Distribution of paired immunoglobulin-like receptor B in the nervous system related to regeneration difficulties after unilateral lumbar spinal cord injury

Wan-shu Peng, Chao Qi*, Hong Zhang, Mei-ling Gao, Hong Wang, Fei Ren, Xia-qing Li

Department of Pathophysiology, Shanxi Medical University, Taiyuan, Shanxi Province, China

Introduction
Repair of the central nervous system (CNS) after injury is known to be difficult (Yiu and He, 2006; Filbin, 2008), and regeneration occurs after peripheral nervous system (PNS) damage (Faroni et al., 2015). Myelin-associated inhibitors exist in the nerve cell membrane, and many of these inhibitors cannot be promptly removed after CNS damage (Akbik et al., 2013; Gou et al., 2014). Myelin-associated inhibitors bind to their respective receptors on neuronal membranes (Llorens et al., 2011). Suppressing or removing these receptors does not restore the regenerative ability of nerve cells in the CNS (Llorens et al., 2011). The paired immunoglobulin-like receptor B (PirB) is a functional receptor of myelin-associated inhibitors for axonal regeneration and synaptic plasticity in the central nervous system, and thus suppresses nerve regeneration. The regulatory effect of PirB on injured nerves has received a lot of attention. To better understand nerve regeneration inability after spinal cord injury, this study aimed to investigate the distribution of PirB via immunofluorescence in the central nervous system and peripheral nervous system 10 days after injury. Immunoreactivity for PirB increased in the dorsal root ganglia, sciatic nerves, and spinal cord segments. In the dorsal root ganglia and sciatic nerves, PirB was mainly distributed along neuronal and axonal membranes. PirB was found to exhibit a diffuse, intricate distribution in the dorsal and ventral regions. Immunoreactivity for PirB was enhanced in some cortical neurons located in the bilateral precentral gyri. Overall, the findings suggest a pattern of PirB immunoreactivity in the nervous system after unilateral spinal transaction injury, and also indicate that PirB may suppress repair after injury.

Abstract
Paired immunoglobulin-like receptor B (PirB) is a functional receptor of myelin-associated inhibitors for axonal regeneration and synaptic plasticity in the central nervous system, and thus suppresses nerve regeneration. The regulatory effect of PirB on injured nerves has received a lot of attention. To better understand nerve regeneration inability after spinal cord injury, this study aimed to investigate the distribution of PirB via immunofluorescence in the central nervous system and peripheral nervous system 10 days after injury. Immunoreactivity for PirB increased in the dorsal root ganglia, sciatic nerves, and spinal cord segments. In the dorsal root ganglia and sciatic nerves, PirB was mainly distributed along neuronal and axonal membranes. PirB was found to exhibit a diffuse, intricate distribution in the dorsal and ventral regions. Immunoreactivity for PirB was enhanced in some cortical neurons located in the bilateral precentral gyri. Overall, the findings suggest a pattern of PirB immunoreactivity in the nervous system after unilateral spinal transaction injury, and also indicate that PirB may suppress repair after injury.

Key Words: nerve regeneration; paired immunoglobulin-like receptor B; myelin inhibitory factor; spinal cord injury; peripheral nervous system; central nervous system; cerebral cortex; dorsal root ganglion; neural regeneration

Funding: This project was supported by the National Natural Science Foundation of China, No. 81171178; the Natural Science Foundation of Shanxi Province in China, No. 2012011036-3; the Research Project of Shanxi Scholarship Council of China, No. 2012-047.

Peng WS, Qi C, Zhang H, Gao ML, Wang H, Ren F, Li XQ (2015) Distribution of paired immunoglobulin-like receptor B in the nervous system related to regeneration difficulties after unilateral lumbar spinal cord injury. Neural Regen Res 10(7):1139-1146.

Materials and Methods
Animals
Six clean male Wistar rats, aged 4–6 weeks and weighing 220–240 g were provided by the Experimental Animal Resources, Shanxi Medical University, China (license No. SCXK (Jin) 2009-0001). Rats were fed normal food and housed in a standard cage in a quiet room (protected from sunlight and noise) under 12-hour light/dark cycles. This study has been approved by the ethics committee at Shanxi Medical University, China. All rats were equally and randomly divided into the model group (left unilateral spinal cord injury group) or sham group (uninjured group). The uninjured right spinal cord and peripheral nerve in the model group served as normal control.

Unilateral SCI
Rats were anesthetized with 1% sterile sodium pentobarbital intraperitoneally. They were then placed face down and the whole spine was exposed. After the animals were shaved (with...
Animals were deeply anesthetized (with 1% sodium pentobarbital (0.4 mL/kg)) and then fixed with 200 mL 4% paraformaldehyde (by cardiac perfusion after 200 mL ice-cold saline perfusion). The two sides of sciatic nerves, the L3-L5 DRG, and injured spinal cord segments (such as the proximal segment (0.3 mm from the center of injury site cranially), the center of injury (0.2–0.3 mm), and the distal segment (0.3 mm from the center of injury site caudally)) were harvested. The frontal cortex, precentral gyrus, postcentral gyrus, and cerebellum were also collected (Paxinos and Watson, 2005). The precentral and postcentral gyri were located by identifying the central sulcus. The precentral and postcentral gyri tissues were collected 0.5 mm from the central sulcus. After post-fixation (with 4% paraformaldehyde) and immersion with 30% sucrose solution overnight, the tissues were embedded in optimal cutting temperature compound and then sectioned into 12-µm-thick slices by a cryostat (Leica-CM1950, Leica Microsystems GmbH, Wetzlar, Germany).

**Immunofluorescence**

Sections (n = 9–10) from each sample were placed on poly-lysine precoated slides and then washed with 0.01 M PBS (3 × 5 minutes) and permeabilized in 0.1% Triton X-100 for 10 minutes at room temperature. Blocking with 5% normal donkey serum for 1 hour at room temperature inhibited non-specific staining. Sections were subjected to the primary antibody goat anti-PirB polyclonal antibody (1:500; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. The sections were then washed with 0.01 M PBS (3 × 10 minutes), followed by incubation with the secondary antibody donkey anti-goat IgG conjugated with Alexa Fluor-488 (1:500; Life Technologies, Shanghai, China) for 1 hour at room temperature (in the dark). Sections were then washed (3 × 5 minutes) with 0.01 M PBS. The sections were subsequently mounted with 25 µL anti-fade gold mounting medium with 4′,6-diamidino-2-phenylindole (Life Technologies). All sections were observed under an upright fluorescent microscope (Olympus, Tokyo, Japan). The presence of PirB was measured based on the optical density value determined by ImageJ software (NIH).

**Statistical analysis**

All data are expressed as the mean ± SEM and were analyzed by one-way analysis of variance followed by the Tukey’s multiple comparison test using Prism graph-pad 5.0 (GraphPad Software, La Jolla, CA, USA). A value of P < 0.05 was considered statistically significant.

**Results**

**General behavior and motor/sensory function in rats with unilateral SCI**

Before rats were subjected to the SCI or sham operation, paw withdrawal thermal latency and motor function (holding power) were both normal. All SCI rats showed paralysis of the left lower limb. The holding power of the ipsilateral limb was totally lost from day 1 up to day 10. The paw withdrawal thermal latency of the ipsilateral limb (left) was significantly (P < 0.05) protracted/lost within the maximal measurement...

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period of 50 seconds compared with the contralateral hindlimb. Furthermore, motor/sensory function in the contralateral limb remained normal (Table 1).

**Distribution of PirB in the PNS and CNS**

In the PNS, PirB was negative in the DRG neurons of normal rats. A few PirB-positive cells were identified in the capsule of DRG, and this appeared to be non-neuronal expression. Additionally, PirB immunoreactivity was not found in a transverse section of the sciatic nerve. Compared with the PNS, some positive cells were observed in both the dorsal and ventral horn areas of the spinal cord. PirB-positive cells were mainly distributed along the meninges spinalis, and weakly positive cells were observed in the deep part of dorsal and ventral horns (Figure 2).

In uninjured rats, PirB immunoreactivity was differentially distributed in the cerebellum, cortex of the frontal cerebrum, and cortex of the precentral and postcentral gyr. Compared with other brain areas, PirB immunoreactivity was distinct in the cerebellum. PirB was heavily distributed in neuronal processes instead of the cytoplasm. Moreover, PirB was diffusely distributed in both cerebellar gray and white matter.

**Table 1** Holding power (g) and paw withdrawal thermal latency (second) test in rat hindlimbs after unilateral spinal cord injury (SCI)

| Time after SCI | Holding power | Paw withdraw thermal latency |
|----------------|---------------|-----------------------------|
|                | Left hindlimb | Right hindlimb              | Left hindlimb | Right hindlimb |
| 24 hours       | 0°            | 53.85±7.12                  | > 50°         | 24.30±0.37     |
| 1 week         | 0°            | 53.40±6.01                  | > 50°         | 23.30±0.54     |
| 2 weeks        | 0°            | 52.95±9.01                  | > 50°         | 22.01±0.59     |
| 3 weeks        | 0°            | 54.55±6.44                  | > 50°         | 23.10±0.28     |
| 4 weeks        | 0°            | 54.55±6.44                  | > 50°         | 23.60±0.31     |

Data are presented as the mean ± SEM. Three rats in each time point. Statistical analysis for comparison of mean values was performed by one-way analysis of variance followed by the Tukey’s multiple comparison test. *P < 0.05, vs. right hindlimb.
In the cortices of the precentral and postcentral gyri of the two cerebral hemispheres, PirB staining was observed in only a couple of neuronal processes. The distribution pattern of PirB staining in the cortices of both frontal cerebral was found to be cytoplasmic and mainly distributed along the cell membrane (Figure 3).

**Unilateral SCI altered the expression of PirB in the spinal cord**

Ten days after left unilateral SCI, cells of the ipsilateral (left) dorsal and ventral horns were positive for PirB. This was also observed in neuronal processes at the proximal, middle and distal segments of the injury site. PirB immunoreactivity on the left side of the spinal cord was observed to be stronger compared with the respective locations of the right contralateral spinal cord. Quantification of PirB immunoreactivity in the proximal, middle, and distal segments of the left (ipsilateral) dorsal horn was 2.15, 5.70, and 2.20 times stronger, respectively, than those respective contralateral segments. Compared with the contralateral segments in the ventral horns, PirB immunoreactivity was 2.94, 2.60 and 5.73 times stronger on the ipsilateral side. PirB immunoreactivity in the ventral horn was relatively stronger than that in the dorsal horn on the ipsilateral side. PirB immunoreactivity in the ipsilateral spinal cord was higher than that in the contralateral spinal cord ($P < 0.05$ or $P < 0.001$; Figure 4).

PirB immunoreactivity in the dorsal or ventral horn of the contralateral spinal cord in injured rats was stronger than in sham surgery rats 10 days after injury (Figure 5). The number of PirB-positive neurons in both cortices of the left and right precentral gyri in the model group was not statistically different compared with sham surgery rats. PirB expression in the cerebellum after injury was not altered compared with sham surgery rats (data not shown).

**The altered expression of PirB in the PNS and CNS occurred away from the injury site**

After unilateral mechanical injury, the variation in PirB immunoreactivity in ipsilateral $L_3$ DRG and sciatic nerve was similar to that of the respective segments on the contralateral side (i.e., increased presence of PirB in the ipsilateral DRG and sciatic nerve). More specifically, PirB immunoreactivity in ipsilateral DRG was mainly distributed along the neuronal or axonal membrane (Figure 6). PirB immunoreactivity in the sciatic nerve was distributed along the axonal membrane. Quantification revealed that PirB immunoreactivity in the ipsilateral DRG and transverse sciatic nerve was significantly ($P < 0.01$) greater than that of the contralateral side. In the injured brain, the increase in PirB immunoreactivity and its cytoplasmic and membrane distribution pattern were observed in the cortex of the precentral gyri (Figure 6C). Interestingly, in the uninjured brain, PirB was only distributed along neuronal processes in this region (Figure 2).

**Discussion**

Myelin-associated inhibitors may play an inhibitory role in axonal regrowth after CNS injury because of myelin-associated inhibitor receptors on the neuronal membrane (Lopez et al., 2011). In addition to the Nogo receptor-p75 neurotrophin receptor Lingo/Troy receptor complex, PirB has been identified as the functional receptor for myelin-associated inhibitors (Kim et al., 2004; Syken et al., 2006; Filbin, 2008; Adelson et al., 2012). Increased expression of PirB at the injury site inhibits nerve regeneration in the brain after hypoxic-ischemic cerebral damage (Adelson et al., 2012; Wang et al., 2012; Wang and Zhi, 2014). However, the total expression pattern of PirB after injury in specific brain regions has not been clearly described. We therefore investigated the distribution and level of expression of PirB in the rat PNS and CNS after 10 days of left SCI. The results showed that PirB expression at the injury site was increased, and this finding corroborates with previous studies (Zhou et al., 2010; Wang et al., 2012; Gou et al., 2014; Israelsson et al., 2014). The present study also showed that the increased expression of PirB occurred remotely from the injury site, but its expression was still relevant to the site of injury based on the anatomical structure or physiological co-relationship. Our findings revealed that the altered expression occurred in many regions except the injury site. The modulation of PirB expression in remote locations after unilateral injury may be indicative of intracellular signaling occurring antegrade and/or retrogradely. These pathways may interfere with the functional status of the CNS after injury.

PirB has also been described and identified on cells of the immune system (Kubagawa et al., 1999; Takai and Ono, 2001; Uehara et al., 2001; Masuda et al., 2005; Nakayama et al., 2012). Importantly, PirB is a dual functional molecule for brain and immunity (Boulanger et al., 2001; Nakamura et al., 2004; Syken et al., 2006). PirB is involved in normal brain development, synaptic plasticity, and neurodegeneration (Llorens et al., 2011; Mironova and Giger, 2013). The intracellular mechanism of PirB has been shown to be similar in both immune and nervous systems. Neon light signaling for T cells has helped to understand the intracellular effects when major histocompatibility complex proteins (such as PirB) communicate with specific peptides/proteins on its cell surface (Takai et al., 2011). Major histocompatibility complex expression on the cell membrane initiates T-cell binding for immune signaling activation (Imada et al., 2009). In the nervous system, PirB mediates inhibitory effects mainly on neutrophils and macrophages (cells that infiltrate the nervous tissue) (Pereira et al., 2004), and also initiates an inhibitory effect by binding to myelin-associated inhibitors on neuronal membranes (Atwal et al., 2008; Filbin, 2008). However, the expression and spatial and developmental regulation of PirB in some subsets of neurons strongly suggests that PirB is involved in neuronal functions under both physiological and pathophysiological conditions (Boulanger, 2009; VanGuilder Starkey et al., 2012). In addition to its inhibitory role in axonal regeneration after CNS injury, PirB has multiple effects on the structure and function of the CNS by interacting with myelin-associated inhibitors. These effects include the restriction development and neuroprotection after stroke (Llorens et al., 2011; Adelson et al., 2012).
neuronal degeneration, and changes in synaptic structures in Alzheimer's disease (Djurisic et al., 2013; Kempf and Schwab, 2013; Kim et al., 2013).

A structural or functional relationship is likely to exist between the NGR-P75<sup>NTR</sup>-Lingo/Troy complex and PirB (Filbin, 2003). PirB-mediated inhibition of axonal re-growth has been thought to be due to the inhibition of Trk by the tyrosine phosphatase SHP-2 (Maeda et al., 1998; Fujita et al., 2011b). Binding of myelin-associated inhibitors to the NGR-P75<sup>NTR</sup>-Lingo/TROY receptor complex initiates the axonal regrowth inhibitory pathways, and has recently been shown to be directly or indirectly related to the Rho-associated coiled-coil containing protein kinase and myosin light chain signaling pathways (Peng et al., 2011; Rolando et al., 2012). SHP-2 is a novel regulator of Rhoa isoenzyme RhoGAP in cultured vascular smooth muscle cells (Bregeon et al., 2009; Kimura and Eguchi, 2009). Rhoa is activated by SHP-2 via Rhoa phosphorylation, and therefore, PirB interacts synergistically with NGR-P75<sup>NTR</sup>-Lingo/TROY (Llorens et al., 2011; Mironova and Giger, 2013). Moreover, PirB itself inhibits the Rho-ROCK signaling pathway (Wang et al., 2012). Three myelin-associated inhibitors have been shown to lose their efficacy for inhibiting axonal regeneration when their respective receptor complex is neutralized or eliminated (Zheng et al., 2005; Fujita et al., 2011a). In the current study, immunoreactivity for PirB was found to be weak in some specific regions in the PNS and CNS of the sham group. The distribution pattern of PirB in the DRG and sciatic nerve appeared to be non-neuronal, but there may have been a neuronal distribution in the cerebral cortex. However, the results did not provide information about the PirB-positive component. Therefore, more details about the distribution pattern of PirB in the nervous system under normal conditions need further verification. After unilateral mechanical lumbar injury in our study, the expression of PirB was activated in selective regions of the PNS and CNS. Remarkably, immunofluorescence staining for PirB was enhanced at the ipsilateral spinal cord 10 days after left SCI. PirB staining in all of the proximal, central, and distal segments of the left spinal cord, including the dorsal horn and ventral horn, was more diffuse and stronger compared with the contralateral segments. PirB immunoreactivity was strongest in the center of the injury. Moreover, the morphology of PirB-positive cells appeared neuron-like in the PNS and CNS. However, the temporal and spatial pattern of PirB immunoreactivity at the injury site in the relevant locations to injury could not be concluded from this study. The degradation of myelin may have occurred during SCI. Therefore, the high level of PirB immunoreactivity at the injury site may have been a cellular response to the local accumulation of myelin-associated inhibitors, and it may have also been activated by these inhibitors.

Myelin-associated inhibitors originate from local injury in the PNS and are quickly engulfed by phagocytes that infiltrate the injury area (Ma et al., 2011). Therefore, the high immunoreactivity level of PirB in the PNS after unilateral SCI in this study should be interpreted differently. Therefore, the stronger expression of PirB in the ipsilateral DRG and sciatic nerve after left SCI in this study represents a signaling pathway from the CNS to the PNS. This can be hypothesized because of the afferent terminals of DGR neurons that extend into the dorsal horn of the spinal cord, and the efferent processes of ventral motors in the spinal cord that are part of the sciatic nerve.

In the present study, the sensory and motor center in the cortex of the bilateral precentral and postcentral gyri showed different expression patterns of PirB after left unilateral SCI. The increased expression of PirB on some neuronal membranes was observed in the cortex of the bilateral precentral gyri but not in postcentral gyri. This finding may suggest that the signal for PirB activation can spread to the bilateral locomotor centers through information exchange at different neural ascending pathways by synapses. The message may then reach both sides of the motor center. Nevertheless, the negative reactivity of PirB in the sensory center that we observed was not expected. The accuracy of the anatomical location, temporal transport of PirB from the spinal cord to the center, and the severity of spinal dorsal horn injury may be the reasons for the lack of immunoreactivity. The expression of PirB at different locations in the CNS at different time periods of injury deserves further investigation.

In summary, the increased expression of PirB at the injury site and at specific neural afferent and efferent pathways suggests the involvement of myelin-associated inhibitors and their receptors for the CNS during injury.

Author contributions: XQL obtained funding, designed the study, provided technical support and revised the manuscript. WSP participated in the immunofluorescent staining and writing the final paper. CQ and HZ contributed equally to animal surgery and sample processing. HZ, MLG, HW and FR contributed equally to sample section and data analysis. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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Figure 5 PirB immunoreactivity in the right (non-injured side) dorsal and ventral horn of rat spinal cord after injury. PirB immunoreactivity (green) in dorsal and ventral horn of injured spinal cord is stronger than that of sham surgery rats 10 days after surgery. Scale bar: 50 µm. PirB: Paired immunoglobulin-like receptor B.

Figure 6 Immunoreactivity for PirB in the PNS and CNS is localized away from the injury site. (A) PirB immunoreactivity (green) in DRG neurons and transverse sciatic nerve is mainly localized on the cellular membrane. (B) Quantification of PirB immunoreactivity in the DRG and sciatic nerve. Data are expressed as the mean ± SEM (3 rats per time point) and were analyzed by one-way analysis of variance followed by the Tukey’s multiple comparison test. ***P < 0.001, vs. contralateral (right). (C) PirB immunoreactivity (green) in the cortex of the bilateral precentral gyri. Scale bars: 50 µm. DRG: Dorsal root ganglion; PirB: paired immunoglobulin-like receptor B.
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