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Up-regulation of Robo1 in dorsal root ganglia after sciatic nerve transection in rats

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Roundabout (Robo) receptors have many important roles including mediating the migration of the growth cone and neurons, promoting axonal growth and development of nerve fascicles, and guiding the growth direction of central nervous system by binding Slit. To better understand the role of Robo in peripheral nervous system, this study investigated the expression profile of Robo1 in the dorsal root ganglia (DRG) of adult rats following sciatic nerve transection (SNT). Adult Sprague-Dawley rats that were untreated (n = 8), or received SNT (n = 40), were analyzed. DRG from each treatment group at days three, seven, 14, 21, and 28 post-SNT were collected and assayed by real-time PCR and immunohistochemistry. Expression of Robo1 and 2 was performed, with staining also evaluated in relation to neuron diameters. We found that both mRNA and protein levels of Robo1 were detected in normal DRG, and these levels increased following SNT. Increases were initially detected at day three post-SNT, then peaked between day seven and 14, then gradually returned to basal levels by day 21 post-SNT. Neither normal, nor SNT DRG exhibited co-localization of Robo1 and 2, and this observation was independent of neuron diameter. These results suggest that Robo1 in DRG is upregulated following sciatic nerve transection in rats.

Key words: Dorsal root ganglion, rat, roundabout 1, sciatic nerve injury.

INTRODUCTION

The roundabout (Robo) families of receptors are members of the immunoglobulin superfamily of cell adhesion molecules (CAM). Robo1-4 contain five Ig subunits, three type III fibronectin repeats, a transmembrane region, and long cytoplasmic tails that contain robo-specific motifs (Hivert et al., 2002; Kidd et al., 1998a, b; Liu et al., 2004; Sundaresan et al., 1998; Jaworski et al., 2010; Pappu and Zipursky, 2010; Zhang et al., 2010). Robo proteins share homology with other CAMs, including neural cell adhesion molecule (NCAM), L\textsubscript{1} protein, and DCC (deleted in colorectal carcinoma), and thus are capable of homophilic and heterophilic binding interactions to promote neurite outgrowth (Doherty et al., 2000; Hivert et al., 2002; Kutcher et al., 2004; Li et al., 1999). Upon binding of Robo proteins by the ligand, Slit, Robo proteins can direct the growth of the central nervous axons and promote cell migration, as well as axon branching and dendrite growth (Brose et al., 1999; Li et al., 1999; Rajagopalan et al., 2000; Wang et al., 1999; Whitford et al., 2002). Robo proteins also contribute to axon tract fasciculation in the developing nervous system (Hivert et al., 2002; Liu et al., 2004; Simpson et al., 2000; Sundaresan et al., 2004; Jaworski et al., 2010; Pappu and Zipursky, 2010). Moreover, both

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the Slit and Robo protein families have been shown to be essential for axon guidance and cell migration in worms, flies, fish and mice (Brose et al., 1999; Brose and Tessier-Lavigne, 2000, Inatani, 2005, Rajagopalan et al., 2000; Wong et al., 2002; Jaworski et al., 2010; Pappu and Zipursky, 2010).

Robo1 was the first Robo gene identified in Drosophila based on a comprehensive screen for factors that regulate growth cone midline crossing in the developing central nervous system (CNS) (Inatani, 2005; Kidd et al., 1998b). To date, four vertebrate Robo homologues have been cloned, Robo 1-4 (Camurri et al., 2004; Kidd et al., 1998b; Lee et al., 2001; Nguyen-Ba-Charvet and Chedotal, 2002). The spatiotemporal expression of vertebrate Robo and Slit genes has been well-characterized during the early embryonic stages (Holmes et al., 1998; Mambetisaeva et al., 2005; Marillat et al., 2002; Rudenko et al., 1999), and expression has also been detected both postnatally and into adulthood (Marillat et al., 2002; Wehrle et al., 2005; Hagino et al., 2003; Kidd et al., 1998a, b; Rajagopalan et al., 2000). Robo1 is specifically expressed in the developing retina and olfactory bulb, in dorsal root ganglia (DRG), and in the spinal cord of zebrafish, chicken, mice and rats (Challa et al., 2001; Lee et al., 2001; Li et al., 1999; Mambetisaeva et al., 2005; Marillat et al., 2002; Nguyen-Ba-Charvet and Chedotal, 2002). These experimental results indicate that the spatiotemporal expression of Robo1 appears to be an important determinant of function during the neuronal development of the brain (Hagino et al., 2003; Kidd et al., 1998a, b; Rajagopalan et al., 2000).

Our previous data demonstrated that both Slit and Robo2 exhibit differential expression patterns in the adult rat spinal cord, DRG, and sciatic nerve before and after sciatic nerve transection (SNT). These results suggest that Slit1 and Robo2 have important roles in regeneration of peripheral nerve injury (Yi et al., 2006). Slit1 is immunohistochemistry.

Therefore, in this study, expression levels and distribution patterns of Robo1 in adult rat DRG were investigated following SNT using real-time PCR and immunohistochemistry.

MATERIALS AND METHODS

Animal models of SNT

All procedures were performed with the approval of the local animal ethics committee, in accordance with university guidelines for animal experiments (Chinese government animal protection and management law). A total of 48 adult Sprague-Dawley rats (250 ± 20 g) were used (Xiayang Center of Experimental Animals, Central South University, Changsha, China) and these were randomly divided into two groups: (1) a normal group (n = 8), and (2) a group that underwent sciatic nerve transection (SNT) (n = 40). For surgery, rats were anesthetized with an intraperitoneal injection of 2% sodium pentobarbital (40 mg/kg) and fixed in a prostrate position. All surgeries were conducted under sterile conditions. The sciatic nerve 10 mm proximal to its division to the tibial and common peroneal nerves was transectioned (Yi et al., 2006). After surgery, all rats were injected subcutaneously ketoprofen at a dose of 5 mg/kg once daily for three days. In order to obtain samples for different timepoints following SNT (that is, three, seven, 14, 21 and 28 days post-SNT), rats were randomly subdivided into five groups (n = 8).

Tissue preparation

For immunohistochemistry, rats were over-anesthetized with pentobarbital, then perfused with saline, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. L4-L6 DRG were carefully dissected and post-fixed in the same fixative for 2 h. DRG were then transferred sequentially to 10, 20, and 30 % sucrose solutions in phosphate buffered saline (PBS) for three days or longer at 4°C for cryoprotection until sectioning. For real-time PCR samples, the rats were over-anesthetized and L4 to L6 DRG were collected into liquid nitrogen and homogenized immediately.

Real-time quantitative PCR

Total RNA was extracted from frozen tissues using TRizol Reagent according to the manufacturer’s instructions (Invitrogen, USA). The quality of the RNA obtained was determined based on 28S/18S rRNA bands detected on electrophoresis gels stained with ethidium bromide. RNA concentrations were determined using NanoDrop spectrophotometry (Thermo Scientific, USA), and cDNA was generated from 500 ng pooled total RNA from each treatment group using SuperScript II reverse transcriptase (Invitrogen, USA) and oligo-dT primers. Real-time quantitative PCR assays were performed using a LightCycler 480 quantitative PCR system (Roche) with SYBR Green (TOYOBO Co., Japan), and 28 s rRNA detected as an internal control. Primers used in the amplification reaction included (shown 5' to 3'): 28S rRNA F, AGC AGCCGACTTATAGCAG; 28S rRNA R, CACTGGGCGATTGAGAGAGGCCTACACAGATG, and R, TAGGGACAGTGGGAATCTCG; rat Robo1 F, TTATAGCAG; and R, TAGGGACAGTGGGAATCTCG; rat Robo1 F, TTATAGCAG. RNA concentrations were determined using NanoDrop spectrophotometry (Thermo Scientific, USA), and cDNA was generated from 500 ng pooled total RNA from each treatment group using SuperScript II reverse transcriptase (Invitrogen, USA) and oligo-dT primers. Real-time quantitative PCR assays were performed using a LightCycler 480 quantitative PCR system (Roche) with SYBR Green (TOYOBO Co., Japan), and 28 s rRNA detected as an internal control. Primers used in the amplification reaction included (shown 5' to 3'): 28S rRNA F, AGCAGCGACTTTAGAAGCTG, and R, TAGGGACAGTGGGAATCTCG; rat Robo1 F, GCAGAGGGCCCTACACAGATG, and R, CACTGGGCGATT-TTATAACAG.

Immunohistochemistry

Free floating sections were washed in PBS / 0.3% Triton x-100 / 5% bovine serum albumin (BSA) for 1 h, thereafter, were incubated with goat anti-rat Robo1 antibody (1:200, Santa Cruz Biotechnology, USA) overnight at 4°C. After three washes in PBS, sections were incubated in biotinylated rabbit anti-goat antibody (1:200, Vector, USA) for 2.5 h, then in avidin biotin complex (ABC) solution (1:100, Vector) for 1 h at RT. Subsequently, sections were rinsed and stained with 0.05% diaminobenzidine (DAB, Vector) in the presence of 0.03% hydrogen peroxide for 5 min. Following additional rinses, sections were mounted on gelatin subbed slides, air dried, dehydrated in ethanol, cleared in xylene, and coverslipped with DPX (Fluka Chemie AG, Switzerland). As a negative control, sections were incubated without primary antibody and processed as described above (Zheng et al., 2008, 2010). For immunofluorescence microscope studies, sections were
In this study, a transection of the peripheral axons of damaged DRG following SNT as indicated. Values represent the mean ± SEM (n = 3). p < 0.05 compared with Nor.

Data collection and statistical analyses

Light microscopy images were captured using a digital camera (Nikon, Tokyo, Japan) at 100 × magnification. Five sections from each specimen and five visual fields for each section were randomly selected. The optical density (OD) value of positively stained cells in each field were quantified using Image Pro Plus 5.0 for Windows, and the number of stained neurons, according to their diameter, were counted. Analysis of digitized images was performed. All data are presented as the mean ± standard error of the mean (SEM). Comparisons between the normal group and the SNT group were made using one-way analysis of variance (ANOVA) followed by Dunnett’s tests. A p-value less than 0.05 was considered statistically significant. All data were analyzed using SPSS 15.0 statistical software for Windows.

RESULTS

Changes in levels of Robo1 mRNA in DRG following SNT

As shown in Figure 1, levels of Robo1 mRNA detected by real-time PCR were observed to increase at day three post-SNT, and peaked between day seven to 14 post-SNT. At day 21, mRNA levels for Robo 1 had returned to normal levels. Furthermore, on days seven and 14, the relative expression of Robo1 compared to the normal group was significantly different (p < 0.05).

Robo1 protein levels in DRG following SNT

Expression of Robo1 was detected in large (25 to 40 µm), medium (25 to 15 µm), and small (< 15 µm) DRG sensory neurons using immunohistochemical methods (Figures 2B and C). In these assays, an increase in levels of Robo1 were detected at day three post-SNT, the highest levels were detected at day seven post-SNT, and Robo1 levels gradually returned to basal levels at day 21 post-SNT (Figures 2D to H). On days three, seven and 14 post-SNT, the OD values of the positively stained cells between SNT samples and normal or contralateral DRG significantly differed (p < 0.05). Moreover, neither large, medium, nor small DRG sensory neuron numbers were observed to change at any of the examined time points following SNT (Figure 3). Co-expression studies of Robo1 and 2 were also performed for samples of normal DRG and damaged DRG following SNT, and no neurons co-expressing Robo1 and 2 were detected (Figure 4).

DISCUSSION

In this study, a transection of the peripheral axons of
DRG neurons resulted in the up-regulation of Robo1 in all sizes of neurons after peripheral nerve injury. However, neither normal nor sensory neurons affected by SNT were observed to co-express Robo1 and 2. In combination, these findings suggest that Robo1 has a role in normal DRG and plays a role following sciatic nerve injury in rats.

Robo1 is up-regulated in primary sensory neurons after peripheral nerve injury

Injured peripheral neurons regain their regenerative capacity by reverting to gene expression patterns that were used during development. These gene programs involve hundreds of genes, and many of these genes that are highly expressed, including injury- and growth-associated molecules such as growth-associated protein 43 (GAP-43), cytoskeleton-associated protein 23 (CAP-23), and some neurotrophic receptors, have been shown to mediate peripheral nerve regeneration following peripheral axotomy (Costigan et al., 2002; McLean et al., 2002; Xiao et al., 2002). Unlike central nerve injury, peripheral branches of sensory neurons remain in contact with target tissues, which provide trophic support (Schwab and Bartholdi, 1996; Smith and Skene, 1997; Woolf et al., 1990). Moreover, injury to the central process has been shown to have a limited effect on gene
expression profiles of DRG neurons, which is characterized by an absence of GAP-43 up-regulation, and limited, or absent, regeneration were observed in the dorsal root and spinal cord, respectively (Chong et al., 1994; Schnell and Schwab, 1990; Schwab and Bartholdi, 1996; Schwaiger et al., 2000). In this study, an increase in Robo1 expression was detected in primary sensory neurons in adult rat DRG following peripheral transection. This pattern of expression is similar to the expression profiles of other growth-associated molecules, and some trophic receptors, during peripheral nerve regeneration (Fu and Gordon, 1997). In combination, these data suggest that Robo1 expression is primarily influenced by the target tissues that provide trophic support.

The functional role of Robo1 in DRG neurons remains unknown. However, Robo receptors have been shown to positively promote axonal elongation and branching, to cause growth cone collapse and guidance axonal growth, and to serve as the receptor of Slit (Hammond et al., 2005; Rajagopalan et al., 2000; Wang et al., 1999).

It has also been hypothesized that Robo receptors form a functional complex at the plasma membrane in response to Slit binding (Yi et al., 2006). This interaction may occur in DRG to affect neurons and the growth cone.

The induction of Robo1 following peripheral injury may also reflect on the early stages of a regenerative program initiated by injured primary sensory neurons.

**Robo1 may have a role in guiding the regeneration of the growth cone of injured peripheral nerves**

During the development of the CNS, an axonal growth cone detected, reacted to environmental cues to direct an axon to the appropriate location. These guidance cues, both attractive and repulsive, act through distinct signaling pathways to reorganize the cytoskeleton in responsive cells (Hornberger et al., 1999). Based on the differential expression of receptor complexes that have been detected, a single guidance cue can have attractive or repulsive effects depending on the intracellular state of the cell and the cross-talk that has occurred between intracellular signaling cascades (Hammond et al., 2005; Hornberger et al., 1999). Slit1 and Slit2 are ligands of Robo receptors that mediate downstream signaling from Robo proteins, and are expressed in Schwann cells of peripheral nerves (Yi et al., 2006). When 3T3 cells were transfected with Robo, neurite outgrowth of Robo-positive

*Figure 3. Quantitation of the number of large (black), medium (light gray), and small (dark gray) DRG sensory neurons observed in normal and SNT samples. No change in the number of DRG sensory neurons was detected at any of the examined time points following SNT. Values represent the mean ± SEM (n = 5).*
neurons, such as retinal neurons and olfactory neurons, were stimulated. In contrast, Robo-negative neurons, such as cerebellar granule cells, were unaffected (Hivert et al., 2002). Similarly, when DRG of chicken embryos, trigeminal neurons of zebrafish, and cultured DRG neurons of adult rat were treated with an antibody raised against the first 1g domain of Robo1/2, or a soluble Robo-Fc chimera, outgrowth was inhibited (Yeo et al., 2004; Yi et al., 2006).

Therefore, in combination, these data suggest that the expression of Robo1 in DRG neurons mediates signaling to guide the growth cone of injured peripheral nerves during regeneration.

Homophilic and/or heterophilic interactions between Robo family members can directly contribute to axon tract fasciculation in the developing nervous system (Hivert et al., 2002; Yeo et al., 2004). Interestingly, our results indicate that Robo1 and 2 are not co-expressed in sensory neurons, thereby suggesting that Robo1 and 2 have different roles in the process of peripheral nerve regeneration following injury.

In summary, our results indicate that Robo1 is expressed in adult rat DRG neurons, and undergoes changes in expression following axotomy. Correspondingly, further studies are needed to address the role of Robo1 expression in DRG during the processes of neuronal survival and axonal regeneration.

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**REFERENCES**


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