Spontaneous Functional Viscerosensory Regeneration into the Adult Brainstem

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The dorsal root entry zone of the vagus nerve (vDREZ) is uniquely characterized by peripheral tissue insertions (PTIs) deep to the brainstem surface, consisting of Schwann cells and a reticulum of astrocytic processes. Because Schwann cells permit peripheral axonal regeneration, the capacity of vagal medullary PTIs to allow centripetal regeneration of visceral afferents after vagal dorsal rhizotomy in adult rats was investigated. The present work shows that vagal axons spontaneously regenerate into appropriate and ectopic brainstem nuclei. They accomplish this by first growing along PTIs but then extend along basal laminas of medullary blood vessels. Electrically stimulated regenerated vagal afferents induced Fos expression (indicating functional connectivity) within appropriate but not ectopic nuclei. The unique structure of the vDREZ can thus support spontaneous functional regeneration of visceral primary afferent axons into the adult CNS.

Key words: vagus nerve; Schwann cells; functional regeneration; synaptogenesis; axon guidance; autonomic nervous system

Introduction

Visceral sensation (enteroception) is, to a significant extent, mediated by axons of the vagus nerve (cranial nerve X). Like those of nearly all somatic and visceral primary afferent neurons, vagal sensory somata lie outside the CNS within a sensory ganglion (nodose) and have peripherally and centrally projecting axons. The latter enter the CNS via the vagal dorsal root entry zone (vDREZ), which differs from those of spinal DREZs in that there are invaginations of Schwann cell-containing peripheral tissue insertions (PTIs) deep to the brainstem surface (Rossiter and Fraher, 1990; Fraher and Rossiter, 1991). Although PTIs extend into the medulla for many hundreds of micrometers, vagal afferents are typically ensheathed by alternating sequences of Schwann cells and oligodendrocytes. PTIs are absent from spinal DREZs, in which the transition from peripheral to central nervous tissue occurs at a single node of Ranvier (the transitional node). Injury to spinal dorsal roots is followed by axonal regeneration up to, but not beyond, the DREZ, attributable in part to the inhibitory nature of CNS astrocyte- and myelin-derived molecules (Ramer et al., 2001). The presence of regeneration-permissive Schwann cells deep to the brainstem surface evokes the intriguing possibility that spontaneous regeneration into the brainstem is feasible after vagal dorsal rhizotomy. The experiments conducted here not only verify this hypothesis but also show regeneration into the brainstem beyond the central limit of PTIs via the basal laminas of medullary arteries. Regenerating

axons enter appropriate and ectopic brainstem nuclei, and, in the case of appropriate reinnervation, synaptic connectivity with second-order neurons is restored.

Materials and Methods

Surgery and transganglionic tracing. All procedures conformed to the guidelines of the Canadian Council on Animal Care and to those of the University of British Columbia. Adult male Wistar rats were deeply anesthetized with ketamine–xylazine (70 and 10 mg/kg, respectively), and the left vagal roots (dorsal and ventral; $n = 21$) were exposed intracranially by drilling a hole in the base of the skull just medial to the posterior lacerated foramen (the exit point of the hypoglossal and vagus nerves) (for details, see Norgren and Smith, 1994). Dorsal and ventral roots were crushed repeatedly with fine forceps. Animals recovered without incident under observation. Survival times were 2 ($n = 8$), 10 ($n = 5$) or 20 ($n = 8$) d. In three additional animals, the nodose ganglion was removed (survival time, 10 d). Two days before being killed, rats were reanesthetized with ketamine–xylazine, and the left cervical vagus nerve was exposed and injected with 0.5 μ l of a 1% solution of the B fragment of cholera toxin B (CTB) (List Biologic, Campbell, CA) via a glass pipette glued to a 2 μ l Hamilton syringe. CTB is trans-ganglionically transported by vagal afferents (Robertson et al., 1992).

Vagal stimulation. To assess functionality of the regenerated visceral afferents, the ipsilateral vagus nerve (cervical) was stimulated electrically, and immunohistochemistry was performed to detect the immediate early gene product Fos, which is upregulated in second-order neurons after C-fiber activation (Hunt et al., 1987). In a separate group of 20 d rhizotomized ($n = 5$), 2 d rhizotomized ($n = 4$), and intact (sham-operated) animals ($n = 5$), cervical vagal nerves were stimulated electrically with silver hook electrodes at C-fiber strength (4 mA, 1 msec pulses, 10 Hz for 10 min; Grass stimulator model S44; Grass Instruments, Quincy, MA). These animals had received vagal CTB injections 2 d previously. Animals were killed (1 ml of 30% chloral hydrate, i.p.) 2 hr after the end of stimulation. All rhizotomized or sham animals underwent surgery on the same day, and their identities were coded. From the rhizotomized group, four rats were randomly selected to be killed 2 d later and received CTB injections on the day of rhizotomy.

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Figure 1. Glial and axonal elements of the vDREZ and reactions to rhizotomy. *a, b*, Astrocytes (red) and Schwann cells (green). The vDREZ has one to three rootlets in the dorsoventral plane, which may or may not have an astrocytic projection into the dorsal root (vDR). *a*, Rhizotomized side. *b*, Intact side. Intrabulbar p75 immunoreactivity is less prominent on the intact side, attributable to (Figure legend continues)

Immunohistochemistry and image analysis. Animals were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. Brainstems were removed and, after blocking in 10% normal donkey serum, 20–40 μ m cryosections were stained immunohistochemically to visualize the following: vagal afferents (CTB, host goat, 1:2000; List Biologic), Schwann cells (p75, host rabbit, 1:500; or mouse, 1:50; Chemicon, Temecula, CA), basal laminas (laminin, host rabbit, 1:1000), astrocytes [glial fibrillary acidic protein (GFAP), host rabbit, 1:1000; Sigma, St. Louis, MO], neurons (neuronal-specific nuclear protein, 1:100; Chemicon), and nuclei of vagally activated second-order neurons (Fos, host rabbit, 1:5000; Oncogene Sciences, Uniondale, NY). Secondary antibodies (host donkey, 1:200; Jackson ImmunoResearch, West Grove, PA) were conjugated to aminomethylcoumarin, Cy3, or fluorescein isothiocyanate. Primary antibodies were left on overnight, and secondaries were left on for 2 hr. All antibodies were presented in PBS with 0.2% Triton X-100 and 0.1% sodium azide. Images were captured using a Zeiss (Oberkochen, Germany) Axioplan 2 microscope with a motorized z-drive. Dual- and triple-channel images were constructed using a Qimaging (Burnaby, British Columbia, Canada) CCD camera and Northern Eclipse (Empix Imaging, Mississauga, Ontario, Canada) software.

Fos-labeled nuclei were counted in the paratrigeminal nucleus (Pa5) (ipsilateral only), nucleus of the solitary tract (nts) (ipsilateral and contralateral), and lateral reticular nucleus (Lrt) (ipsilateral and contralateral) in five randomly selected 40- μ m-thick sections per animal. The sham-operated and 20 d rhizotomized rats' identities were coded to prevent observer bias. Images were taken either from the level of the vDREZ (rostral Pa5) or at the level of the area postrema (AP) (caudal Pa5, nts and Lrt). Differences in the number of Fos-positive nuclei between rhizotomized and sham-operated rats in each brainstem region were assessed using a one-way ANOVA. Statistical significance was set at $p \leq 0.05$.

Results

Peripheral tissue insertions into the brainstem express p75 and laminin

The glial and axonal elements of the vDREZ were examined immunohistochemically (Fig. 1) at 2 and 20 d after rhizotomy for p75, which labels predominantly Schwann cells (but also some vagal axons) for GFAP, which labels astrocytes, and for laminin, which is a component of the basal laminas deposited by Schwann cells and arterial endothelial cells. The structure of the vDREZ is highly variable: there are often, but not always, glial domes that project into the proximal part of the vagal dorsal root (Fig. 1*a, b*), similar to those present in spinal DREZs (Ramer et al., 2001). Likewise, the presence of PTIs was variable (Fig. 2): these were present

where glial domes were absent, extended from the surface of the brainstem to the division between the inferior cerebellar peduncle (icp) and the spinal trigeminal tract (sp5) (mean length, $630 \pm 50 \mu\text{m}$; $n = 5$), and were often tapered toward their medial ends. Laminin was highly expressed in the dorsal root but was not as highly expressed in PTIs (Fig. 1*c–f*), suggesting that the PTI Schwann cells may be phenotypically distinct from those in the roots. To ensure that p75 expression was localized to Schwann cells (and not entirely to vagal axons), I removed the nodose ganglion in three animals (see Fig. 5*a,c*).

Because vagal afferents take up and transport CTB to their central terminals (Robertson et al., 1992), CTB was injected into the vagus nerve 2 d before the animals were killed. In intact animals (Fig. 1*g*), axons could be followed from the vDREZ to the nts, and, in more caudal sections, terminals were observed in Pa5 (Fig. 1*h*). In rhizotomized animals, as in intact rats, dorsal vagal motoneurons were retrogradely labeled by CTB as a result of efferent regeneration of vagal motoneurons into the cervical vagus nerve (Fig. 1).

PTIs permit axonal regeneration into the brainstem

At 10 and 20 d after rhizotomy, axons had regenerated deep to the brainstem surface, but occurred only where PTIs were present (Fig. 2*a–f*). At rostral levels (close to the vDREZ), but not more caudally (at the level of the AP), vagal afferents had regenerated to Pa5 by 10 and 20 d after rhizotomy but were absent at 2 d (Fig. 3*b–e*). In none of the rhizotomized animals were CTB-labeled axons present in the nts, indicating not only complete rhizotomy but also the inability of regenerating axons to reach deeper brainstem nuclei. In three of eight cases, regenerating axons were also observed in the external cuneate nucleus (eCu) (Fig. 3*f–i*), indicating that regenerating axons did not always specifically reinnervate appropriate nuclei.

Regenerated axons make functional connections in the brainstem

In intact animals ($n = 5$), electrical vagal stimulation resulted in the appearance of Fos-positive nuclei in ipsilateral and contralateral nts and Lrt, as well as in caudal and rostral Pa5 (Fig. 4*a,d,g,j,m,p,s*). Two days after vagal rhizotomy, only occasional Fos-positive nuclei were present ($n = 4$), verifying again the completeness of the rhizotomy (Fig. 4*b,e,h,k,n,q,s*). At 20 d after rhi-

Figure 2. Vagal axons regenerate into the brainstem along PTIs. *a–f*, Twenty days after vagal dorsal rhizotomy, CTB-filled axons (*a, d*) have regenerated deep to the brainstem surface (*d*), but only where there is a p75-positive PTI (arrows in *b* and *e*). Both sections (*c, f*) were taken from the same animal. *g–i*, Regenerating (GAP-43-expressing) axons (*g, i*, arrows) penetrate the brainstem but do not necessarily colocalize with laminin (arrowhead in *h*). Scale bar, 100 μm .

zotomy ($n = 5$), Fos-positive nuclei were present not only within Pa5 but also within both ipsilateral and contralateral nts (Fig. 4*c,f,i,l,o,r,s*). Basal Fos levels were low (equivalent to 2 d rhizotomized animals; data not shown), and no Fos expression was ever observed in contralateral Pa5 in intact or rhizotomized rats (data not shown). Despite a significant difference between ipsilateral nts in intact and 20 d rhizotomized rats, there was no difference in contralateral nts.

Medullary vasculature permits regeneration beyond PTIs

Because PTIs only extend $630 \pm 50 \mu\text{m}$ deep to the brainstem surface, the mechanism by which vagal axons reached Pa5 was unclear, as this nucleus lies approximately twice that distance from the vDREZ. The brainstem at this level is invested by numerous arteries of the ventrolateral medullary (vlm) arterial system, as has been demonstrated by intraarterial injections of a mixture of neoprene latex and ink (Scremin, 1995). Although the morphology of the vessels studied here resembles that of vlm arteries, they will be referred to as “vessels” or “vasculature” because some may in fact be veins. Many of these penetrate the brainstem surface dorsal to the vDREZ. They take a medial-to-ventromedial course initially, converging with PTIs at the border between the icp and sp5 (Fig. 5*a,c,g*) but then turn dorsally and follow the division between the two white matter tracts and ramify within dorsal nuclei (Fig. 5*b*) such as Pa5 and eCu. Axons penetrating the brainstem surface via PTIs subsequently grew along these vessels where they met PTIs (Fig. 5*d*). Within Pa5, regenerating CTB-labeled axons were concentrated around arteries (Fig. 5*e,f*).

Discussion

This is the first demonstration of spontaneous functional regeneration of sensory axons into the adult CNS. Even olfactory axons, which are well known for their ability to grow into the olfactory bulb (a CNS structure), do so only after the parent neuron

to the lack of reactivity of PTI-associated Schwann cells (see also Fig. 3*a,c–e*). *c–e*, Rhizotomized side. *f*, Intact side. Schwann cells of the PTI (green) express less laminin (blue) than those of the vagal dorsal root (arrowheads in *c* and *d*). *g*, Vagal sensory neurons (nodose; inset) and dorsal root axons transport CTB and can be followed from the vDREZ to the nts in intact rats. *h, i*, CTB labeling of the nts and Pa5 at the level of the AP in an intact rat (*h*) and one rhizotomized 20 d earlier (*i*). No CTB-labeled axons are apparent in either the nts or the Pa5 at this level. Dorsal vagal motoneurons are apparent in both the intact and rhizotomized animals. Those in the rhizotomized rats have regenerated into the cervical vagus. Scale bars: *a–f*, 100 μm ; *g–i*, 200 μm . Sp5, Spinal trigeminal nucleus.

Figure 3. Appropriate and ectopic reinnervation of brainstem nuclei by regenerating CTB-labeled vagal afferents and functional reconnection. *a*, Normal labeling of brainstem just caudal to the level of the vDREZ. The nts and Pa5 are indicated. 4v, Fourth ventricle. *b*, Normal labeling of Pa5. *c–e*, CTB labeling of Pa5 2, 10, and 20 d after vagal rhizotomy, respectively. *f, g*, Labeling of brainstem structures at the level of the vDREZ in an intact animal (*f*) and one 20 d after rhizotomy (*g*). *h*, The eCu is normally devoid of vagal afferents. *i*, After rhizotomy and regeneration, some vagal afferents enter the eCu. Scale bars: *a, f, g*, 200 μ m; *b–e, h, i*, 50 μ m.

has died and been replaced by another (G. A. Graziadei and P. P. Graziadei, 1979; P. P. Graziadei and G. A. Graziadei, 1979; Graziadei and Monti Graziadei 1980). In this respect, the olfactory system is not one that exhibits axonal regeneration but, more

Figure 4. Functional reinnervation as determined by Fos expression. Top, Schematic representation of brainstem areas searched for Fos-positive nuclei (modified from Paxinos and Watson, 1997). *a–r*, Representative images of Fos expression in contralateral nts (*a–c*), ipsilateral nts (*d–f*), caudal Pa5 at the level of the AP (*g–i*), rostral Pa5 at the level of the vDREZ (*j–l*), contralateral Lrt (*m–o*), and ipsilateral Lrt (*p–r*). *s*, Quantification of Fos-positive nuclei in intact rats, those rhizotomized 20 d earlier, and those rhizotomized 2 d earlier. Fos expression after electrical vagal stimulation is reduced in the ipsilateral nts 20 d after rhizotomy compared with intact rats but not in caudal or rostral Pa5. Scale bar (in *r*): *a–r*, 100 μ m.

accurately, neuronal regeneration. The present work shows that a unique feature of the vDREZ, PTIs, permit the initial penetration of the brainstem, but regenerating axons are then conveyed to both appropriate and ectopic nuclei by medullary blood vessels. That vagal afferents fail to penetrate astrocytic glial domes where they are present at the vDREZ suggests that nodose ganglion neurons do not differ from spinal sensory neurons in their propensity for centripetal regeneration, but that the local environment dictates regenerative success or failure. X-irradiation of the spinal cords in young rats leads to the death of astrocytes and their replacement by Schwann cells, which, later in life, can support the regeneration of spinal dorsal root axons into the spinal cord (Sims and Gilmore, 1994a,b). It is likely that the X-irradiation procedure produces an environment not unlike the medullary PTI. It is also notable that the glial barrier between the PTI and the brainstem neuropil is deficient in places (Rossiter and Fraher, 1990), which would facilitate regeneration across the glia limitans. The collaboration between PTIs and medullary vasculature in supporting regeneration is also of potential clinical importance: the DREZ is not only devoid of intraspinal Schwann cells but also is highly avascular (Fraher, 1992, 2000), indicating that the promotion of angiogenesis after dorsal root or CNS damage may facilitate regeneration and functional recovery. The properties of PTI Schwann cells need to be more thoroughly investigated because of their potential analogous role to olfactory ensheathing cells, which also exist both outside and within the CNS (Doucette, 1984) and which are thought to mediate axonal growth from PNS to CNS. At least one feature of PTI Schwann cells distinguishes them from Schwann cells in the vagal dorsal rootlet outside the CNS, which is that they have a significantly different myelin–axon relationship (Fraher and Rossiter, 1991): they have a shorter internodal length, and the thickness of the myelin they produce is intermediate between oligodendrocyte myelin and that of peripheral Schwann cells.

Vagal axons regenerated to Pa5 but not to the nts. The observation that Fos expression was equivalent in intact and regenerated rats was perhaps surprising given that the density of terminals in the regenerated animals was less than that in intact rats. This may be accounted for by an denervation-induced increased sensitivity of Pa5 neurons to vagally released neurotransmitter: a supersensitivity to

Figure 5. Path taken by regenerating vagal afferents. *a*, Labeling of Schwann cells of the PTI (red) and bulbar neurons (green) 3 d after nodose ganglionectomy. *b*, Bulbar blood vessels of the vlm vascular system, showing blood supply to Pa5. Many of these travel between the icp and sp5 (see *g*). *c*, Close association of the PTI (3 d nodose ganglionectomized) with vlm vessels (asterisks). *d*, CTB-labeled axons in transit from a PTI (arrowheads) to a vlm vessel (asterisk). *e*, *f*, Axons growing along vlm vasculature (asterisks) leave blood vessels and enter Pa5 neuropil. Scale bars: *a*, *b*, 75 μ m; *c*, *d*, 50 μ m; *e*, *f*, 40 μ m. *g*, Schematic showing the path of regenerating axons (solid red line). Dotted red line indicates the normal path of central vagal afferents to nts. bv, Blood vessel; Sp5, spinal trigeminal nucleus.

glutamate has been reported in the nts after nodose ganglion removal (Colombari and Talman, 1995). It was also surprising that Fos expression was the same between rhizotomized and sham-operated rats in contralateral (but not ipsilateral) nts and in ipsilateral and contralateral Lrt. Pa5 projects to both the ipsilateral and contralateral nts and bilaterally to the lateral reticular nucleus, the nucleus ambiguus, and the rostroventrolateral reticular nucleus (Caous et al., 2001; de Sousa et al., 2001), which are involved in cardiorespiratory reflexes, thermoregulation, gastric sensation, and visceral nociception (Berthoud and Neuhuber, 2000). This extensive innervation by Pa5 neurons of other brainstem structures, combined with the efferents to the nts from diverse regions, may underlie the unexpected and extensive Fos labeling in the contralateral nts and Lrt. The fact that vagal afferents failed to directly reinnervate the ipsilateral nts is likely to underpin the reduced Fos expression there, and the remaining Fos-positive nuclei in ipsilateral nts may well have been activated through direct and indirect projections of Pa5. The approximately equivalent number of Fos-positive nuclei in ipsilateral and contralateral nts (both targets of the ipsilateral Pa5) supports this notion.

Teleologically, the vital role of the vagus nerve in the maintenance of homeostasis may underlie its ability for some central regeneration. The extensive appearance of Fos in higher-order neurons suggests a partially redundant polysynaptic relaying of vagus-derived signals arriving in Pa5.

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