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Dynamic expression pattern of leucine-rich repeat neuronal protein 4 in the mouse dorsal root ganglia during development

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HIGHLIGHTS

- The expression pattern of Lrrn4 was investigated in the DRGs during development.
- We used Lrrn4 reporter knock-in mice to characterize Lrrn4-expressing cells.
- The expression of Lrrn4 was dynamically changed in developing DRG neurons.
- The expression of Lrrn4 may correlate to the synaptogenesis in the spinal cord.

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ABSTRACT

A member of leucine-rich repeat neuronal protein (Lrrn) family, Lrrn4, is a type I transmembrane protein and functions as a cell adhesion molecule. In our previous report, Lrrn4 is expressed in a subset of small-sized dorsal root ganglion (DRG) neurons of the adult mice. In the present study, we investigated the expression pattern of Lrrn4 in the developing DRGs. The expression of Lrrn4 was first observed in 7% of total DRG neurons at embryonic day (E) 13.5, gradually increasing to 44% at E17.5, reached the maximum level between E17.5 and postnatal day (P) 7, decreased drastically after P7, and became the adult level by P14. Interestingly, the expression of Lrrn4 was mainly observed in TrkC-positive neurons at E13.5, and the predominant expression was shifted from TrkC-positive neurons to TrkA-positive neurons between E15.5 and E17.5. As the central afferents of TrkC-positive and TrkA-positive neurons begin to penetrate into the spinal cord to form synapse with secondary neurons at E13.5 and E15.5, respectively, the time course of Lrrn4 expression may suggest the contribution of Lrrn4 to synaptic formation. In addition, some cell adhesion molecules containing leucine-rich repeat are identified as synaptic adhesion molecules, suggesting that the spatiotemporal expression pattern of Lrrn4 contributes to the development of synaptic function in the DRG neurons.

1. Introduction

Dorsal root ganglion (DRG) neurons convey sensory information from the periphery to the spinal cord [8]. Distinct subtypes of DRG neurons are classified by the expression of neurotrophic factor receptors, such as receptor tyrosine kinase (Trk) family and Ret [14]. The Trk family consists of TrkA, TrkB, and TrkC, which are predominantly expressed in the nociceptive, mechanoreceptive, and proprioceptive neurons, respectively [6,14]. In the nociceptive neurons, TrkA and Ret, which is the common receptor subunit for the ligands of glial cell-derived neurotrophic factor family, are expressed in the peptidergic and non-peptidergic neurons, respectively [7].

Although the expression of TrkA, TrkB, TrkC, and Ret is restricted to the subpopulation of mature neurons in the DRGs of adult mice, their expression patterns alter drastically during the development [6,7]. During the embryonic development, TrkA is expressed in 80% of DRG neurons between embryonic day (E) 13 and postonatal day (P) 0; TrkB is expressed in 40% of DRG neurons at E11 and their proportion drops to 10% at E13; TrkC is expressed in almost all DRG neurons at E11.5 and the prevalence drops to less than 10% at E13; Ret is expressed in less than 10% of DRG neurons until E15 and their population gradually increases to be 45%

Abbreviations: CAM, cell adhesion molecule; CNS, central nervous system; E, embryonic day; DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LRR, leucine-rich repeat; L, lumbar; Lrrn, leucine-rich repeat neuronal protein; Lrrn4$^{+/-}$, Lrrn4 heterozygous; P, postnatal day; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.1% Triton X-100; RT, room temperature; Trk, receptor tyrosine kinase.

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at P0. After birth, their prevalence becomes the adult level until P21 [6,7].

During the development of the neuronal circuits, cell adhesion molecules (CAMs) are involved in various cellular events such as neuronal survival, axon guidance, axonal fasciculation, synapse formation, and synaptic maturation [1]. CAMs consist of several protein families such as integrin family, cadherin family, immunoglobulin superfamily, and leucine-rich repeat (LRR) superfamily [1,4,11]. Recent studies have demonstrated that several members of LRR superfamily are localized at synaptic sites and are involved in various aspects of synaptogenesis including transsynaptic adhesion of pre- and post-synaptic neurons [11].

A member of LRR superfamily, leucine-rich repeat neuronal protein (Lrrn) 4 is a type I transmembrane protein and is identified as a new member of Lrrn family [2]. In the central nervous system (CNS), Lrrn4 is expressed in the various regions including the hippocampus and cortex and is involved in hippocampus-dependent memory retention [2]. In contrast to the CNS, Lrrn4 is exclusively expressed in a subset of small-sized nociceptive neurons in the adult DRGs [3]. In the present study, we examined the expression pattern of Lrrn4 in the DRGs using β-galactosidase staining in Lrrn4-heterozygous ($Lrrn4^{+/-}$) mice generated by the replacement of Lrrn4 gene with β-galactosidase gene during embryonic and postnatal development.

2. Materials and methods

2.1. Mice

In the present study, we used C57BL/6J mice (CLEA Japan, Tokyo, Japan). Previously, $Lrrn4^{+/-}$ mice have been generated by replacing the exon 1 of Lrrn4 gene to β-galactosidase gene and backcrossed to C57BL/6J mice at least 6 times [2]. These mice were kept under a 12-h light/dark cycle with food and water *ad libitum*. At all times, the experiments were performed under the control of the Animal Research Control Committee in accordance with the Guidelines for Animal Experiments of Wakayama Medical University, Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6), and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80-23, revised 1978). All efforts were made to minimize the number of animals used and their suffering.

2.2. Northern blotting

Northern blot analysis was performed with some modifications as previously described [3]. Briefly, total RNA was isolated from the DRGs of various stages of postnatal mice. Total RNA was fractionated in a 1.2% agarose gel containing 2.4% formaldehyde and then transferred to a positively charged nylon membrane (Roche Diagnostics, Indianapolis, IN, USA). The blotted membrane was hybridized with the Lrrn4 cDNA fragment labeled with [α-^{32}P] dCTP. After the hybridization, the membrane was washed with 2× SSC (1× SSC: 44.6 mmol/L NaCl, 5 mmol/L trisodium citrate, pH 7.0) and 0.1% sodium dodecyl sulphate solution at 68 °C for 20 min and exposed to X-ray films.

2.3. Tissue preparation

Tissues were prepared from embryonic (E13.5, E15.5 and E17.5), postnatal (P0, P7 and P14), and adult (P56) mice. For β-galactosidase staining and immunohistochemistry, postnatal and adult mice were deeply anesthetized and transcardially perfused with ice-cold 0.85% NaCl followed by ice-cold modified Zamboni's fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate-buffered saline [PBS]). The lumbar (L) 4/5 DRGs were quickly removed and postfixed in the same fixative at 4 °C for 3 h. Embryos were obtained from pregnant mice under deep diethyl ether anesthesia. These embryos were immersed in the modified Zamboni's fixative at 4 °C for 3 h. Then, all specimens were immersed in 20% (w/v) sucrose in 0.1 M PBS for 16 h and then they were embedded in the O.C.T. medium (Miles, Elkhart, IN, USA), and frozen rapidly in cold *n*-hexane on dry ice.

2.4. β-Galactosidase staining

Frozen sections were cut on a cryostat at 6-μm thickness. After the washing with PBS-T (0.1 M PBS with 0.1% Triton X-100), the sections were incubated in the β-galactosidase staining solution (5 mM potassium hexacyanoferrate [II], 5 mM potassium hexacyanoferrate [III], 2 mM $MgCl_2$, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal] in 0.1 M PBS) at 37 °C overnight. After the washing with PBS-T, these sections were counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, UK).

2.5. β-Galactosidase staining combined with immunohistochemistry

After the β-galactosidase staining, the sections were preincubated in 0.1 M PBS containing 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) at room temperature (RT) for 1 h, followed by the incubation with rabbit anti-TrkA polyclonal antibody (diluted at 1:100; 06-574; Upstate Biotechnology, Lake Placid, NY, USA), goat anti-TrkB polyclonal antibody (diluted at 1:1600; AF1494; R&D Systems, Minneapolis, MN, USA), goat anti-TrkC polyclonal antibody (diluted at 1:400; AF1404; R&D Systems), goat anti-Ret polyclonal antibody (diluted at 1:100; AF482; R&D Systems) at 4 °C for 16 h. After the washing with PBS-T, they were incubated with biotinylated donkey anti-goat or rabbit immunoglobulin G antibodies (diluted at 1:400; Jackson ImmunoResearch) at RT for 1 h, followed by the incubation with horseradish peroxidase-conjugated streptavidin (Dako, Carpinteria, CA, USA). The peroxidase reaction was developed with 0.06% 3-amino-9-ethylcarbazole (Dako) in 50 mM Tris buffer (pH 7.7) containing 0.03% hydrogen peroxide. The specificity of the anti-TrkA, anti-TrkB, and anti-TrkC antibodies was described in our previous study [13]. Jongen et al. have shown the specificity of the anti-Ret antibody [9]. In addition, the characteristics of these primary antibodies were described in Supplemental information.

2.6. Quantitative analysis

The quantitative analysis of β-galactosidase staining was performed by the modified method for immnohistochemistry [3]. For cell counting, four embryos at each stage and L4/L5 DRGs from four mice at each postnatal stage were pooled and processed for the sectioning. The embryos and DRGs were sectioned at 6 μm. For embryonic DRGs, four sections that were 60 μm apart were selected. For each DRGs from postnatal and adult mice, five sections that were 120 μm apart were selected. To visualize all neuronal profiles through the development, we performed β-galactosidase staining followed by the nuclear staining with Nuclear Fast Red. To characterize the Lrrn4-expressing cells, we performed β-galactosidase staining combined with immunostainings, and then the nuclei were visualized with hematoxylin only for the cell counting (Supplemental Fig. 1). We counted only cells with clearly visible nuclei and nucleoli. The images were processed using the software Photoshop Element 3.0 (Adobe, Seattle, WA, USA).