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Research report

Upregulation of Ryk expression in rat dorsal root ganglia after peripheral nerve injury

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ABSTRACT

To study changes of Ryk expression in dorsal root ganglia (DRG) after peripheral nerve injury, we set up an animal model of unilateral sciatic nerve lesioned rats. Changes of Ryk protein expression in DRG neurons after unilateral sciatic nerve injury were investigated by immunostaining. Changes of Ryk mRNA were also tested by semi-quantitative PCR concurrently. We found, both at the level of protein and mRNA, that Ryk could be induced in cells of ipsilateral DRG after unilateral sciatic nerve lesion. Further investigation by co-immunostaining confirmed that the Ryk-immunoreactive (Ryk-IR) cells were NeuN-immunoreactive (NeuN-IR) neurons of DRG. We also showed the pattern of Ryk induction in DRG neurons after sciatic nerve injury: the number of Ryk IR neurons peaked at 2 weeks post-lesion and decreased gradually by 3 weeks post-lesion. The proportions of different sized Ryk IR neurons were also observed and counted at various stages after nerve lesion. Analysis of Ryk mRNA by RT-PCR showed the same induction pattern as by immunostaining. Ryk mRNA was not expressed in normal or contralateral DRG, but was expressed 1, 2 and 3 weeks post-lesion in the ipsilateral DRG. Ryk mRNA levels increased slightly from 1 to 2 weeks, decreased then by 3 weeks post-lesion. These results indicate that Ryk might be involved in peripheral nerve plasticity after injury. This is a novel function apart from its well-known fundamental activity as a receptor mediating axon guidance and outgrowth.

1. Introduction

Ryk is a RTK-related receptor that differs from other members of this family at a number of conserved residues in the activation and nucleotide-binding domains and lacks detectable catalytic activity [6,9,11,24–26]. The interesting feature in the extracellular domain of RYK is the presence of a Wnt inhibitory factor (WIF) module [19], suggesting the possibility that Ryk may bind to one of the Wnt family members.

The analysis of Ryk-deficient mice has recently demonstrated an absolute requirement for Ryk in normal development and morphogenesis of craniofacial structures and the limbs [8]. Several studies have now uncovered a role for Ryk–Wnt interactions in axon guidance during the mammalian central nervous system (CNS) development. These have identified Ryk interacting with Wnt1/Wnt5a as a key chemorepulsive axon guidance receptor in the establishment of major axon tracts, such as the corpus callosum and corticospinal tract (CST) [12,13]. In addition, Ryk-Wnt3 interaction is pivotal to the topographic mapping of retinal ganglion cell (RGC) axons onto the lateral region of the optic tectum within the embryonic chick brain [20]. Moreover, Ryk also was reported to be required for Wnt3a-mediated neurite outgrowth of the embryonic dorsal root ganglia, even without obvious deficiency in DRG neurite outgrowth in Ryk siRNA transgenic mice [14].

Although it is still a long way to go for fully understanding the Ryk function during the embryogenesis, but in order to truly overcome the CNS injury, we need to learn more about the basic developmental mechanism and molecules involved in the adult context. Considering Ryk functions in several important aspects of axons guidance and DRG neurite outgrowth at developmental stages, in this study we observed at changes in Ryk expression in dorsal root ganglia (DRG) neurons after peripheral never injury, and tried to provide insights for further study of its functional role in axonal plasticity.

2. Materials and methods

2.1. Animal models

All animal experiments conformed to the regulations of the Animal Research Committee of Chinese Academy of Sciences in accordance with the Guidelines on

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Animal Experiments at Institute of Biophysics, Chinese Academy of Sciences, which is based on the NIH Guidelines for the Use and Care of Laboratory Animals. Male Sprague–Dawley (SD) rats weighing 200–250 g were supplied by a regional vendor, Vital River Inc. (Beijing, China) and used as experimental animals. The rats were anesthetized with pentobarbital (25 mg/kg, Sigma–Aldrich Inc., Saint Louis, MO, USA) administered intraperitoneally and surgery was done to expose the right branch of sciatic nerve, using hemostatic forceps the sciatic nerve was clamped three times (10 s clamped with 10 s interval each time). After the operation a 2 mm injured area along the sciatic nerve could be seen, the skin was sutured and the animal was allowed to recover. Then the rats were kept in favorable environment and sacrificed at 7, 14 and 21 days post-surgery.

2.2. Immunohistology

At 7, 14 and 21 days after sciatic nerve injury, seven rats from each group were sacrificed with an overdose of pentobarbital (Sigma–Aldrich Inc.). Bilateral L4–L6 dorsal root ganglia were dissected out after the animals were perfused with 4% PFA (Sigma–Aldrich Inc.). The ganglia from the uninjured side or from normal rats were used as negative controls. The ganglia were post-fixed overnight at 4 °C, then washed with 1× PBS and transferred to 30% sucrose for 24 h. The tissues were embedded in Tissue-Tek O.C.T (Electron Microscope Science, Hatfield, PA, USA) and quickly frozen on dry ice. The ganglia were sectioned longitudinally at 20 μm with a freezing microtome (Leica Microsystems, Wetzlar, Germany). Serial sections were collected and put on gelatin-coated slides until dry. All sections were incubated with blocking buffer (PBS containing 5% normal goat serum, 1% bovine serum albumin and 0.3% Triton X-100, all from Sigma–Aldrich Inc.) for 1 h. For Ryk immunostaining, sections were then incubated with Ryk polyclonal antibody (rabbit anti-mouse IgG, 1:1000) which was produced using two peptide antigens from N-terminal amino acid residues (N-TWHAKSKVEYKLGF-C) [10] and C-terminal amino acid residues (N-YLKDGYRIAQPINCP-C) of the Ryk protein. A Trk A polyclonal antibody (rabbit anti-mouse IgG, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer was used for Trk A immunostaining. For Ryk and NeuN co-immunostaining sections were incubated with a mixture of NeuN monoclonal antibody (mouse anti-mouse IgG, 1: 500, Chemicon International Inc., Temecula, CA, USA) and Ryk polyclonal antibody (rabbit anti-mouse IgG, 1:1000). All incubations with primary antibodies were carried out at 4 °C overnight. After washing with PBS and incubating with blocking buffer, sections for Ryk or Trk A immunostaining were incubated with Cy-3 goat anti-rabbit IgG (1:1000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) or Cy-3 goat anti-mouse IgG (1:1000, Jackson ImmunoResearch Laboratories Inc.). Sections for Ryk and NeuN co-immunostaining were incubated together with Cy-3 goat anti-rabbit IgG (1:1000, Jackson ImmunoResearch Laboratories Inc.) and Cy-2 goat anti-mouse IgG (1:1000, Jackson ImmunoResearch Laboratories Inc.). All the secondary antibodies were incubated for 2 h at room temperature. Then all slides were mounted on gelatin-coated slides with Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL, USA), air-dried, coverslipped, and observed under a fluorescent microscope (Nikon Instruments Inc., Melville, NY, USA).

2.3. In situ hybridization and immunostaining on adjacent sections

The 1 kb Ryk cDNA fragment that includes 500 bp nucleotides of 3′ untranslated region and 500 bp of coding region at the carboxy terminus was cloned into pBluescript (Stratagene, Agilent Technologies Company, Cedar Creek, TX, USA) from rat E13.5 cDNA by RT-PCR, and used as a probe for hybridization. The Ryk antisense cRNA, labeled with DIG RNA Labeling Kit (Roche Applied Science Inc., Penzberg, Germany), was used as a probe. The Ryk sense cRNA was used as a negative control probe. Dorsal root ganglia were fixed in 4% PFA, and frozen sections 5–10 μm thick were prepared after equilibration in 10–20% sucrose and embedding in Tissue-Tek O.C.T (Electron Microscope Science). *In situ* hybridization was carried out as previously described [4].

To combine the NeuN immunostaining and Ryk *in situ*, we serially sectioned each DRG into 5–10 μm sections. All sections were collected and labeled with serial number according to the sectioning order. Then all the odd-numbered sections were used for NeuN immunostaining and all the even-numbered sections for Ryk *in situ*. Pictures of adjacent section pairs were merged using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). Each pair included one section used for Ryk *in situ* (even number "*n*") and one section used for NeuN immunostaining (odd number "$n-1$" or "$n+1$").

2.4. Semi-quantitative reverse transcription-polymerase chain reaction

At various time stages after sciatic nerve lesion, rats were decapitated while under pentobarbital anesthesia, and the L4–L6 dorsal root ganglia ipsilateral and contralateral to the nerve lesions were collected on dry ice and stored at −70 °C until RNA extraction could be performed. Each sample consisted of six ganglia from two rats (the pooled ipsilateral or contralateral L4–L6 ganglia from two rats). Three ipsilateral and three contralateral samples were analyzed for each time point. Total RNA from the ganglia samples was extracted by the guanidinium-thiocyanate method with TRIZOL-reagent (Invitrogen Corporation, Carlsbad, CA, USA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with SuperScript™ II RT-PCR system (Invitrogen Corporation). The primer sequences were as follows: Ryk 5′-ccacaggcaatttcccaacaagatc-3′(forward), 5′ gttgattggctgggctattcggtaacc-3′ (reverse, 399 bp product). Beta-actin: 5′-tagaagcatttgcggtgcacg-3′ (forward), 5′-tgcccatctatgagggttacg-3′(reverse, 640 bp product). Trk A: 5′-ctggtgcattcccttctctgtgg-3′ (forward), 3′-ggtctctgatgtgctgttagtg-5′ (reverse, 329 bp product). The following PCR conditions were used to determine the linear range of the *Ryk* gene amplification: 2 min at 94 °C, 5 cycles of 20 s at 94 °C, 30 s at 60 °C and 1 min at 68 °C, 5 cycles of 20 s at 94 °C, 30 s at 55 °C and 1 min at 68 °C, and 30 cycles of 20 s at 94 °C, 30 s at 46 °C and 1 min at 68 °C, followed by 7 min at 68 °C. The following PCR conditions were used to determine the linear range of the *beta-actin* gene amplification: 2 min at 94 °C, 25 cycles of 30 s at 94 °C, 30 s at 62 °C and 1 min at 72 °C, followed by 10 min at 72 °C. The following PCR conditions were used to determine the linear range of the *Trk A* gene amplification: 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C, followed by 10 min at 72 °C. For the results of relative quantitation to be meaningful, the PCR products had been measured only for 20 cycles, which we found it was exponential phase of *Ryk*, *TrkA* and *beta-actin gene* amplification. Amplified bands were visualized by agarose electrophoresis, bands were scanned from photographs, and the relative densitometric ratios of target to beta-actin were obtained using the CS Analyzer 2.0 program (ATTO Bioscience, Rockville, MD, USA). The normalization of *Ryk* or *TrkA* with *beta-actin* was performed within individual groups at various time points post-lesion (1 week, 2 weeks and 3 weeks).

2.5. Quantitation

The number of TrkA-immunoreactive (TrkA-IR) neurons in the DRG were stereologically counted in a physical disector, fractionator paradigm [2,7]. In brief, the ganglia were sectioned, a section separation, *k*, was chosen, and the first sample section was selected randomly between the first and the *k*th section, the *R*th section. Then, sections *R* and $R+1$, $R+k$, $R+k+1$, etc. were used as double disector pairs. Each chosen section was divided into four equal parts and one-quarter from each section was randomly chosen. Profiles of stained neurons were located in each section and then checked in the facing section, and those found in one section, but not the other was counted ($Q-$). The total number (N_t) of TrkA-IR neurons per ganglion was estimated by multiplying the number of tops ($\sum Q-$) by 4 (since 1/4th of each section was counted) by section separation $k/2$ (since double dissectors were used).

Cell-size was estimated as the average profile diameter measured by the NIH Image Analysis System. The average diameter of each neuron was determined by dividing the sum of the long and short diameters by two ((major axis + minor axis)/2). Neurons are referred to as "small" (10–25 μm), "medium" (26–50 μm), and "large" (51–70 μm) based on their diameters.

3. Results

3.1. Induction of Ryk-IR neurons in adult DRG after sciatic nerve injury

We screened for Ryk immunoreactivity in adult DRG by immunostaining with anti-Ryk polyclonal antibody. Trk A immunostaining was used as a positive control to guarantee we were studying an animal model of unilateral sciatic nerve injury (Fig. 1B–D) [21].

No positive staining against Ryk antibody was observed in DRG on either side obtained from normal rats or contralateral DRG from rats after unilateral sciatic nerve injury (Fig. 1E). Strong induction of Ryk protein could be detected in ipsilateral DRG at 1 week post-lesion and was continually expressed until 3 weeks post-lesion (Fig. 1F–H). By co-immunostaining with Ryk and NeuN antibodies, we identified that the Ryk-IR cells are DRG neurons (Fig. 1I–L). The Ryk antibody immuno-labeled neurons were scattered throughout the post-lesion ganglia. We also noticed there were no obvious changes in the Ryk-IR density of DRG neurons at various stages post-lesion (Fig. 1F–H). We found the numbers of Ryk-IR neurons significantly fluctuated throughout post-lesion, especially from 2 weeks to 3 weeks post-lesion (Fig. 1G–H and K–L).

3.2. Numbers of Ryk-IR neurons in DRG after sciatic nerve lesion

We quantified the numbers of Ryk-IR neurons in the ipsilateral L4–L6 DRG of unilateral sciatic nerve injured rats at various times after injury, using contralateral L4–L6 DRG or bilateral DRG in intact