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

Xin Li, Yao-hua Li, Shun Yu, Yaobo Liu

A R T I C L E   I N F O

Article history:
Received 22 January 2008
Received in revised form 4 April 2008
Accepted 9 May 2008
Available online 4 September 2008

Keywords:
Ryk
Dorsal root ganglia
Sciatic nerve
Lesion
Induction

A B S T R A C T

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.

© 2008 Elsevier Inc. All rights reserved.

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
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., St. 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 C.O.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 0.1% 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-TWHASKRYEYKGFC) [10] and C-terminal amino acid residues (N- YLKDGYRIAQPINCP-C) of the Ryk protein. A Trk A polyclonal antibody (rabbit anti-Trk A, 1:1000) which was produced using two peptide antigens from N-terminal amino acid residues (N-TWHASKRYEYKGFC) [10] and C-terminal amino acid residues (N-YLKDGYRIAQPINCP-C) of the Trk protein. A rabbit anti-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 NeuN immunostaining were incubated together with Cy-3 goat anti-rabbit IgG (1:10000, 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 Biotechnol- ogy 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. The number of TrkA-immunoreactive (TrkA-IR) neurons in the DRG were stereologically counted in a physical dissector, 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 kth section, the (k+1)th section. Then, sections R and R+1, R + k, R + k + 1, etc. were used as double dissector 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) 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 dissector 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-injury and was continually expressed until 3 weeks post-injury (Fig. 1F–H). By co-immunostaining with Ryk and NeuN antibodies, we identified that the Ryk-IR cells are DRG neurons (Fig. 11–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-injury (Fig. 1F–H). We found the numbers of Ryk-IR neurons significantly fluctuated throughout post-injury, especially from 2 weeks to 3 weeks post-injury (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
rats as negative controls. As shown in Fig. 2, the number of Ryk-IR neurons reached 5242 ± 145 per ganglion at 1 week post-lesion, then slightly increased to 5470 ± 293 at 2 weeks post-lesion, and remarkably reduced to 3846 ± 250 at 3 weeks post-lesion. As a positive control we noticed the number of Trk A-IR neurons showed a linear decline from 1 to 3 weeks post-lesion (Fig. 2). We did not find the substantial loss or regeneration of NeuN-IR neurons by checking through from day 1 to day 30. The numbers of NeuN-IR neurons constantly kept around 12,000 without significant fluctuation, as 11,955 ± 467 at 1 week post-lesion, 12,026 ± 501 at 2 weeks post-lesion, 11,949 ± 488 at 3 weeks post-lesion in comparison to 11,946 ± 334 in intact animals (Fig. 2).

### 3.3. Size of Ryk-IR neurons and the proportions of different sized Ryk-IR neurons in DRG after sciatic nerve lesion

We also determined the average size of Ryk-IR neurons and the proportions of different sized Ryk-IR neurons in ipsilateral L4–L6 DRG at various times post-lesion (Trk A group as a positive control, Fig. 3). At each time point, 100 TrkA-IR or Ryk-IR neurons were randomly picked up and their diameters were measured. We found that after lesion the average diameter of Ryk-IR neurons was 24.36 ± 1.20 μm at stage of 1 week, 25.16 ± 1.19 μm at stage of 2 weeks and 23.42 ± 1.09 μm at stage of 3 weeks. The average diameter of Trk A-IR neurons was 22.64 ± 1.04 μm in normal DRG, compared to 19.71 ± 0.93 μm at 1 week post-lesion, 18.66 ± 1.23 μm at 2 week post-lesion and 18.029 ± 1.08 μm at 3 weeks post-lesion (Fig. 3B). We found most of Ryk-IR neurons were as big as 15–35 μm in diameter throughout the induction and their proportion was more than 80% in total of Ryk-IR neurons. We also noticed that the proportions of Ryk-IR medium sized neurons (25–35 μm in diameter) increased gradually after the induction, this correlated with a reduction of Ryk-IR small-sized neurons (10–25 μm in diameter) (Fig. 3A). The trend of changes for different sized Ryk-IR neurons is displayed in Fig. 3A.
Fig. 3. Size histograms of TrkA-IR and Ryk-IR neurons in the L4–L6 dorsal root ganglia of the normal rat and at various postoperative time points in sciatic nerve injured rats. (A) The left lane shows the proportions of differently sized TrkA positive cells in ipsilateral DRG after unilateral sciatic nerve injury as positive control. The right lane shows the proportions of differently sized Ryk positive neurons in ipsilateral DRG after unilateral sciatic nerve injury. At each time point, the size of 100 TrkA-IR or Ryk-IR neurons was measured. Neuron size is expressed as the average diameter of the soma, and the number of neurons is expressed as percent of total neurons in each group. (B) shows average diameter of Ryk-IR or TrkA-IR neurons in control DRG and various time points post lesion. Results are given as means ± S.E.M. (n = 7), and the statistical analyses were performed by the t-test. Asterisks indicate values that show a significant difference from the normal/contralateral values (P < 0.05). *P < 0.05 compared with control groups (contralateral DRG or normal DRG).
Fig. 4. Expression of Ryk mRNA in the ipsilateral dorsal root ganglia at various postoperative time points in unilateral sciatic nerve injured rats. Experimental groups included group I: contralateral or normal DRG; groups II–IV: ipsilateral DRG at 1, 2 and 3 weeks after sciatic nerve lesion induction, respectively. No Ryk mRNA expression was detected in contralateral DRG or normal DRG. (A) shows RT-PCR amplification of Ryk cDNA (399 bp) and Trk A (329 bp) at various time points after sciatic nerve lesion with beta-actin as positive control (640 bp); (B) shows quantification of PCR values. Values are means ± S.E.M. of Ryk/beta-actin ratios or Trk A/beta-actin ratios in ipsilateral DRG compared to contralateral or normal DRG as negative control. Statistical analyses were performed using repeated measures ANOVA followed by the paired t-test for contralateral/ipsilateral or unpaired t-test for control/lesion. Asterisks indicate values that have significant differences from the normal/contralateral values (P < 0.05). * P < 0.05 compared with control groups (contralateral DRG or normal DRG); (C) Top panel shows the combined images of in situ with Ryk antisense RNA probe and immunostaining with NeuN antibody by overlapping and merging adjacent section pairs in Photoshop. The Ryk mRNA expressed cells were coincidentally matched with NeuN-Ab labeled neurons at various time points post-lesion (white arrow). Bottom panel shows the images of in situ with Ryk sense RNA probe. Scale bar: 50 μm.
3.4. Expression of Ryk mRNA in DRG neurons after sciatic nerve lesion

We tested the induction of Ryk mRNA expression in ipsilateral DRGs after sciatic nerve lesion by normalizing the density ratios of Ryk RT-PCR products to beta-actin. We found the pattern of Ryk mRNA induction in ipsilateral DRG was very similar to the induction at the protein level, comparable to results obtained by immunostaining (Fig. 4A). The expression of Ryk mRNA was first observed in the ipsilateral DRG at 7 days after injury. The expression level increased 15% at 2 weeks after injury, compared with the density ratio at 1 week (Fig. 4B). The level of Ryk expression decreased about 40% in the ipsilateral DRG at 3 weeks after lesion induction compared to the level observed at 2 weeks after lesion induction (Fig. 4B).

In order to determine if the mRNA changes in Ryk expression are specific to DRG neurons, combined Ryk in situ and NeuN immunostaining were performed as described in the methods section. We reconstructed the combined images of in situ and immunostaining by overlapping and merging adjacent section pairs in Photoshop, we found all the Ryk mRNA expressing cells could be coincidentally matched with NeuN antibody labeled neurons throughout the induction at various time points post-injury (Fig. 4C, white arrow).

4. Discussion

The present study is focused on the numbers and the size of DRG neurons that express Ryk after peripheral nerve injury. We know most small and medium-sized sensory neurons express Trk A in adult DRG [15,17]. In Trk A (−/−) mutant mice, 82% of lumbar DRG neurons, including all of the small cell population as well as some medium-sized neurons, are lost, including virtually all with a nociceptive phenotype [22,23]. Furthermore, Shen et al. showed that the numbers of Trk A-IR neurons in adult DRG decline after peripheral nerve injury [21]. Therefore, we used Trk A-IR in DRG as a positive marker for our ipsilateral sciatic nerve injury animal model. Using this method, we could observe significant changes in Trk A expression level in our animal model.

Based on this animal model we found that Ryk could be induced in DRG neurons after peripheral nerve lesion at both mRNA level and protein levels. This induction was first observed 1 week after injury and lasted for more than 3 weeks. Throughout the induction we identified that all of Ryk-IR cells were NeuN-IR neurons. Furthermore, we did not observe any obvious change in signal intensity in these Ryk-IR DRG neurons post-injury. In order to exclude the possibility of immune cells infiltration or neuron apoptosis, we counted the total numbers of cells and neurons in DRG at control and various stages post-injury, we did not observe the total number of cells or neurons in lesioned ganglia showing a substantial increase or reduction in comparison to uninjured ganglia by a statistical test (t-test). We also excluded the neuron apoptosis by immunostaining with active caspase-3 antibody (data not shown). Therefore, as we concluded above, both Ryk protein and mRNA were induced in DRG neurons after peripheral nerve lesion, and this induction with fluctuation of Ryk-IR neuron number did not lead to any neuron loss or immune cells invasion.

We know the different peripheral fibers belong to different size neurons. The peripheral C fibers belong to small neurons, peripheral Aβ fibers belong to medium-sized neurons and Aδ/Aβ fibers belong to large neurons in DRG. A lot of evidence showed that different-diameter neurons represent various electrophysiological characters with different receptors and transmitters. Generally, C/Aδ fibers mediated sensory transductions with noxious stimulation and Aδ/Aβ fibers mediated sensory transductions with innocuous stimulation. We saw most of Ryk-IR neurons were as big as 15–35 μm in diameters throughout the induction and their proportion was more than 80% in total of Ryk-IR neurons. We also noticed that the proportions of Ryk-IR medium-sized neurons (25–35 μm in diameters) increased gradually after the induction, this correlated with a reduction of Ryk-IR small-sized neurons (10–25 μm in diameter). Hereby, all these results matched with the general principle: small and medium-diameter neurons respond to a noxious stimulation with transduction mediated by Aδ/C fibers. It also provides the hints that Ryk induction correlates with the functional reaction of small and medium-sized neurons to noxious stimulation.

The mechanism of this induction and the possible influence on function of DRG neurons are not known. Recent studies have identified the Ryk receptor as a key chemorepulsive axon guidance receptor in the projection of major axon tracts, such as the corticospinal tract (CST) and corpus callosum [12,13]. In previous studies, repulsive axon guidance molecules such as members of Eph and Semaphorin families have been reported around or within the lesioned site at later stages after CNS injury [1,3,5,16,18]. These evidences have largely supported the hypothesis that repulsive axon guidance molecules may negatively affect adult axon growth and may thus be a possible cause of the failure of CNS or peripheral nerve regeneration, although such a link has not been fully experimentally proven. Here, if we assign Ryk as a chemorepulsive receptor, as the results from the current study showed that Ryk is an induced factor expressed within days after peripheral nerve injury, it is easy to be assumed that Ryk may function as inhibitory axon guidance molecule during development, and it may also function to inhibit the axon population in the adult nervous system. However, on the other side Ryk was also discovered as mediating Wnt3a stimulation towards outgrowth DRG neurite at E13 [14]. More generally, guidance cues have differing effects dependent on context of matrix molecules. As shown in Wnt3 mediated RCG axons projection, Wnt3 repulsion is mediated by Ryk, expressed in a ventral-to-dorsal decreasing gradient, whereas attraction of dorsal axons at lower Wnt3 concentrations is mediated by Frizzled(s) [20]. Hereby, we think it is not the time to draw the conclusion that the Ryk induction may play an inhibitory or facilitative effect towards lesioned axons if we do not know which ligand could be induced or bind to by Ryk receptor. But we believe this is a new observation that indicates an association between peripheral nerve injury and Ryk. The expression of Ryk by different sized neurons in DRG at early, intermediate and late times after peripheral nerve injury suggests that cell–cell signal mediated by guidance molecule/receptor interactions may substantially play a host of important functions at multiple time points after peripheral nerve injury. In the future to identify the axon guidance molecules and their signal mechanism involved in regeneration may allow us to assess whether the principles that govern axons pathfinding during development can also apply to the regeneration of axons in adulthood. Given the lessons learned from the molecular control of axon pathfinding during development, significant opportunity may exist to utilize the rich biology of axon guidance molecules to enhance functional recovery in neurological trauma and disease.

Conflict of interest

There is no conflict of interest.

Acknowledgements

We thank Dr. Daria Bancescu, Dr. Javid A. Dar and David Quach for proof-reading. This study was supported by two
NSFC (Natural Sciences Foundation of China) project grants (Nos. 30640006, 30771063), and partly supported by the funding for outstanding researcher from Beijing Municipal Government (No. 20061D0501800264).

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.brainresbull.2008.05.011.

**References**


