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Characterization of trkB immunoreactive cells in the intermediolateral cell column of the rat spinal cord

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Abstract

The objective of the present study was to characterize the trkB receptor immunoreactive (-ir) cells in the intermediolateral cell column (IML) of the upper thoracic spinal cord. Small trkB-ir cells (area = 56.1 $\pm$ 4.4 μm^2) observed in the IML showed characteristics of oligodendrocytes and were frequently observed in close apposition to choline acetyltransferase (ChAT)-ir cell bodies. Large trkB-ir cells (area = 209.3 $\pm$ 25.2 μm^2) showed immunoreactivity for the neuronal marker NeuN, indicating their neuronal phenotype, as well as for ChAT, a marker for preganglionic neurons. TrkB and ChAT were co-localized in IML neurons primarily in cases that had received *in vivo* administration of nerve growth factor (NGF). These findings reveal two different cell types, oligodendrocytes and neurons, in the IML of the spinal cord that show trkB immunoreactivity, suggesting their regulation by BDNF and/or NT-4. In addition, there is evidence that NGF may play a role in the regulation of trkB-ir preganglionic neurons in the IML.

Keywords

preganglionic neurons; BDNF; NT-4; oligodendrocytes; sympathetic

The regulation of neurons and glial cells in the spinal cord is poorly characterized. TrkB expressing cells have been reported in the spinal cord, suggesting that they are regulated by either brain derived neurotrophic factor (BDNF) and/or neurotrophin -4 (NT-4). For example, in the lower intermediolateral cell column (IML; T7–T10), pre-ganglionic neurons have been shown to express trkB mRNA, are trkB immunoreactive (-ir) [17], and appear to be dependent on neurotrophin-4 (NT-4) derived from adrenal medullary chromaffin cells for survival [16]. However, retrograde transport of NT-4 from the target could not be demonstrated in these preganglionic neurons, and thus the exact mechanisms involved in the NT-4-mediated survival of the lower intermediolateral cell column are not known. Preganglionic neurons located in the IML of the upper thoracic cord may be regulated by brain-derived neurotrophic factor (BDNF) [5] and/or NT-4 [16], though these phenomena have not been well documented.

In addition to preganglionic neurons, trkB-ir glial cells have been reported in the motor areas of the lumbar spinal cord [19] and were shown to be altered by exercise [13]. The extent to which the trkB-ir glial cells were present in the upper thoracic cord has not been described.

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The goal of the present study was to characterize the trkB-ir cells in the IML of the upper thoracic spinal cord, a region that provides preganglionic innervation to the superior cervical ganglion (SCG). The effects of exogenous NGF on trkB immunoreactivity also was investigated. Portions of this study have been published in abstract form [14].

Adult Sprague Dawley rats (3 months; n=6) were anesthetized with an overdose of sodium pentobarbital (intraperitoneal 125 mg/kg) and then perfused with 4% paraformaldehyde in 0.1M PO₄ buffer following a pre-rinse with 0.9% saline. Following perfusion, the spinal cord was blocked to incorporate the lower cervical and upper thoracic segments of the spinal cord (C8-T4) and post-fixed for one hour. Additional rats (n=6) received a 2 week *in vivo* administration of NGF peptide into the right lateral ventricle as previously described [8,9,10, 21]. NGF infused into the ventricular system enters the subarachnoid space where it bathes the perivascular axons associated with the extracerebral blood vessels [8,9,10]. We have shown previously that infused NGF accumulates in the SCG following *in vivo* intracerebroventricular infusion [18], presumably by retrograde transport along the axons of SCG postganglionic neurons that innervating the extracerebral blood vessels. The procedures used in this study were approved by the Miami University IACUC and every effort was taken to minimize pain and discomfort to the animals.

Frozen sections (35 µm) of the spinal cord were cut on a sliding microtome and mounted on glass microscope slides. Mounted frozen sections were treated with a solution of 0.6 % Triton-X, 1% normal donkey serum (NDS; Jackson Labs) in 0.1M phosphate-buffered saline (PBS) at 4°C for 24 hours, then incubated at room temperature in 3% NDS-PBS for 30 minutes. Sections were then incubated for 48 hours in primary antibody (goat anti-ChAT, Chemicon AB144P; 1:50; rabbit anti-trkB, Santa Cruz Biotechnology 794, sc-12; 1:200; mouse anti-RIP: 2',3'-cyclic nucleotide 3'-phosphodiesterase, Developmental Studies Hybridoma Bank, University of Iowa, 1:250, marker for oligodendrocytes as shown in Watanabe *et al.* [20]; mouse anti-NeuN, Chemicon MAB377; 1:1000, a neuronal marker [12]). The primary antibodies were diluted in 1% NDS-PBS. Following a series of five minute washes with PBS, sections were incubated for two hours in appropriate secondary antibodies (Alexa 488 donkey anti-goat; Alexa 594 donkey anti-rabbit; Alexa 488 donkey anti-mouse; Alexa 555 donkey anti-rabbit; Alexa 647 donkey anti-mouse, Molecular Probes; all at 1:200 dilution), which were diluted in PBS. Sections were rinsed with PBS and then PB and coverslipped using Gel/Mount medium (Biomedica Corp). As a control, tissues were processed with the omission of primary antibodies. To test for specificity of the trkB antibody, the antibody was preabsorbed with excess trkB peptide (Santa Cruz Biotechnology 794: sc-12 P) overnight and then applied to select tissues. No immunostaining was observed in this competition study. In addition, primary sensory neurons showed trkB immunoreactivity that is consistent with that observed by other investigators [23]. The trkB antibody is specific for the cytoplasmic domain (i.e. full-length trkB receptor) and does not recognize the truncated trkB receptor [19].

Images were captured using an Olympus FV500 confocal microscope and Fluoview v4.3 software. Using ImageQuant software, somal areas were measured from cell bodies outlined manually from confocal images at 400x magnification. In addition, the number of large and small cells was calculated by counting the total number of immunoreactive cells from three different images from each animal, using similar fields of view at 400x magnification. Data were analyzed using student t-tests. Significance was reported at p<0.05.

The intermediolateral column (IML) at spinal cord levels C8-T4 was the focus of this study (Fig. 1) as this is a primary location of preganglionic neurons that terminate onto the SCG [1]. Based on somal area, trkB immunoreactivity in the IML was localized to two distinct cell populations. The first cell type was small (mean somal area = 56.1 ± 4.4 µm²) and relatively numerous throughout the gray and white matter in the vicinity of the IML (Fig. 1–Fig 4). Such

cells have been described by others in the lumbar and thoracic spinal cord [13,19]. Frequently, the small trkB-ir cells were in close apposition with ChAT-ir cell bodies (Fig. 2), a phenomenon observed in both non-infused and NGF-infused tissues. The small cells expressed RIP (Fig. 3), a marker for oligodendrocytes, indicating their glial nature. No significant change in the mean number of small, trkB-ir cells in the IML was observed in the NGF-infused tissues (non-infused = 13.3 ± 3.0 ; NGF = 12.5 ± 1.2), suggesting that these cells were not influenced by NGF administration.

The second trkB-ir cell type in the IML was relatively large (Fig. 2, Fig 4; mean somal area = $209.3 \pm 25.2 \mu\text{m}^2$) and the overall number of large trkB-ir cells was not altered by NGF administration (non-infused = 8.0 ± 2.0 ; NGF = 6.9 ± 0.86). These cells also were immunopositive for NeuN, a neuronal marker (Fig. 4), confirming their neuronal phenotype. Though not observed in non-infused cases, large trkB-ir cells frequently showed immunoreactivity for ChAT (Fig. 2), a marker for preganglionic neurons, in the NGF-infused cases, where approximately 39% of the trkB-ir neurons also were ChAT-ir.

The results of the present study reveal the presence of small trkB-ir cells in the IML, which, as suggested by their somal area and RIP immunoreactivity, appeared to be oligodendrocytes. These glial cells frequently were found in close apposition to ChAT-ir preganglionic cell bodies, suggesting a relationship between these two cell types. The exact nature of the relationship between these oligodendrocytes and preganglionic cell bodies is unknown, although interactions between neurons and oligodendrocytes have been described [15], presumably to facilitate myelination. Whether the anatomical relationship observed in the current study represents a similar interaction or a previously undescribed relationship is unknown at present. Though Macias *et al.* [13] showed numerous trkB-ir glial cells surrounding large ventral horn motoneurons in the lumbar spinal cord, a potential interaction between these two cell types was not discussed. In the current study, no obvious differences in the number of these cells were observed following NGF administration. These findings are consistent with previous work showing that trkB associated with oligodendrocytes was not affected by NGF [11].

The presence of the full length trkB receptor on oligodendrocytes in the spinal cord was first described by Skup *et al.* [19] and their use of the Santa Cruz Biotechnology 794 antibody to characterize full length trkB receptors, rather than the truncated form [7], on oligodendrocytes was discussed at length. These trkB-ir glial cells were well characterized in the ventral horn of the lumbar spinal cord [13,19] as well as in other lumbar cord areas [13], where they appear to be altered by exercise [13,19]. We show here that this same cell type is present in the IML of the upper thoracic spinal cord.

The sympathetic preganglionic cell bodies located in the spinal cord have been well characterized as expressing the biosynthesizing enzyme ChAT [2,4,22], yet the regulation of these neurons is not well studied. Expression of trkB receptor protein in large cholinergic neurons in the IML suggests that preganglionic neurons in the thoracic spinal cord are regulated by BDNF and/or NT-4 [6]. There is evidence that BDNF (described in mouse [5]) and/or NT-4 (described in rat [16,17]) may provide trophic support to sympathetic preganglionic neurons, though the mechanisms through which BDNF or NT-4 may act to influence preganglionic activity are unclear. Although it was determined that both trkB and NT-4 were important for development and maintenance of sympathetic preganglionic neurons innervating the adrenal medulla, few trkB mRNA cells were labeled in the spinal cord following fluorogold administration from the adrenal medulla [16], indicating that the survival activities of NT-4 were likely not due to retrograde transport. These investigators suggested that a G-protein system may be activated rather than direct transfer of information from the axon terminal via a neurotrophin. The axonal transport of NT-4 from the anterior eye chamber to the SCG has

been demonstrated [3], thus it is possible that NT-4 derived from peripheral targets might influence preganglionic neurons that innervate the SCG. Macias *et al.* [13] reported neither BDNF-ir cell bodies nor BDNF mRNA in the intermediate zone of the lumbar spinal cord, suggesting that BDNF may not be involved in the regulation of preganglionic neurons at the lumbar level.

What mechanism might be responsible for the upregulation of trkB in preganglionic neurons following NGF administration? While large trkB-ir neurons were present in the IML of both control and NGF-infused cases, trkB co-localization with ChAT was primarily observed following NGF infusion. The increase in trkB/ChAT double labeled neurons in the IML following NGF infusion suggests that these neurons were somehow influenced by exogenous NGF. It is unclear whether the increased NGF protein in the SCG, which is typical following NGF infusion [18], influenced the trkB-ir preganglionic neurons, or whether enhanced NGF at the peripheral targets, such as the extracerebral blood vessels, may have enhanced NT-4 or BDNF transport along the postganglionic neuron to the SCG, which in turn would affect the preganglionic innervation. Regardless of the specific mechanism, the current findings provide evidence that enhanced NGF in the periphery may affect the protein expression of trkB in preganglionic neurons in the upper thoracic spinal cord.

In summary, in the present study, two different trkB-ir cell types, neurons and oligodendrocytes, were identified in the IML of the upper spinal cord, indicating their regulation by either BDNF or NT-4. The trkB-ir oligodendrocytes, which were not affected by NGF administration, frequently were observed closely apposed to large trkB and ChAT-ir cell bodies, suggesting a potential relationship between these two cell types. Further, large trkB-ir neurons were found to be immunoreactive for ChAT, a marker for preganglionic neurons, particularly in the NGF-infused cases, providing evidence that NGF protein may influence the activity of preganglionic neurons in the spinal cord.

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Figure 1.

A. Coronal section through the upper thoracic spinal cord, along with diagrammatic inset, indicate the specific area of the intermediolateral cell column (IML) examined in the present study. Also, in this micrograph, note the lack of trkB immunoreactivity following the omission of trkB antibody from the staining protocol (control). B. Small trkB-ir cells (arrows) were observed throughout the IML of the upper thoracic spinal cord in both non-infused (shown here) and NGF cases (shown in Fig. 2–Fig. 4 below). C. As a positive control for the trkB antibody, primary sensory neurons showed trkB immunoreactivity (arrows) in this frozen section taken from the adult rat. Scale = A. 200 μ m; B. 25 μ m; C. 50 μ m.

Figure 2. Large trkB-ir cells (red in A.), which were also immunoreactive for ChAT (green in B.), were evident in the spinal cord from cases receiving *in vivo* NGF administration. Co-localization of trkB and ChAT, such as that shown in the merged image in C. (arrows), only were observed in NGF-infused cases. D. The boxed area in C., shown at higher magnification, depicts the typical relationship between a small trkB-ir cells (red) and a trkB/ChAT-ir pre-ganglionic cell body (red/green). Note the small trkB-ir cell (arrows) in close apposition to the trkB/ChAT-ir cell body (asterisk). Scale, A.–C. = 50 μ m; Scale, D. = 10 μ m.

Figure 3. Small trkB-ir cells (arrows) in the IML of the upper thoracic spinal cord were immunoreactive for trkB (red in A.) and RIP (blue in B.), a marker for oligodendrocytes. Co-localization of trkB and RIP is evident (arrows) in the merged image in C., indicating the glial nature of these cells. Not all trkB-ir cells (arrowhead) were immunoreactive for RIP. Scale = 50 μ m.

Figure 4.

A. Both large (asterisks) and small (arrows) cells show immunoreactivity for trkB in this coronal section taken from the spinal cord of an NGF-infused rat. B. and C. Most of the large cells in this field also showed immunoreactivity for NeuN, a neuronal marker (green), indicating their neuronal phenotype, whereas no small trkB-ir cells were positive for NeuN. Scale = 25 μ m.