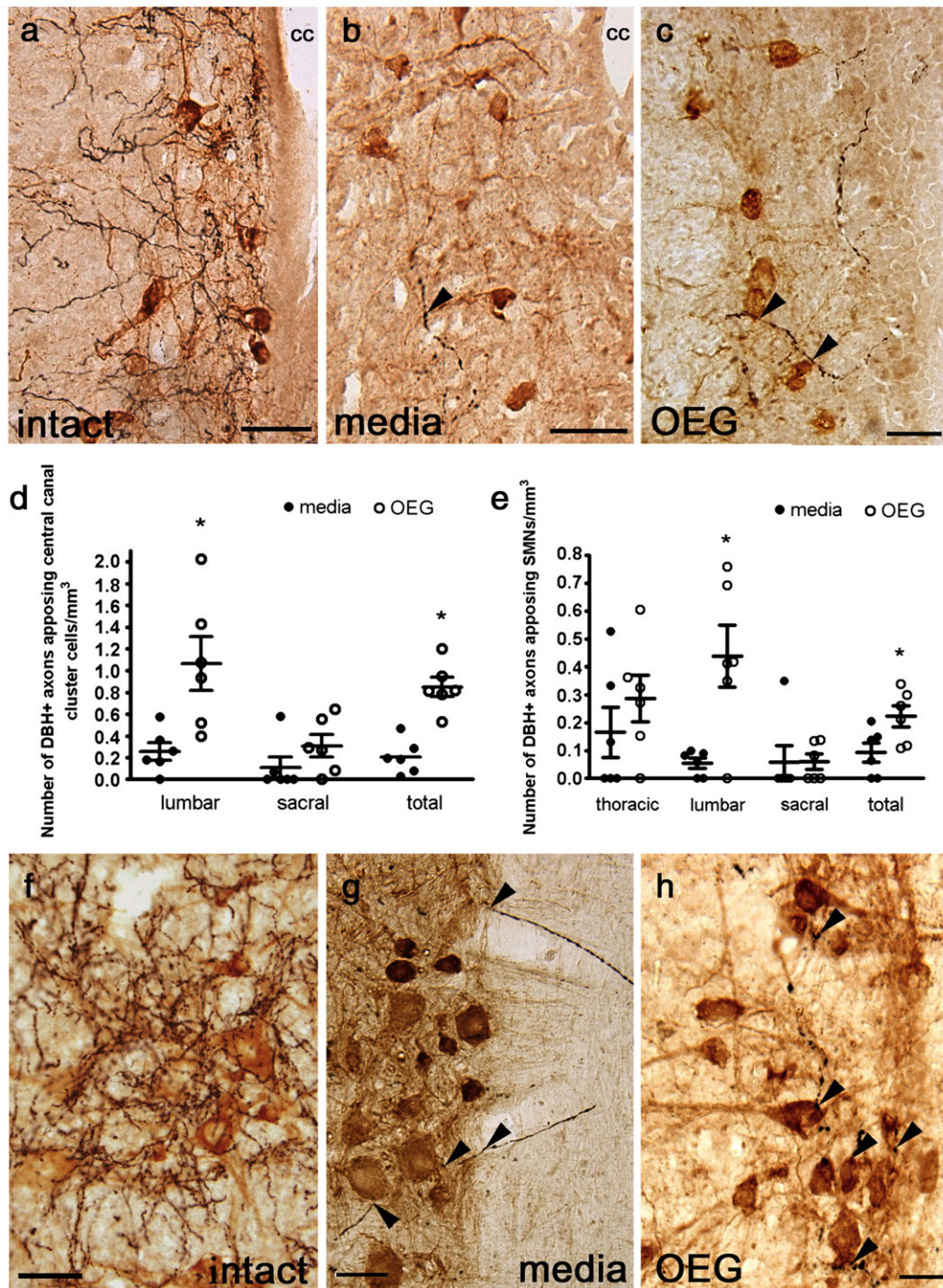
#### *NA axons appose parasympathetic preganglionic neurons in adult spinal rats*

In intact spinal cords, DBH-positive axons densely innervate cholinergic sympathetic (Fig. 1a) and parasympathetic preganglionic neurons (PPN; Fig. 6e; Kohno et al., 1988). Although DBH appositions along PPNs in the spinal cord of media- and OEG-injected rats were less dense than in intact rats, they were frequent and extensive (Figs. 6f, g). DBH-positive axons often coursed within and between clusters of PPN somata. In addition, we occasionally detected DBH appositions to cholinergic sympathetic preganglionic neurons in the thoracic and upper lumbar segments (data not shown). Thus, the presence of DBH-positive axons along preganglionic neurons may influence autonomic control in adult spinal rats.

#### **Discussion**

In this study we asked if the DBH-labeled axons observed caudal to the transection in adult spinal rats are derived from a source other than regeneration of coerulespinal axons, vary in density depending on the transplantation or training status, and appose motor-associated cholinergic neurons. Coerulespinal axons degenerate in the caudal stump after a complete spinal cord transection (Commissiong, 1984), yet a small amount of noradrenaline remains in the caudal stump of the spinal cord (Roudet et al., 1994). Our results suggest that this low level of noradrenaline is derived from peripheral NA axons that associate with spinal blood vessels. Based on current and previous results (McNicholas et al., 1980; Amenta et al., 1987; Hildebrand et al., 1997), we conclude that the presence of NA axons in the spinal cord caudal to the lesion is not conclusive evidence of coerulespinal regeneration even after a complete spinal cord transection. Additionally, the overall density of NA axons in the entire caudal stump was comparable in media- and OEG-injected rats despite the better locomotor performance of OEG-injected spinal rats (Kubasak et al., 2008). Nevertheless, NA varicosities





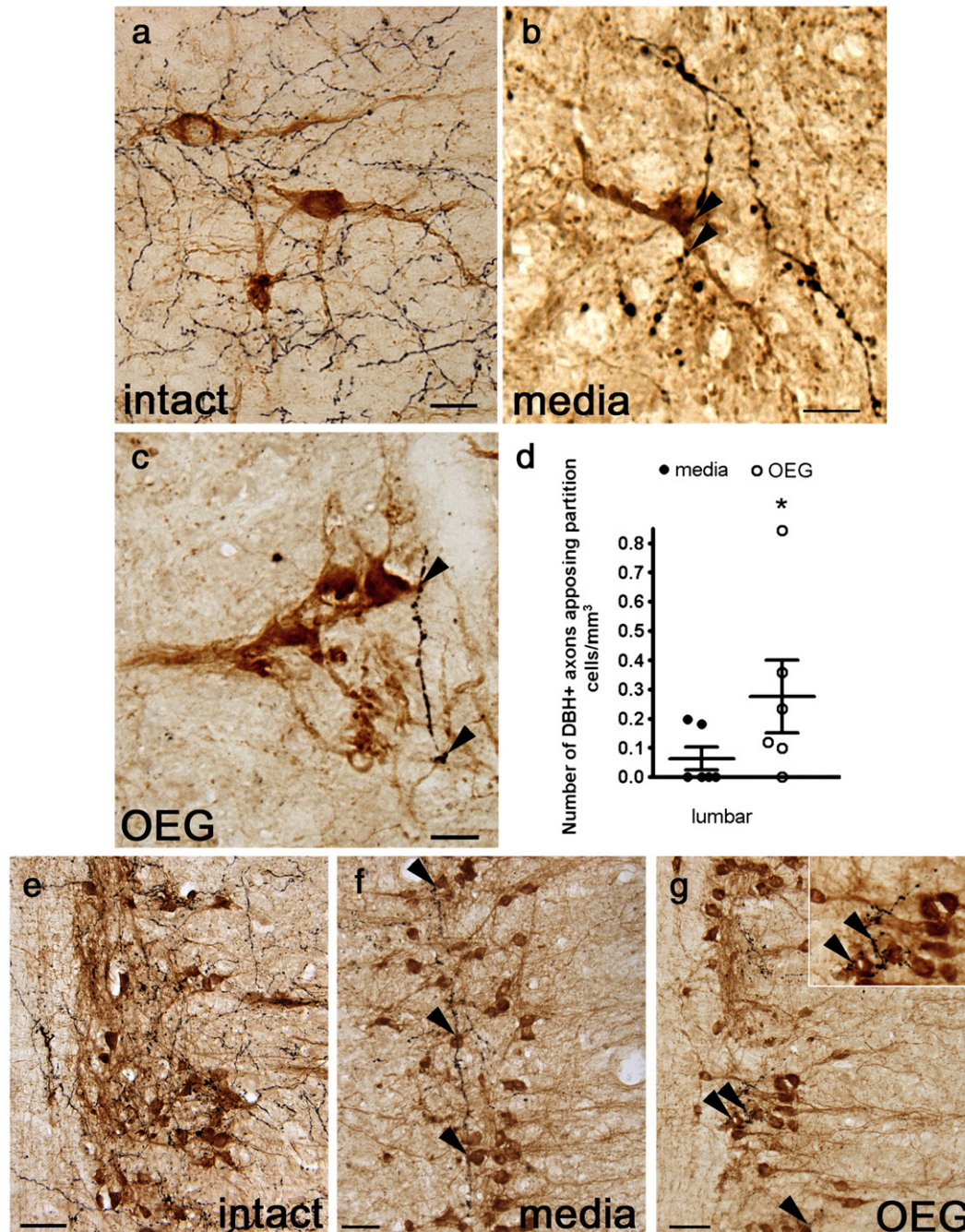
**Fig. 5.** Noradrenergic (NA) axons labeled with dopamine  $\beta$ -hydroxylase (DBH) appose central canal cluster cells (a–c) and somatic motor neurons (f–h) in the lumbar spinal cord from intact, media-, and OEG-injected spinal rats. (a–c) Many DBH-positive fibers (black) course near central canal cluster cells (brown) in a sagittal section from an intact rat (a) with fewer fibers found in spinal rats (b, c; arrowheads). cc = central canal (d) OEG-injected rats contain significantly more appositions of DBH-positive fibers along central canal cluster cells than media-injected rats at lower lumbar ( $P = 0.006$ ) but not at sacral ( $P = 0.09$ ) levels. When data from both segments were combined, OEG-injected rats contained more DBH-positive varicosities apposing central canal cluster cells than media-injected rats ( $P = 0.003$ ). (e) The number of DBH-labeled fibers apposing SMNs at the lumbar level was greater in OEG- than media-injected rats ( $P = 0.014$ ). When data from all segmental levels were combined, OEG-injected rats contained more DBH-positive varicosities apposing SMNs than media-injected rats ( $P = 0.017$ ). (f–h) Numerous NA axons appose SMNs in an intact rat (f). DBH-positive axons (arrowheads) associate with blood vessels that course within the white matter and then appose SMNs (brown) in a media-injected rat (g). Varicose NA axons are associated with SMNs in an OEG-injected spinal rat (h). Bars in (d, e) indicate mean  $\pm$  SEM for 6 media-injected and 6 OEG-injected rats. \*significant difference between media- and OEG-injected rats. Scale a–b, f–h = 50  $\mu$ m, Scale c = 25  $\mu$ m.

associated more often with partition cells, central canal cluster cells, and SMNs in OEG- than media-injected rats. These interactions may contribute to the greater recovery of locomotor ability reported previously in OEG- compared to media-injected rats (Kubasak et al., 2008).

NA axons enter the caudal stumps of the transected spinal cords along several pathways

Sympathetic postganglionic neurons synthesize noradrenaline and innervate various peripheral tissues, including the vasculature. In





**Fig. 6.** Dopamine  $\beta$ -hydroxylase (DBH)-labeled axons appose cholinergic partition cells in sagittally sectioned lower lumbar spinal cord and parasympathetic preganglionic neurons (PPNs) in the sacral cord of an intact rat, and in media- and OEG-injected spinal rats. (a–c) Many DBH-labeled axons (black) course near partition cells (amber-brown) in intact rats (a). DBH-positive axons (arrowheads) also course near partition cells in the caudal stump of media- (b) and OEG-injected (c) spinal rats. (d) The density of DBH-labeled axons that appose partition cells in the lumbar spinal cord is significantly higher in OEG- than media-injected rats ( $P = 0.004$ ). (e–g) DBH-labeled axons, presumably from the locus coeruleus, densely innervate cholinergic PPNs (brown) in intact rats (e). DBH-positive axons (arrowheads) course among clusters of PPN in media- (f) and OEG-injected (g) spinal rats. Area in (g) marked by double arrowhead is enlarged (inset) to illustrate the proximity of DBH-labeled axons to PPNs. Bars in (d), mean  $\pm$  SEM for 6 media- and 6 OEG-injected rats. \*significant difference between media- and OEG-injected rats. Scale a–c, e–f = 50  $\mu$ m.

addition, DBH-immunoreactive axons course along cranial arteries and are abolished by a superior sympathetic ganglionectomy (Crutcher, 1981; Cohen et al., 1992). In the spinal cord, TH- and DBH-positive postganglionic sympathetic axons enter the ventral roots along blood vessels and also are found in the meninges (Fig. 2e; Hildebrand et al., 1997). Sympathetic fibers penetrate and sprout within the spinal cord (McNicholas et al., 1980; Commissiong, 1984) and the dorsal roots (Beattie et al., 1997; Brook et al., 2000) in response to injury. Therefore, NA axons associated with blood vessels

or dorsal roots may have sprouted in response to injury in the present study. The central- and peripheral-derived NA axons differ in their morphology with the former being thin with regularly spaced varicosities, and the latter being thick and more uniform in diameter (Crutcher, 1982). With regeneration and reinnervation, however, targets seem to determine the morphology of the NA axons regardless of their origins (Bjorklund and Stenevi, 1971; Olson and Seiger, 1976; Bjorklund and Stenevi, 1977). Therefore we did not attempt to distinguish central- versus peripheral-derived NA axons based on

axonal thickness. Our results suggest that future studies will need to perform tracing experiments to unambiguously distinguish regenerating coerulespinal axons from the peripheral innervation of NA axons.

#### *Dopaminergic, but not NA, interneurons are found in the caudal stump of adult spinal rats*

Intrinsic spinal catecholaminergic neurons are a potential source of NA axons in spinal rats as they are frequently detected in lower vertebrates (Heathcote and Chen, 1993, 1994) and occasionally in mammals, including rodents (Mouchet et al., 1986; Cassam et al., 1997). We confirmed previous reports that TH-positive interneurons are found in restricted regions of the cervical and sacral spinal cord (Singhaniyom et al., 1983; Dietl et al., 1985; Mouchet et al., 1986; Cassam et al., 1997). We did not, however, find intraspinal NA neurons in the spinal cord caudal to the transection site of our Wistar Hannover spinal rats. Our results are similar to those of Mouchet et al. (1986), but differ from those of Cassam et al. (1997). Using Wistar rats, Cassam et al. (1997) detected DBH-positive cell bodies 14 days after a complete spinal cord transection, a significantly shorter time than our paradigm of 7 months. One possible explanation is that a transient up-regulation of NA interneurons may occur early after the injury. These differences also may be due to the rat strain or even sub-strain as these factors appear to influence spinal NA innervation (Clark et al., 1991; Clark and Proudfoot, 1992). Nevertheless, our finding of dopaminergic, but not NA, neurons in the spinal cord below the lesion is consistent with biochemical assays indicating that there is a normal level of dopamine after a chronic complete spinal cord transection (Commissiong, 1985).

#### *Noradrenaline in the caudal stump may contribute to locomotor recovery in adult spinal rats*

While adult cats with a complete spinal cord transection can learn to plantar step bipedally with training (Lovely et al., 1986; Giroux et al., 2001), adult rodents with a complete spinal cord transection require interventions such as locus coeruleus grafts (Yakovlev et al., 1989; Gimenez y Ribotta et al., 1998a; Gimenez y Ribotta et al., 1998b), or pharmacological and/or electrical interventions (Guertin, 2004; Ichihama et al., 2005; Lavrov et al., 2006; Musienko et al., 2008; Courtine et al., 2009) that likely activate central pattern generation in the lumbosacral cord to recover bipedal plantar stepping ability. Administration of noradrenaline depolarizes the membrane potential and reduces after hyperpolarization of rat motoneurons *in vitro* (Elliott and Wallis, 1992; Fedirchuk and Dai, 2004). As noradrenaline utilizes non-synaptic release, i.e., volume transmission, it can diffuse from a few microns up to a millimeter in the extracellular space (Beaudet and Descarries, 1978; Ridet et al., 1993; Hentall et al., 2003). Therefore, NA varicosities near motor-associated cholinergic neurons in the caudal stump potentially could contribute to the recovery of stepping ability in OEG-injected rats. Lopez-Vales et al. (2006a,b) reported a correlation between NA fiber density in the lumbar spinal cord and functional recovery and found NA axons apposed SMNs in OEG- but not media-injected adult spinal rats. In contrast, we did not find a correlation between NA fiber density in the caudal stump and hindlimb stepping ability, and observed NA axons apposing SMNs regardless of transplantation status. Nonetheless, we did detect significantly more DBH-positive varicosities associated with central canal cluster and partition cells, as well as SMNs in the lumbar spinal cord, in OEG- than media-injected rats. In addition, NA axons may also interact with other populations of locomotor pattern generating neurons (MacLean et al., 1995; Kjaerulff and Kiehn, 1996) that contribute to locomotor improvement in OEG-injected rats. While most *in vivo* studies transplanting OEG focus on how they promote axonal regeneration (Cheng and Olson, 1995; Chen et al., 1996;

Ramon-Cueto et al., 1998; Ramon-Cueto et al., 2000; Lu et al., 2001; Lu et al., 2002; Fouad et al., 2005; Lopez-Vales et al., 2006a, 2007; Kubasak et al., 2008), our results indicate that they also promote the reorganization of NA axons that interact with the existing spinal locomotor networks in the caudal stump. Future pharmacological receptor blocking experiments will be important to examine the functional role of noradrenaline in the caudal stump during locomotion.

SMNs develop supersensitivity to noradrenaline after a complete spinal cord transection that can lead to muscle spasms, a form of hyper-reflexia (Rank et al., 2007). The supersensitivity to sensory input is partially mediated by a sodium channel persistent inward current (PIC) which is completely eliminated by the blockade of 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub> and NA $\alpha_1$  receptors, suggesting that endogenous sources of noradrenaline and 5-HT below the level of the lesion are necessary for the induction of sodium PICs in spinal rats (Harvey et al., 2006). Even a small amount of noradrenaline in the caudal stump could contribute to an improvement in locomotor ability since noradrenaline could modulate afferent input, a critical component for successful stepping, and could also directly excite SMNs. In fact, an early study of spinal cord injury suggested that plasma DOPA could be converted into catecholamines and potentially modulate locomotor activity of spinal rats (Andén et al., 1972).

While the hindlimb stepping ability of OEG-trained rats was significantly better than that of OEG-untrained spinal rats (Kubasak et al., 2008), we did not find any correlation between long-term training and the NA fiber density or NA varicosities associated with any motor-associated cholinergic neurons. It is possible that 20 min/day, 5 days/week for 6 months of hindlimb step training was not sufficient to induce activity-dependent sprouting and reorganization within the adult rat spinal cord caudal to the lesion. Alternatively, the training effect may be mediated by other mechanisms such as (1) the sprouting and reorganization of other neurotransmitter pathways; (2) changes in synaptic strength among the locomotor networks including motor-associated cholinergic neurons; (3) decreased inhibitory influences of GABAergic and glycinergic innervation on SMNs (de Leon et al., 1999; Tillakaratne et al., 2002); and/or (4) changes in the NA receptor expression levels that contribute to locomotor recovery.

#### *Other possible functions of NA in the caudal stump of spinal rats*

Since we detected NA axons in the superficial dorsal horn as well as intermediate laminae where autonomic preganglionic neurons are located, noradrenaline in the lower thoracic, lumbar, and sacral spinal cord may contribute to physiological functions related to nociception and autonomic control after complete coerulespinal denervation. Descending coerulespinal pathways, and pontine A5 and A7 cells also innervate the superficial dorsal horn (Schroder and Skagerberg, 1985; Fritschy et al., 1987; Kwiat and Basbaum, 1992) and modulate nociception (Yeomans et al., 1992; Lu and Perl, 2007) and therefore, extensive arborization of NA axons in the superficial dorsal horn may contribute to pain modulation after a spinal cord injury.

Noradrenaline normally contributes to the pontine micturition center, as the locus coeruleus and A5 cells project their axons bilaterally to PPN in the sacral cord (Vizzard et al., 1995; Nuding and Nadelhaft, 1998). In addition, NA $\alpha_1$  receptors are activated during bladder voiding reflexes and these functions are partially mediated by a rhythmic motor pattern that can elicit motor output in paralyzed and deafferented adult rats (McKenna et al., 1991; Carro-Juarez and Rodriguez-Manzo, 2000; Carro-Juarez et al., 2003).

#### **Conclusions**

In combination with previous reports, we demonstrate that the presence of NA axons in the spinal cord caudal to a complete spinal cord transection in adult rats cannot be interpreted as evidence for



coeruleospinal regeneration. Whether from peripheral or supraspinal sources, however, these NA axons could contribute to the recovery of hindlimb stepping ability after a complete spinal cord transection. These NA axons may influence locomotion by activating SMNs directly or indirectly through the excitation of cholinergic or other interneurons that then terminate on SMNs. Regeneration of other descending motor pathways as well as reorganization of the locomotor circuitries within the caudal stump also could contribute to functional recovery (Edgerton et al., 2004).

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