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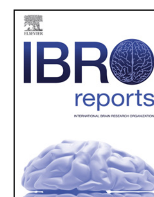

Neonatal spinal injury induces de novo projections of primary afferents to the lumbosacral intermediolateral nucleus in rats

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ABSTRACT

Complete spinal transection in adult rats results in poor recovery of hind limb function and severe urinary bladder dysfunction. Neonatal rats with spinal cord transection, however, exhibit spontaneous and significant recovery of micturition control. A previous study in which biotinylated-dextran amine (BDA) was used as an anterograde tracer demonstrated that primary afferent fibers from the fifth lumbar dorsal root ganglion (DRG) project more strongly and make more terminals in the ventral horn after neonatal spinal cord transection at the mid-thoracic level. In the present study, we injected BDA into the sixth lumbar (L6) DRG of neonatally spinalized rats to label primary afferent fibers that include visceral afferents. The labeled fibers projected to the intermediolateral nucleus (IML) in the intermediate zone on ipsilateral side of the L6 spinal segment, whereas no projections to the IML were observed in sham-operated or intact rats. The BDA-labeled fibers of neonatally spinalized rats formed varicose terminals on parasympathetic preganglionic neurons in the IML. These findings suggest that some primary afferent projections from the L6 DRG to the IML appear after neonatal spinal cord transection, and these de novo projections might contribute to the recovery of autonomic function such as micturition following spinal cord injury in the neonatal stage.

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1. Introduction

Spinal cord injury (SCI) in the adult causes sensorimotor and autonomic dysfunction, even leading to death in some cases (Krassioukov et al., 2007). Despite numerous studies aimed at enhancing recovery from impairments caused by SCI, no therapeutic strategies for SCI have been established (Mathias and Low, 2014). In experimental studies, thoracic spinal cord transection in adult rats and cats leads to severe bladder dysfunction in addition to paraplegia at spinal levels below the injury site (de Groat et al., 1998). Severe dysuria in rodents with complete SCI is usually managed by researchers manually pressing on the urinary bladder twice a day (Herrera et al., 2010).

Rats that undergo spinal cord transection at the neonatal period, however, exhibit far less motor dysfunction compared to those at the adult stage (Stelzner et al., 1975; Yuan et al., 2013; Takiguchi et al., 2015). We recently demonstrated that compensatory projections of primary afferent fibers to the intermediate zone and the ventral horn in the lumbar spinal segment are strengthened after neonatal spinal cord transection at the thoracic level, based on experiments in which biotinylated dextran amine (BDA), an anterograde tracer, was administered into the dorsal root ganglion (DRG) (Takiguchi et al., 2015). This plastic change in the primary afferent projections caused by neonatal spinal cord transection might be involved in the ameliorative effects on motor function because axonal regeneration of descending tracts through the injury site has not been confirmed in either the neonatal SCI model or the adult SCI model.

In contrast to motor function, the mechanisms underlying the recovery of autonomic function after neonatal SCI have not been thoroughly investigated. In adult rats, several brain regions are important for controlling micturition. Among them, the pontine micturition center, also referred to as Barrington's nucleus, is considered mainly responsible for regulating micturition (de Groat et al., 1998). The descending projections from Barrington's nucleus to the lumbosacral spinal cord form by postnatal day (P5) in neonatal rats (Sugaya et al., 1997). When supraspinal projections from the pontine micturition center are interrupted in neonatal rats, primary afferent projections to the spinal cord may increase to compensate for urinary bladder dysfunction. In the present study, therefore, we examined whether primary afferent projections to the intermediolateral nucleus (IML) in the sixth lumbar (L6) spinal

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segment, which contains parasympathetic preganglionic neurons (PPNs), are increased in neonatally spinalized rats. We labeled primary afferent fibers by administering BDA into the L6 DRG, which contains visceral afferents from the urinary bladder. We then examined chemical markers of DRG neurons incorporating the BDA using double-labeling immunohistochemistry.

2. Materials and methods

2.1. Materials

Wistar rats neonates (n = 45, Japan SLC, Hamamatsu, Japan) and Wistar rat dams (n = 4, Japan SLC) were used in the present study. All experimental procedures were performed according to the standards established by the NIH Health Guide for the Care and Use of Laboratory Animals and the Policies on the Use of Animals and Humans in Research. The protocols were approved by the Institutional Animal Care and Use Committee of the Animal Research Center, Yokohama City University Graduate School of Medicine.

2.2. Neonatal spinal cord transections

The spinal cords of P5 rat pups (n = 13; neo-ST rats) that were randomly selected from each four litters were completely transected at the eighth thoracic (T8) level, as previously described (Takiguchi et al., 2015). Briefly, under anesthesia with isoflurane gas (1.0%-2.0%), spinal cords were completely transected with small spring microscissors following partial laminectomy (T7-T9). Once bleeding stopped, the muscles, fascia, and skin were closed in layers using 6-0 nylon sutures. The rats were gently cleaned with gauze soaked in 70% ethanol to eliminate the smell of blood, then returned to their home cages after recovery from anesthesia. Sham-operated rats (n = 13) from four litters underwent laminectomy, but no spinal transection. Intact rats (n = 19) from four litters underwent no surgical treatments. These three groups were randomly allocated in almost equal proportions from each litter. All rat pups were returned to their dams. All rat pups were weaned at 3 weeks after surgery (P26) and housed individually in polycarbonate cages in a room maintained at $25 \pm 1\,^{\circ}C$, with a 05:00 on/19:00 off light cycle. Neo-ST rats exhibited severe motor disturbances of the hind limbs, but they could sweep their limbs by extensively moving their joints. They could also urinate by themselves.

2.3. BDA administration

At P33, BDA was administered into the L6 DRG to label primary afferent fibers by a protocol similar to that reported previously (Takiguchi et al., 2015). All rats (n = 45) were anesthetized with isoflurane and the left L6 DRG was exposed following unilateral laminectomy. BDA (0.5 μL; MW 10 kDa, 10% in distilled water, D1956, Life Technologies, Carlsbad, CA, USA) was injected using a glass pipette attached to a micromanipulator (MNM-333, Narishige, Tokyo, Japan). The tip of the pipette was 50 μm in diameter, and the micropipette was connected to a 1.0-μL Hamilton syringe by polyethylene tubing (size 5, Igarashi Ika Kogyo Co., Ltd., Tokyo, Japan). After the injection, the muscles, fascia, and skin were sutured in layers using 4-0 nylon.

2.4. Tissue preparation

Tissue processing was performed according to a modified protocol as previously described (Takiguchi et al., 2015). One week after the BDA injection, the rats were deeply anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde (4% PFA) in 0.1 M phosphate buffer (PB). The spinal cord and L6 DRG were dissected and postfixed with 4% PFA overnight at 4 °C. The tissues were cryoprotected in 25% sucrose in 0.1 M PB for 2 days and embedded in O.C.T. compound by immersion in 2-methylbutane (isopentane) cooled by liquid N_2. Sections of the DRGs and spinal cords including the L6 segment were cut at a thickness of 50 μm with a cryostat (CM3050 S, Leica, Nussloch, Germany) and stored in 0.01 M phosphate-buffered saline (PBS) at 4 °C. To visualize DRG immunofluorescence, 20-μm thick sections were cut using the same cryostat and mounted on gelatin-coated microscope slides.

2.5. BDA histochemistry

Some serial sections of the spinal cord were used for BDA histochemistry. BDA was visualized with a Vectastain Elite ABC standard kit (PK-6100, Vector Laboratories, Inc., Burlingame, CA, USA). The nickel-enhanced method was used as previously described (Takiguchi et al., 2015).

2.6. Double-labeling Immunohistochemistry

Some serial sections of the L6 spinal segment were mounted on microscope slides. The sections were post-fixed in 4% PFA in 0.1 M PB (pH 7.4) for 15 min at room temperature. After rinsing in 25 mM PBS, the sections were incubated in PBS containing 0.1% Tween 20 (PBST) for 30 min. The sections were then washed in PBS and placed in blocking solution, 5% (w/v) Block Ace (Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan) at room temperature for 60 min. Following the blocking procedure, the spinal cord sections were incubated with goat polyclonal anti-choline acetyl transferase (ChAT; 1:100, Millipore, Temecula CA, USA) overnight at 4 °C. The next day, the sections were rinsed in PBST and PBS, and subsequently incubated for 90 min at room temperature in DyLight 488-conjugated donkey anti-goat IgG (H + L) (1:400, 705-485-147, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) After several washes, the sections were incubated with Alexa Fluor 555-conjugated streptavidin (1:150, S32355, Life Technologies). The slides were coverslipped with slow-fade reagent (SlowFade Gold antifade reagent, S36936, Invitrogen, Carlsbad, CA, USA).

Cryosections of the DRGs on the slides were post-fixed in 4% PFA in 0.1 M PB (pH 7.4) for 15 min at room temperature. After rinsing in 25 mM PBS, the sections were incubated in PBST for 30 min. Next, the sections were washed in PBS and placed in blocking solution at room temperature for 60 min. Following the blocking procedure, the sections were incubated with goat polyclonal anti-calcitonin gene-related peptide (CGRP; 1:200, 1720–9007, AbD Serotec, Kidlington, UK), or mouse monoclonal anti-neurofilament 200 kDa (RT-97; 1:200, GeneTex Inc., Irvine, CA, USA) overnight at 4 °C. The next day, the sections were rinsed in PBST and PBS, and subsequently incubated for 90 min at room temperature in DyLight 488-conjugated donkey anti-goat IgG (H + L) (1:400, 705-485-147, Jackson ImmunoResearch Laboratories) or DyLight 488-conjugated donkey anti-mouse IgG (H + L) (1:400, 715-485-150, Jackson ImmunoResearch Laboratories). After several washes, the sections were incubated with Alexa Fluor 555-cojugated streptavidin (1:150, S32355, Life Technologies). The slides were coverslipped with slow-fade reagent (SlowFade Gold antifade reagent, S36936, Invitrogen).

The specificity of the antibodies was verified by incubation with 0.5% normal mouse serum (Jackson ImmunoResearch Laboratories), or 0.5% normal goat serum (Jackson Immuno Research Laboratories) instead of the primary antibodies.

2.7. Image acquisition and analysis

Z-stack Images of the DRG and spinal cord were digitally photographed using a Keyence BIOREVO microscope (BZ-9000,

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