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Distribution and phenotype of TrkB oligodendrocyte lineage cells in the adult rat spinal cord

Aminata P. Coulibaly^{a,b}, Matthew R. Deer^c, and Lori G. Isaacson^{a,b,c}

^aCenter for Neuroscience and Behavior, Miami University, Oxford, OH, 45056

^bGraduate Program in Cell, Molecular, and Structural Biology, Miami University, Oxford, OH, 45056

^cDepartment of Biology, Miami University, Oxford, OH, 45056

Abstract

The distribution and phenotype of a previously undescribed population of nonneuronal cells in the intact spinal cord that expresses TrkB, the cognate receptor for brain derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4), were characterized by examining the extent of co-localization of TrkB with NG2, which identifies oligodendrocyte progenitors (OPCs) or CC1, a marker for mature oligodendrocytes (OLs). All TrkB nonneuronal cells expressed Olig2, confirming their role in the OL lineage. Similar to OPCs and OLs, TrkB cells resided in gray and white matter of the spinal cord in similar abundance. Less than 2% of TrkB cells expressed NG2, while over 80% of TrkB cells in the adult spinal cord co-expressed CC1. Most OPCs did not express detectable levels of TrkB, however a small OPC pool (~5%) showed TrkB immunoreactivity. The majority of mature OLs (~65%) expressed TrkB, but a population of mature OLs (~36%) did not express TrkB at detectable levels, and 17% of TrkB nonneuronal cells did not express NG2 or CC1. Approximately 20% of the TrkB nonneuronal population in the ventral horn resided in close proximity to motor neurons and were categorized as perineuronal. We conclude that TrkB is expressed by several pools of OL lineage cells in the adult spinal cord. These findings are important in understanding the neurotrophin regulation of OL lineage cells in the adult spinal cord.

Keywords

oligodendrocyte precursor cells; OPCs; NG2 cells; perineuronal oligodendrocytes; satellite oligodendrocytes

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Corresponding Author Information: Lori G. Isaacson, Center for Neuroscience and Behavior, Graduate Program in Cell, Molecular, and Structural Biology, Department of Biology, Miami University, 280 Pearson Hall, Phone: 513-529-3142, FAX: 513-529-6900, isaacslg@miamioh.edu.

Aminata P. Coulibaly, coulibap@miamioh.edu

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1. INTRODUCTION

Oligodendrocytes (OLs) are supportive cells in the central nervous system (CNS) that are responsible for the myelination of axons and the establishment of proper communication between neurons in the brain and spinal cord. The dysregulation or loss of OLs has been implicated in disorders such as multiple sclerosis (Lee et al., 2012), schizophrenia (Karoutzou et al., 2008), amyotrophic lateral sclerosis (Lasiene and Yamanaka, 2011), X-linked Charcot-Marie Tooth (Sargiannidou et al., 2009), mood disorders (Roy et al., 2007) and the lack of recovery following brain injury or stroke (Dewar et al., 2003). Therefore it is important to understand the biology of these cells in uninjured adult tissues.

OLs are derived from a defined group of precursors known as oligodendrocyte progenitor cells (OPCs; Cohen et al., 1996; Scarisbrick et al., 2000; Nishiyama et al., 2002; Nishiyama et al., 2009). During development and into adulthood, factors such as neurotrophins and neurogulins regulate the proliferation and differentiation of OPCs, which express the proteoglycan marker nerve/glial antigen (NG2; Nishiyama et al., 2009), into mature OLs. In turn mature OLs are characterized by a down regulation of NG2 and up regulation of the adenomatous polyposis coli (APC; commonly known as CC1) antigen (Kessaris et al., 2008; McTigue and Tripathi, 2008; Nishiyama et al., 2009).

Oligodendrocytes that express TrkB, the cognate receptor for brain derived neurotrophic factor (BDNF), have been reported in the adult spinal cord (Macias et al., 2007; McCartney et al., 2008; Coulibaly and Isaacson, 2012; Skup et al., 2002), suggesting that a least some cells in the OL lineage are responsive and/or regulated by BDNF. Indeed, numerous reports have indicated that BDNF can affect the cells in the OL lineage (Van't Veer et al., 2009; VonDran et al., 2010). For instance, following spinal cord injury, the presence of BDNF grafts led to increased OPC proliferation and differentiation into OLs (McTigue et al., 1998). BDNF infusion into the rat spinal cord following injury led to decreased OL apoptosis (Koda et al., 2002).

Though TrkB nonneuronal cells have been described in the intermediolateral cell column (IML; Coulibaly and Isaacson, 2012) and ventral horn (Macias et al., 2007; Skup et al., 2002) of the adult spinal cord, the distribution of TrkB cells and their overlap with cells in the OL cell lineage have not been investigated. The overall objective of this study was to characterize in detail the location and phenotype of TrkB cells in the adult spinal cord.

Perineuronal TrkB cells within the spinal cord gray matter (GM), which reside in close association with neuronal cell bodies, have been reported (Coulibaly and Isaacson, 2012; Skup et al., 2002), yet their frequency and phenotype in the spinal cord have not been studied. Therefore, perineuronal cells were included in our analysis of the spinal cord TrkB populations.

Two levels of the spinal cord, the cervical and thoracic cord, were examined in the present study. The thoracic level was analyzed to assist in our understanding of TrkB cells at the level of the sympathetic intermediolateral cell column. For comparison, the cervical level was examined at the level of the phrenic nucleus. This level was selected due to its

important role in respiration studies (Kuzuhara and Chou, 1980; Reviewed in Zimmer et al., 2007).

The findings of this study revealed that, similar to OPCs and OLs, TrkB nonneuronal cells in the spinal cord were present in the gray matter (GM) and white matter (WM) in equal proportions. In addition, these cells typically exhibited characteristics of mature OLs, while TrkB cells that expressed markers for OPCs were relatively rare. Similarly, the majority of TrkB perineuronal cells in adult spinal cord expressed the OL phenotype and comprised approximately 15%-20% of all TrkB cells in the ventral horn. Portions of this study have been previously published in abstract form (Coulibaly and Isaacson, 2013).

2. RESULTS

2.1. Distribution of TrkB cells in the adult rat spinal cord: comparison with OPCs and mature OLs

In order to determine whether TrkB nonneuronal cells were of the OL lineage, TrkB cells were co-labeled with Olig2 (Fig. 1.C.), a transcription factor that is expressed by cells in the OL cell lineage (Nishiyama et al., 2009). TrkB cells exhibited a thin rim of TrkB immunoreactivity surrounding a rounded nucleus (Fig. 1.B.; Skup et al., 2002) and all of the TrkB nonneuronal cells also expressed Olig2 (Fig. 1.C.). Frequent TrkB cells that did not express Olig2 exhibited characteristics of neurons and are denoted by asterisks in Figure 1.C. Some Olig2 cells did not express TrkB (Fig. 1.C.) and likely represented the NG2 or CC1 populations that did not express detectable levels of TrkB (see below).

The density of TrkB nonneuronal cells in the spinal cord and their location throughout the GM and WM of the spinal cord were examined. The density of TrkB cells in the cervical segment averaged 143 cells/mm² in the WM and 204 cells/mm² in GM (Fig. 2.A.). In the thoracic segment, TrkB density averaged 240 cells/mm² in the WM and 232 cells/mm² in the GM (Fig. 2.A.). No significant differences were observed at either segment when TrkB cell density was compared between WM and GM (Fig. 2.A.). Further, when TrkB cell density was compared between the cervical and thoracic segments, no differences were observed.

The density and distribution of TrkB cells were compared with that of mature OLs and OPCs. Similar to TrkB cells, mature OLs showed CC1 immunoreactivity along the perimeter of a rounded cell body (Fig. 1.B.). In the cervical segment, CC1 cells averaged 240 cells/mm² in the WM and 286 cells/mm² in GM (Fig. 2.B.). In the thoracic segment, 258 cells/mm² and 276 cells/mm² were observed in the WM and GM, respectively (Fig. 2.B.). No significant differences were observed at either segment when the density of CC1 cells was compared between WM and GM (Fig. 2.B.). Further, no differences were observed when CC1 cell density was compared between the two segments (Fig. 2.B.).

NG2 cells were characterized by a ring of immunoreactivity around the cell nucleus and fine immunoreactive processes that emanated from the cell body (Ligon et al., 2006; McTigue et al., 2001; Fig. 1.B.). The values for NG2 cell density in the spinal cord generally were lower than TrkB and CC1 cell density. NG2 density averaged 35 cells/mm² in the WM and 41

cells/mm² in the GM in the cervical segment (Fig. 2.C.). In the thoracic segment, NG2 density averaged 58 cells/mm² in WM and 73 cells/mm² in GM (Fig. 2.C.). No significant differences were observed in either segment when NG2 cell density was compared between the GM and WM (Fig. 2.C.). However, NG2 cell density in the cervical GM was significantly less when compared to the thoracic segment GM (Fig. 2.C.).

2.2. Expression of TrkB by OL lineage cells in the adult spinal cord

In order to determine the overlap between the TrkB and mature OL populations, double label immunohistochemistry was carried out using CC1 and TrkB antibodies. A substantial overlap between CC1 and TrkB pools was observed in both the cervical and thoracic segments of the spinal cord (Fig. 3.A., B., C.), yet CC1 only (Fig. 3.B., C.) and TrkB only (Fig. 3.C.) cells also were observed. In the cervical segment, 88% of the cells expressed CC1 (CC1⁺/TrkB⁻ or CC1⁺/TrkB⁺) and 64% expressed TrkB (TrkB⁺/CC1⁻ or TrkB⁺/CC1⁺; Fig. 3.C.). As shown in the basic Venn diagram in Figure 3.C., the overlap between the two populations was substantial with 52% of the population labeled with both CC1 and TrkB (Fig. 3.C.). However, 36% of the cells were CC1 only (CC1⁺/TrkB⁻) and 12% expressed only TrkB (TrkB⁺/CC1⁻). As shown in the stacked Venn diagram on the right panel of Figure 3.C., within the TrkB population, 81% of the TrkB cells co-expressed CC1 (TrkB⁺/CC1⁺). In the thoracic segment (Fig. 3.A., B.), 86% of the cells expressed CC1 (CC1⁺/TrkB⁻ or CC1⁺/TrkB⁺) while 70% expressed TrkB (TrkB⁺/CC1⁻ or TrkB⁺/CC1⁺; Fig. 3.C., basic Venn). Also as shown in the basic Venn, 57% of the population expressed both CC1 and TrkB (CC1⁺/TrkB⁺), 30% of the cells were CC1 only (CC1⁺/TrkB⁻) while 14% expressed only TrkB (TrkB⁺/CC1⁻; Fig. 3.C.). Similar to the cervical segment, within the TrkB population, 81% of the TrkB cells co-expressed CC1 (TrkB⁺/CC1⁺; Fig. 3.C., stacked Venn), while 19% were TrkB⁺/CC1⁻.

In order to determine the overlap between TrkB and OPC populations, double label immunohistochemistry was carried out using NG2 and TrkB antibodies. As shown in the confocal images in Figure 4.A. and Figure 4.B. and the Venn diagrams in Figure 4.C., there was little overlap between the NG2 and TrkB pools. In the cervical segment, 19% expressed NG2 (NG2⁺/TrkB⁻ or NG2⁺/TrkB⁺), while 82% expressed TrkB (NG2⁻/TrkB⁺ or NG2⁺/TrkB⁺; Fig. 4.A., B.). As shown in the basic Venn diagram in Figure 4.B., overlap between the TrkB and NG2 pools (NG2⁺/TrkB⁺) was only 1.3%. 18% of the population expressed NG2 only (NG2⁺/TrkB⁻) while 81% expressed only TrkB (TrkB⁺/CC1⁻; Fig. 4.B.). The stacked Venn diagram on the right panel of Figure 4.B. revealed that, within the TrkB population, only 1.6% of the TrkB cells co-expressed NG2 (TrkB⁺/NG2⁺; Fig. 4.B.). In the thoracic segment, 26% of the NG2/TrkB labeled population expressed NG2 (NG2⁺/TrkB⁻ or NG2⁺/TrkB⁺), while 75% expressed TrkB (NG2⁻/TrkB⁺ or NG2⁺/TrkB⁺; Fig. 4.B., basic Venn). As shown in the basic Venn, only 1.6% of the cells were NG2⁺/TrkB⁺ (Fig. 4.B.) and 25% of the cells expressed only NG2 (NG2⁺/TrkB⁻) while 74% expressed only TrkB (TrkB⁺/CC1⁻; Fig. 4.B.). The stacked Venn diagram depicts that, within the TrkB population, only 2.1% of the TrkB cells co-expressed NG2 (TrkB⁺/CC1⁺; Fig. 4.B.).

2.3. Clustering of OL lineage cells in the adult spinal cord

While analyzing the distribution of OL lineage cells, it was observed that mature OLs tended to cluster or aggregate in groups of 2 or more cells, particularly in the gray matter. In order to better characterize this phenomenon, the clustering of OL lineage cells in the spinal cord was quantified. Clustering was exhibited primarily by CC1 and TrkB cells (Fig. 5.A., B.; See also figure 3B and 6B for examples of clustering), particularly in the GM, and this phenomenon was observed at both the cervical and thoracic levels. The proportion of cells in clusters ranged from 11-22% in the cervical segment (WM=11%; GM=22%; Fig. 5.B.) and 7-23% in the thoracic segment (WM=7%; GM=23%; Fig. 5.B.). In the thoracic segment the number of GM clusters (both TrkB and CC1) was significantly higher compared with the number in the WM (Fig. 5.B.). No difference was observed when comparing the number of clusters between the two spinal segments.

2.4. Perineuronal OL lineage cells in the adult spinal cord

As described in previous studies (Skup et al., 2002; Macias et al., 2007; McCartney et al., 2008; Coulibaly and Isaacson, 2012), TrkB cells frequently were observed in close proximity to neuronal cell bodies in the IML and the ventral horn and thus were categorized as perineuronal. In order to determine the overlap of these cells with OL lineage cells markers, the expression of NG2, CC1 and/or TrkB by perineuronal cells and their association with ChAT immunoreactive (-ir) ventral horn neurons were analyzed. Approximately 57% of the ChAT-ir neurons in the ventral horn of the cervical and thoracic cord was associated with at least one perineuronal cell (Fig. 6). CC1 and TrkB perineuronal cells both resided in close proximity to the neuronal cell body membrane (Fig. 6. A. B.) but perineuronal NG2 cells rarely were observed (Fig. 6.C.). Approximately 12% of the CC1 population was perineuronal while the number of TrkB perineuronal cells ranged from 15%-20% in the cervical and thoracic segments. Though some overlap existed between the CC1 and TrkB perineuronal populations, it was not complete and approximately 65% and 75% of the TrkB perineuronal cells co-expressed CC1 in the respective cervical and thoracic segments. In the cervical segment, 5% of the NG2 population was perineuronal, while no perineuronal NG2 cells were observed in the thoracic segment. Both segments showed fine NG2-ir processes in close proximity to the perimeter of the ventral horn motor neurons (Fig. 6.C.).

3. Discussion

3.1. Phenotype of TrkB nonneuronal cells in the intact spinal cord

TrkB cells in the rat spinal cord were first described by Skup et al. (2002), who noted their presence throughout the cord, and reported their co-localization with OL markers such as GalC, RIP, and/or NG2. Subsequent studies from our laboratory confirmed that the TrkB cells were nonneuronal (McCartney et al., 2008), that many of the cells expressed CC1 (Coulibaly and Isaacson, 2012), and that they did not express markers for astrocytes or microglia (Skup et al. 2002; Coulibaly, unpublished). Others have reported little colocalization of full length TrkB and GFAP in uninjured tissues, though reactive astrocytes can express full length TrkB following CNS injury (McKeon et al., 1997) and in models of multiple sclerosis (Colombo et al., 2012). Further, all TrkB nonneuronal cells co-expressed

Olig2, a transcription factor that has been shown by others to be expressed by OL lineage cells (Miller, 2002), indicating that they are part of the OL cell lineage.

Plasticity of TrkB cells in the spinal cord has been reported. For example the TrkB population in the ventral horn has been shown to exhibit plasticity following exercise (Skup et al., 2002), and TrkB cells in the IML, which houses sympathetic preganglionic neuronal cell bodies, were increased following distal transection of preganglionic axons (Coulibaly and Isaacson, 2012), possibly to contribute to neuronal survival and regeneration (Coulibaly et al., 2013).

In our assessment of the TrkB population in the spinal cord of intact uninjured animals, we expected that most cells in the OL cell lineage would express TrkB. Indeed, VonDran and colleagues (2010) demonstrated that both OPCs and mature OLs in the mouse basal forebrain expressed TrkB and that the cells in the OL lineage were dependent upon BDNF for survival. In addition, OPCs, which give rise to OLs during development (Miller, 2002) and in the adult CNS (Nishiyama et al., 2009; Barnabe-Heider et al., 2010), can be induced to differentiate in the presence of BDNF both *in vitro* (VonDran et al., 2010; VonDran et al., 2011) and *in vivo* (McTigue et al., 1998).

Yet our analysis revealed that only a relatively small proportion of OPCs in the adult spinal cord expressed TrkB at detectable levels. Horner and colleagues (2002) reported that ~3% of the NG2 population in the spinal cord was in the cell cycle over a 12 day period, while 97% of the cells were quiescent or carrying out other activities. These numbers compare favorably with the small proportion of NG2⁺/TrkB⁺ cells observed in our study. Because BDNF appears to regulate OPC proliferation and differentiation, we propose that the OPCs showing detectable levels of TrkB in the present study may represent the pool that has committed to either self-renew or to differentiate into OLs (Barnabe-Heider et al., 2008). Rather than exist in a quiescent state, cells in this stage would be actively dividing and/or maturing into OLs similar to the model proposed by Baumann and Pham-Dinh (2001). It should be noted that at least some of the OPCs that were not expressing detectable levels of TrkB may have been involved in functions other than renewal or OL differentiation and/or might be regulated by other stimulatory molecules, such as glutamate, FGF, PDGF, NGF and/or other neurotrophins (Miller, 2002; Nishiyama et al., 2009). Regardless of their exact function, our data support the existence of a heterogeneous NG2 cell population in the adult spinal cord GM and WM. Our results are supported by previous findings that the population of NG2 cells is heterogeneous in the adult spinal cord (Horner et al., 2002).

As expected, a majority of the CC1 cells co-expressed TrkB, suggesting that a large proportion of mature OLs are regulated by BDNF and/or NT-4. Yet a significant subset (~36%) of mature OLs either expressed TrkB at very low levels or did not express TrkB. It is possible that a subset of the mature OL subpopulation within the spinal cord loses responsiveness to, or possibly is not regulated by, BDNF or NT4.

When considering the phenotype of the TrkB population in the spinal cord, 81% of the TrkB cells expressed the mature OL marker, CC1, while less than 2% of TrkB cells expressed the OPC marker NG2. Therefore approximately 17% of the TrkB cells did not express

detectable levels of NG2 or CC1. Our studies as well as others show no localization of TrkB in other nonneuronal cells such as astrocytes or microglia (Skup et al., 2002; Garraway et al., 2011) and these TrkB only are not in the size range of neurons. While it is possible that these cells expressed NG2 or CC1 below the level of detection of our antibodies, they also may represent a subpopulation of TrkB cells in transition from the precursor (NG2⁺/TrkB⁺) to the mature stage (CC1⁺/TrkB⁺). Indeed, the presence of an “immature OL” stage, one that occurs between the precursor and mature stages, in which NG2 is down-regulated, but detectable levels of CC1 are not evident, has been suggested (Baumann and Pham-Dinh, 2001; Miller, 2002; Nishiyama et al., 2009).

3.2. Distribution of OL lineage cells in the adult spinal cord

OLs typically are known for their role in myelination and thus would be expected to be most prevalent in the white matter. However the results of the present study suggest that TrkB cells as well as OPCs and OLs are found in similar proportions in WM and GM throughout the spinal cord. The equal distribution of OPCs has been reported previously in the spinal cord (Horner et al., 2002) as well as the brain (Staugaitis and Trapp, 2009). To our knowledge, we provide the first report of a similar distribution of TrkB cells throughout the gray and white matter of the intact adult spinal cord.

Little is known regarding the function of OLs that reside in the GM. They may simply participate in the myelination of descending axonal tracts that pass through spinal cord GM (Peters et al., 1970; Shinoda et al., 1986). However, functions for OLs other than myelination have been reported. For example, OLs may play a role in neuroprotection (Taniike et al., 2002) and/or provide important metabolic support to CNS neurons in the form of lactate (Lee et al., 2012).

Approximately 20% of the TrkB population in the GM ventral horn was located within the perineuronal space of motor neurons and categorized as perineuronal. Further, ~75% of these TrkB perineuronal cells expressed the mature OL marker. In the cerebral cortex, perineuronal OLs in association with cortical neuronal cell bodies comprised ~44% of the total number of OLs (Takasaki et al., 2010). Though the role of perineuronal OLs in the spinal cord is not yet known, it is possible that they provide metabolic support to the neurons, as observed in the mouse cortex (Takasaki et al., 2010), and/or possibly protect the neurons from apoptosis (Taniike et al., 2002). Interestingly, the number of cortical perineuronal OLs was found to be significantly reduced in individuals with schizophrenia and mood disorders (Vostrikov et al., 2007).

Though rare, perineuronal OPCs were observed in the cervical segment and numerous NG2-ir processes were observed in close proximity to the perimeter of ventral horn motor neurons in both segments. We postulate that the processes emanate from nearby OPCs rather than from vascular pericytes. Though NG2 is also expressed by vascular pericytes, which in turn might be the source of these processes, most reports indicate that vascular NG2 cells interact only with blood vessels and blood brain barrier elements in the CNS (For review, see Sa-Pereira et al., 2012; Lange et al., 2013).

Specific interactions between ventral horn neurons and OPCs have not been described, yet others have reported NG2 cells in GM as well as WM CNS regions (Dimou et al., 2008; Horner et al., 2000), and have postulated that they may serve roles other than differentiation into OLs. In the WM, NG2 cells can make contact with nodes of Ranvier (Butt et al., 1999), and they express functional GABA and glutamate receptors (Bergles et al., 2000; Gallo et al., 2008), indicating a possible role in the propagation of action potentials. NG2 cells can receive synaptic input from neurons (Bergles et al., 2000; Karadottir et al., 2008; Kukley et al., 2008; for review see Maldonado et al., 2011), and it has been proposed that NG2 processes located in close proximity to the synaptic cleft can produce action potentials as a result of neurotransmitter spillover (Maldonado et al., 2011). NG2 cells adjacent to neurons in the brain GM may play a role in signal integration (Mangin et al., 2008).

It is unclear why perineuronal OPCs would be observed in the cervical segment, but not in the thoracic segment. Differences in brain regions also have been reported, in which OPCs associated readily with interneurons of the dentate gyrus (Mangin et al., 2008), while no perineuronal OPCs were observed in the cerebral cortex (Takasaki et al., 2010). Mangin and colleagues (2008) suggested that perineuronal OPCs in the dentate gyrus played a role in fine tuning the local neuronal network. Whether these cells play a similar role in the cervical spinal cord is a topic for further investigation.

NG2 cells never were observed in clusters, while TrkB and CC1 cells frequently were observed in small aggregates or clusters of 2 or more, particularly in the GM. The singular distribution of NG2 cells, which has been described previously in the spinal cord (Dawson et al., 2003) as well as the cerebral cortex (Hill et al., 2011; Dawson et al., 2000; Dawson et al., 2003; Staugaitis and Trapp, 2009), has been attributed to contact inhibition properties between the NG2 cells (Staugaitis and Trapp, 2009), and in turn provides a network of uniformly distributed NG2 cells throughout both the GM and WM. This singular distribution, together with the Notch pathway and interactions with other CNS elements, results in the creation of individual niches or domains in the CNS and may facilitate the proliferative abilities of the OPCs (McTigue et al., 1998). Such contact inhibition may be specific to the progenitor, undifferentiated status of the NG2 cells. It is unknown whether the clustering serves a role in mature OL function.

3.3 Density of OL lineage cells in the adult spinal cord

In the upper thoracic segment the density of OPCs averaged 58 and 73 NG2 cells/mm² in the WM and GM, respectively. This compares favorably with Schonberg et al. (2012) who reported 34 and 80 NG2 cells/mm² respectively in the WM and GM of the lower thoracic segment. In addition, we observed that the NG2 cell density in the GM of the cervical segment was significantly less than that of the thoracic segment. Indeed, NG2 cell density reportedly is quite variable throughout the CNS. For example, Wilson and colleagues (2006) reported an overall WM density of 171 NG2 cells/mm² in the CNS. On the other hand, Levine and colleagues (1993) reported 14 NG2 cells per every 40,000 mm² in the normal cerebellum. Such variability might reflect different region-specific functions for NG2 cells throughout the brain and spinal cord. Further, no differences were observed in the WM

versus GM distribution of any of the OL lineage cells examined in this study, suggesting that these cells may serve functional roles in both the WM and GM.

It is interesting to note that the density of mature OLs and TrkB cells in the spinal cord was approximately 10 fold higher than the NG2 cell density. Barnabe-Heider et al. (2010) reported that the number of OLs in the spinal cord increased over time, while the number of OPCs did not. This activity could explain the higher density of mature OLs compared with OPCs observed in the present study.

3.4. Summary and Conclusions

Though TrkB nonneuronal cells in the spinal cord were previously classified as OLs, little was known regarding their distribution and/or phenotype. The results of the present study reveal that the nonneuronal TrkB cells expressed Olig2, verifying that they are indeed OL lineage cells. These TrkB cells were distributed uniformly throughout the white and gray matter of the adult spinal cord. The widespread distribution of TrkB cells suggests that BDNF and/or NT-4 utilization by these cells may occur throughout all regions of the adult spinal cord. Local sources of BDNF might originate from neurons (Lu et al., 2005), nonneuronal cells such as astrocytes (Friedman et al., 1998; Dougherty et al., 2000) and/or microglia (Dougherty et al., 2000). Consistent with previous findings (Horner et al., 2002; Staugaitis and Trapp, 2009), OPCs and mature OLs also showed equal distribution in the WM and GM.

Interestingly, the TrkB population did not overlap completely with either OPC or OL populations. TrkB cells exhibited morphological characteristics more similar to mature OLs, and TrkB was expressed by a substantial proportion of mature OLs, while few OPCs expressed detectable levels of TrkB.

Only a small proportion of OPCs expressed detectable levels of TrkB, and this TrkB expressing pool of OPCs may represent a small population of OPCs preparing for renewal or the differentiation and maturation into OLs. While the majority of OLs expressed TrkB, a subset of mature OLs did not express TrkB, suggesting that some mature OLs reduce TrkB expression. Further, approximately 17% of TrkB cells expressed neither NG2 nor CC1. This 'TrkB only' population may represent an immature stage of OL maturation that has down-regulated NG2, but not yet up-regulated mature markers such as CC1.

Mature OLs were observed to form clusters with like cells in the spinal cord, and these clusters were more abundant in the GM. This cellular association suggests a communication between OL lineage cells and a supportive glial network to neurons. Indeed, a recent study demonstrated that mature OLs can provide lactate as an energy source to nearby neurons (Lee et al., 2012).

Approximately 56-58% of ventral horn motor neurons associated with perineuronal cells of the OL lineage. The majority of nestling cells expressed both CC1 and TrkB. The role of these nestling OLs is unknown, but they may play a neuroprotective role (Taniike et al., 2002). Our data suggest that BDNF/NT-4 (known ligands of TrkB) may play a role in the oligodendrocyte-neuronal interactions.

While OPC cell bodies were rarely observed nestling with neurons, their processes frequently were in close proximity to neuron cell bodies. The presence of NG2-immunoreactive processes found in close proximity to the neuronal cell bodies indicates a possible interaction between the OPCs and neuronal cell bodies in the spinal cord GM.

4. Experimental Procedure

4.1. Tissue preparation and immunohistochemistry

Adult female Sprague Dawley rats (Harlan labs, Indianapolis, IN; n=7) approximately 3 months of age were housed in a 12:12 hour light:dark cycle at Miami University Animal Facility. All experiments were done in accordance with a protocol approved by the Miami University Institutional Animal Care and Use Committee.

Animals were anesthetized with sodium pentobarbital (125 mg/kg), then transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Spinal cords were removed and the fourth cervical (C4) and first thoracic (T1) segments were cut at 18µm in the coronal plane using a MICROM HM 550 series cryostat. Sections were mounted on Superfrost microscope slides (Fisher Scientific), allowed to dry, and then incubated overnight in 0.1M PBS-0.2% Triton-X solution, followed by incubation with mouse anti-CC1 (1:500, Abcam #ab16794; for OLs, Bhat et al., 1996; De Nicola et al., 2009), mouse anti-NG2 (1:200, Millipore #MAB5384; for OPCs, Nishiyama et al., 2002), goat anti-olig 2 (1:100, R&D Systems #AF2418; Barnabe-Heider et al., 2010), and/or rabbit anti-TrkB (1:200, sc-12; Santa Cruz Biotechnologies, directed against the full length receptor) antibodies diluted in 0.1M PBS for 24 hours at 4°C. After rinsing, sections were incubated in Alexa Fluor conjugated antibodies (Molecular Probes; 1:200) directed toward the primary host antibody for 2 hours. Sections were then rinsed and coverslipped using fluorescent mounting medium with DAPI (Vector Labs).

4.2 Quantitative analysis

Images were captured with a Zeiss 710 confocal laser scanning microscope and Zen 2009 software. Using the microscope 'Tile' function, a single mosaic of the entire spinal cord (n=7) was automatically 'stitched' from partial images or 'tiles' that were each acquired at 200X magnification in a 4x7 configuration. The number and distribution of TrkB-ir, NG2-ir, CC1-ir profiles (referred to in this study as TrkB, NG2, CC1 cells) were analyzed within the white matter (WM) and gray matter (GM) areas of the spinal cord from tiled images that were magnified to approximately 500X on a computer monitor. A standardized measuring box (approx. 100 µm²) was used to characterize the number and location of OPCs and OLs that expressed TrkB in the spinal cord. The size of the box was determined using the approximate width of the dorsal horn as a guide. The box was placed over 14 gray and white matter regions of the spinal cord. In the WM, the box was placed over the lateral funiculus (LH) and the ventral funiculus (VF) just ventral to the ventral horn on both sides, and a second central region of the VF (Fig. 1.A.) as well as a central region of the dorsal funiculus (DF; Fig. 1.A.). In the GM, the box was placed bilaterally over the dorsal horn (DH) just ventral to Lamina II, the lateral horn (LH) and ventral horn (VH) and a region adjacent to central canal (CC; Fig. 1.A.). The number of cells within each region expressing each

marker was expressed as a proportion of number of cells in the entire cord expressing that marker. Counts were combined from left and right sides for bilateral regions while the VF was totaled from the two sides and a third, more central region. The values were converted to mm^2 for comparison across regions and demonstration in the figures and Venn diagrams (basic and stacked).

Several criteria were used to exclude TrkB neurons from the analysis. Neurons in the ventral horn that expressed TrkB were easily identified due to their large size (See Fig. 5). TrkB neurons in the lateral horn averaged $\sim 1,100 \mu\text{m}^3$ in volume (Coulibaly et al., 2013) compared with TrkB nonneuronal cells with an average volume of $\sim 53 \mu\text{m}^3$ (unpublished information). The minor proportion of small neurons in Lamina II of the dorsal horn that express TrkB (Salio et al., 2005) were not included in our analysis as the measuring box for the dorsal horn encompassed only the lowermost portion of Lamina II and included primarily Laminae III, IV, V. In addition to their larger size, the identity of TrkB neurons was verified by labeling with the NeuN marker (McCartney et al., 2008) a marker that shows specificity for neurons (Mullen et al., 1992). It should also be noted that the DAPI labeled nuclei exhibited by neurons typically were larger and paler when compared to dense, bright blue nuclei of the glial cells (See Figs. 1, 5, 6).

For the proportion of cells expressing CC1 ($\text{CC1}^+/\text{TrkB}^-$ and $\text{CC1}^+/\text{TrkB}^+$) within the double labeled CC1/TrkB population, as shown in the basic Venn (Fig. 3.C.), the total number of CC1^+ cells was divided by the total number of immunolabelled cells ($\text{CC1}^+/\text{TrkB}^-$ and $\text{CC1}^-/\text{TrkB}^+$ and $\text{CC1}^+/\text{TrkB}^+$). To determine the proportion of CC1 only cells, the number of CC1 only cells ($\text{CC1}^+/\text{TrkB}^-$) was divided by the total number of all CC1 immunolabeled cells ($\text{CC1}^+/\text{TrkB}^-$ and $\text{CC1}^-/\text{TrkB}^+$ and $\text{CC1}^+/\text{TrkB}^+$; Fig. 3.C., basic Venn). For the proportion of overlap between the two populations ($\text{CC1}^+/\text{TrkB}^+$), the number of $\text{CC1}^+/\text{TrkB}^+$ cells was divided by the total number of immunolabeled cells ($\text{CC1}^+/\text{TrkB}^-$ and $\text{CC1}^-/\text{TrkB}^+$ and $\text{CC1}^+/\text{TrkB}^+$; Fig. 3.C., basic Venn). To determine the proportion of cells expressing TrkB within the double labeled population as shown in the basic Venn ($\text{TrkB}^+/\text{CC1}^-$ and $\text{TrkB}^+/\text{CC1}^+$), the total number of TrkB^+ cells was divided by the total number of immunolabelled cells ($\text{TrkB}^+/\text{CC1}^-$ and $\text{TrkB}^+/\text{CC1}^+$ and $\text{TrkB}^-/\text{CC1}^+$). For the proportion of TrkB only cells, the number of $\text{TrkB}^+/\text{CC1}^-$ cells was divided by the total number of TrkB cells ($\text{TrkB}^+/\text{CC1}^-$ and $\text{TrkB}^+/\text{CC1}^+$; Fig. 3.C., basic Venn).

To determine the proportion of TrkB cells within the TrkB population that co-expressed CC1, the number of $\text{TrkB}^+/\text{CC1}^+$ cells was divided by the total number of TrkB^+ cells ($\text{TrkB}^+/\text{CC1}^-$ and $\text{TrkB}^+/\text{CC1}^+$; Fig. 3.C., stacked Venn). Within the TrkB population, the proportion of TrkB cells within the TrkB population that did not express CC1 was determined by dividing the number of TrkB only cells ($\text{TrkB}^+/\text{CC1}^-$) by the total number of TrkB immunolabeled cells ($\text{TrkB}^+/\text{CC1}^-$ and $\text{TrkB}^+/\text{CC1}^+$; Fig. 3.C., stacked Venn). Similar analyses were carried out for the NG2 and TrkB data shown in Figure 4.

To assess any differences in cell density between WM and GM, the mean number of profiles per mm^2 in the WM and GM was compared across animals ($n=7$) using Student's t-test with significance reported at $p < 0.05$. NG2-ir vascular cells such as pericytes exhibiting nuclear staining in the shape of a quarter circle (McTigue et al., 2001) and associating with blood

vessels (McTigue et al., 2001; Ligon et al., 2006) were excluded from the analysis. All NG2 non-vascular cells, regardless of their unipolar, bipolar, or multipolar morphologies (Horner et al., 2000), were included in the analysis.

The proportion of cells that were observed in direct apposition in a cluster of two or more cells in the GM (DH, LH, and VH) and WM (DF, LF, and VF) matter was calculated ($n=5$) at both cervical and thoracic segments. The proportions from GM and WM were compared across animals using a Student's t-test with significance reported at $p < 0.05$.

For the analysis of perineuronal cells, sections of the ventral horn were triple-labeled with TrkB/CC1/ChAT or TrkB/NG2/ChAT as shown in Figure 6. The images were acquired at 400X magnification and then magnified to 800X on the computer monitor for quantitative analysis. The proportion of ChAT-ir ventral horn neurons in association with at least one perineuronal OL lineage profile was calculated ($n=7$). For the number of cells in apposition to ventral horn motor neurons, the number of TrkB, CC1, or NG2 cell bodies that were directly apposed to a ventral motor neuron was expressed as a proportion of the total cells expressing that marker. The frequency of CC1⁺/TrkB⁺ or NG2⁺/TrkB⁺ profiles in the perineuronal cell population also was determined.

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Highlights

- A majority of mature oligodendrocyte (OLs) in spinal cord expressed TrkB
- Few oligodendrocyte precursor cells (OPCs) expressed detectable levels of TrkB
- TrkB cells resided in gray and white matter of the spinal cord in similar abundance
- A population of TrkB nonneuronal cells did not express markers for OLs or OPCs
- TrkB ‘perineuronal’ cells nestling with neuronal cell bodies typically were mature OLs

Figure 1.

Localization and identification of OL lineage cells in the adult rat spinal cord. **A.**

Diagrammatic representation of the adult rat spinal cord showing the location of the measuring boxes that were used in this study. DH, dorsal horn; LH, lateral horn; VH, ventral horn; CC, central canal; DF, dorsal funiculus; LF, lateral funiculus; VF, ventral funiculus. **B.** Confocal micrographs depicting the characteristic immunolabeling used to identify TrkB, CC1, and NG2 cells in the spinal cord. **C.** Double immunolabeling with TrkB (red) and Olig2 (green) revealed that all nonneuronal TrkB-immunoreactive (-ir) cells also expressed Olig2 (white arrows). Olig2 cells that were not TrkB-ir (yellow arrow) may represent mature OLs (CC1⁺/TrkB⁻) and OPCs (NG2⁺/TrkB⁻). TrkB-ir neurons (yellow asterisks), identified by larger cell volume, were observed in the gray matter and did not express Olig2. In merged images (far right, B., C.), DAPI was used to demonstrate the cellular nature of the staining. Scale bar in B. = 5 μ m; Scale bar in C. = 20 μ m.

Figure 2.

Density of OL lineage cells within white matter (WM) and gray matter (GM) in cervical (C4) and thoracic (T1) segments of the adult rat spinal cord. Comparison of cell densities between the WM and GM within segments revealed no differences in the number of TrkB (A.), CC1 (B.), or NG2 (C.) cells. In addition, no differences were observed in the number of TrkB and CC1 cells when the cervical and thoracic segments were compared. However, the density of NG2 cells in the GM in the thoracic segment was significantly higher when compared with GM density in the cervical segment. Data shown as mean $\pm$ SEM. *, significance reported at $p < 0.05$. WM, white matter, GM, gray matter.

Figure 3.

The majority of mature OLs expressed TrkB at detectable levels. **A.-A.''** Confocal micrographs of coronal sections through the T1 segment of the spinal cord reveal the co-localization of CC1 and TrkB in nonneuronal cells (yellow arrows). Note one cell expressing CC1 only (green arrows), but only faintly immunoreactive for TrkB. Scale bar = 50 μ m. **B.-B.''** Most cells expressed both CC1 and TrkB (yellow arrows), yet cells expressing CC1 only (green arrows) also were observed. DAPI was used to demonstrate the cellular nature of the staining. Scale bar = 20 μ m. **C.** Basic Venn diagrams (left panel) represent the percentage of cells that expressed CC1 only (green), TrkB only (red), or CC1

and TrkB (yellow) in cervical (C4) and thoracic (T1) segments. Stacked Venn diagrams (right panel) reveal that ~81% of the TrkB cells also expressed CC1 in both of the segments.

Figure 4.

Little overlap between OPC and TrkB populations in the intact spinal cord. **A.-A.ʹʹ** Confocal micrographs of coronal sections at T1 segment reveal the lack of co-localization between NG2 and TrkB cells. NG2 cells (green arrows) and TrkB cells (red arrows) showed no overlap. DAPI was used to demonstrate the cellular nature of the staining. Scale bar = 50 μ m. **B.** Basic Venn diagrams (left panel) represent the percentage of cells expressing NG2 only (green), TrkB only (red), or NG2 and TrkB (yellow) in the spinal cord in cervical (C4) and thoracic (T1) segments. Stacked Venn diagrams (right panel) reveal that ~2% of the TrkB cells also expressed NG2 in both of segments.

Figure 5.

Clusters of TrkB and CC1 cells were observed primarily in the GM of the spinal cord. **A.-A.ʹʹ** Both TrkB (red) and CC1 (green) cells in the ventral horn (delineated by yellow lines) frequently were observed in aggregates or clusters of two or more cells (white arrows). Motor neurons (yellow asterisks) abutted by CC1⁺/TrkB⁺ cells were present. DAPI used to demonstrate the cellular nature of each cell type. Scale bar = 50 μ m. **B.** Graphs showing the number of TrkB (upper) and CC1 (lower) cells that were observed in clusters at cervical (C4) and thoracic (T1) segments of the spinal cord. In the thoracic segment, the number of

CC1 and TrkB clusters was significantly higher in the GM compared with WM. Data shown as mean $\pm$ SEM. *, significance at $p < 0.05$ when comparing WM and GM at T1.

Figure 6.

A population of OL lineage cells resided in close apposition to motor neurons in the ventral horn of adult rat spinal cord and was categorized as perineuronal. **A.-A."** The majority of perineuronal cells expressed both CC1 and TrkB (yellow arrows). CC1 cells that were not in close proximity to motor neuron cell bodies (*) also were observed (white arrows). **B.-B."** TrkB perineuronal cells (white arrows) are shown in close proximity to ChAT-ir ventral horn motor neurons (*). Yellow arrows indicate the TrkB nonneuronal cells that were not in direct apposition to neuronal cell bodies. **C.-C."** NG2 cell cell bodies (yellow arrows) rarely were observed in direct apposition to ventral horn neuronal cell bodies (i.e. perineuronal), yet NG2-ir processes (white arrows) frequently were observed in close proximity to the ventral horn neuronal cell bodies (*). Scale bar = 50 μ m.