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MICRORNA MACHINERY RESPONDS TO PERIPHERAL NERVE LESION IN AN INJURY-REGULATED PATTERN

D. WU,[a] M. RAAFAT,[a] E. PAK,[a] S. HAMMOND[b] AND A. K. MURASHOV[a]*

[a]*Department of Physiology, East Carolina University, Greenville, NC 27834, USA*

[b]*Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC 27599-7090, USA*

Abstract—Recently, functional and potent RNA interference (RNAi) has been reported in peripheral nerve axons transfected with short-interfering RNA (siRNA). In addition, components of RNA-induced silencing complex (RISC) have been identified in axotomized sciatic nerve fibers as well as in regenerating dorsal root ganglia (DRG) neurons *in vitro*. Based on these observations, and on the fact that siRNA and microRNA (miRNA) share the same effector enzymes, we hypothesized that the endogenous miRNA biosynthetic pathway would respond to peripheral nerve injury. To answer this question, we investigated changes in the expression of miRNA biosynthetic enzymes following peripheral nerve crush injury in mice. Here, we show that several pivotal miRNA biosynthetic enzymes are expressed in an injury-regulated pattern in sciatic nerve *in vivo*, and in DRG axons *in vitro*. Moreover, the sciatic nerve lesion induced expression of mRNA-processing bodies (P-bodies), which are the local foci of mRNA degradation in DRG axons. In addition, a group of injury-regulated miRNAs was identified by miRNA microarray and validated by real-time quantitative PCR (qPCR) and *in situ* hybridization analyses. Taken together, our data support the hypothesis that the peripheral nerve regeneration processes may be regulated by miRNA pathway.

Key words: miRNA, P-body, sciatic nerve, axon growth, regeneration, axon varicosities.

One of the unique features of the peripheral nervous system (PNS) is its ability to regenerate axons after injury (Yoo et al., 2010). To initiate a regenerative response, the PNS neuron must shift its physiology from synaptic transmission and maintenance of structure to the growth of the axon (Benowitz and Yin, 2007). Shifting to the growth phenotype requires both activation of a growth program through gene transcription as well as activation of local signaling cascades that regulate axon assembly (Snider et al., 2002; Vogelaar et al., 2009). Recent observations suggest that the microRNA (miRNA) pathway may be involved in regulation of these processes (Hengst et al., 2006; Murashov et al., 2007).

The miRNA pathway is an important layer of post-transcriptional gene regulation (Jackson et al., 2010). MiRNAs are initially processed in the nucleus by biosynthetic enzymes, Drosha and DGCR8/Pasha, whereas in cytoplasm, Dicer cleaves them into the mature miRNAs (Bernstein et al., 2001; Lee et al., 2003). MiRNAs then trigger formation of RNA-induced silencing complex (RISC), which is implicated in a sequence-specific translational repression called RNA interference (RNAi) (Bagga et al., 2005; Tan et al., 2009). Several subunits of RISC have been conclusively identified to date: Argonaute2 (Ago2) nuclease (Hammond et al., 2001; Meister and Tuschl, 2004), fragile X mental retardation protein (FMRP) (Ishizuka et al., 2002), p100 (Caudy et al., 2003), TRBP (Chendrimada et al., 2005), PACT (Lee et al., 2006), and RCK/p54 (Chu and Rana, 2006). Recent studies showed that Argonaute proteins interact with the RNA-binding protein GW182 (Sen and Blau, 2005; Eulalio et al., 2009a,b; Takimoto et al., 2009). Observations revealed that GW182-containing foci, known as GW bodies (GWBs), coincide with mRNA-processing bodies (P-bodies) where GW182 colocalizes with the decapping complex (Dcp1, Dcp2), Rap55 and Ro52 (Sen and Blau, 2005; Bhanji et al., 2007; Eulalio et al., 2009a,b; Takimoto et al., 2009). Importantly, depleting GW182 suppressed silencing of miRNA targets. Therefore, these observations suggest that in addition to RISC, target silencing by miRNAs is regulated by P-bodies.

Recent studies revealed the differential expression of several miRNAs after traumatic injury in CNS, including the brain and spinal cord (Lei et al., 2009). Reduction in the expression of several sensory organ-specific miRNAs was also observed in the injured ipsilateral dorsal root ganglion (DRG) following spinal nerve ligation (SNL) (Aldrich et al., 2009). These evidences suggest that miRNAs are likely to be important mediators of plasticity. Functional RNAi machinery has been reported in axons of PNS neurons (Yoo et al., 2010). Components of RISC were observed in severed sciatic nerve fibers and cultured DRG axons (Hengst et al., 2006; Murashov et al., 2007). Based on these observations and on the fact that short-interfering RNA (siRNA) and miRNA share the same effector enzymes, it is logical to presume that the endogenous miRNAs machinery could be involved in the regulation of molecular response to peripheral nerve injury.

*Corresponding author. Tel: +1-252-744-3111; fax: +1-252-744-3460.
E-mail address: murashoval@ecu.edu (A.K. Murashov)
Abbreviations: Ago2, argonaute2; DRG, dorsal root ganglia; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FISH, fluorescent *in situ* hybridization; FMRP, fragile X mental retardation protein; GWBs, GW bodies; HRP, horseradish peroxidase; miRNA, microRNAs; PNS, peripheral nervous system; qPCR, real-time quantitative PCR; RNAi, RNA interference; RISC, RNA-induced silencing complex; SGs, stress granules; siRNA, short-interfering RNA; TUJ1, beta III tubulin antibody; VR, varicosities.

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In the current study, we asked whether miRNA might be one of the pathways that respond to peripheral nerve crush. Here, we show an injury-regulated expression of several pivotal miRNA biosynthetic enzymes in murine sciatic nerve *in vivo*, and in DRG axons *in vitro*. In addition, a group of miRNAs, which were expressed in an injury-regulated pattern in regenerating sciatic nerve and DRG was identified by miRNA microarrays, qPCR, and *in situ* hybridization. These data provide further evidence in support of the hypothesis that the peripheral nerve regeneration processes may be regulated by miRNA pathway.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on 8-week-old CD-1 mice, obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were housed one per cage under standard laboratory conditions, with a 12-h light/dark schedule and unlimited access to food and water. All experimental procedures and the care of the animals were administered according to the guidelines set forth by the Animal Care and Use Committee of East Carolina University, an AAALAC-accredited facility. In the approved animal use protocol, we included a detailed description of the research being conducted, minimized the number of animals being used in the study, minimized pain and distress to animals in the study, and proved there is not an alternative model to the use of a living animal.

Conditioning nerve lesion

Anesthesia was induced using i.p. ketamine (18/mg/mL)–xylazine (2 mg/mL) mixture (0.05 ml/10 g of body weight). The procedure followed a protocol described previously (Islamov et al., 2004). Exposure of the right sciatic nerve was performed with sterile surgical instruments. Approximately 5 mm of nerve was exposed from the sciatic notch to the trifurcation of the nerve. The exposed sciatic nerve was crushed in the mid-thigh for 15 s with a fine hemostat. The wounds were closed with 3M Vetbond tissue adhesive and mice were allowed to recover for 4, 5, or 7 days. For the following experiment, the injured ipsilateral nerves were called injured nerves and the ipsilateral DRGs were called injured DRGs. The contralateral nerves were called naive nerves and DRGs from contralateral side were called naive DRGs. Nerves and DRGs from intact animals were called intact nerves and intact DRGs.

Sciatic nerve collection

After specified time periods, animals were euthanized and sciatic nerves were quickly dissected out, snap frozen in liquid nitrogen, and stored at −80 °C. The naive nerve was taken from the contralateral side (sciatic nerve from left side). The excised crush sample was taken from the injury side approximately 5 mm in both directions from the point of injury. The intact nerve sample was taken from mice at the matched age without sciatic nerve crush surgery.

Dissociated DRG culture

Mouse L4/5 DRG neurons were collected 5 days after a conditioning sciatic nerve crush from both the naive side and injured side. DRGs were dissociated with collagenase and 0.25% trypsin in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Eugene, OR, USA). The dissociated DRGs were plated on poly-L-lysine and laminin (Invitrogen) coated plates. DRGs were grown in DMEM/F12 containing 10% horse serum, L-glutamine, and N2 supplement at 37 °C for 18 h. Cytosine β-D-arabinofuranoside and 5,6-dichlorobenzimidazole riboside (Sigma, Saint Louis, MO, USA) were added to the final concentration of 10 μM and 80 μM, respectively in the growth medium to inhibit the growth of glial cells.

Protein lysates

Proteins were extracted from the sciatic nerves collected at specific time points. To obtain adequate proteins for these experiments, at least five animals were pooled per time point. Samples of sciatic nerves were homogenized in ice-cold homogenization buffer, containing protease inhibitor cocktail (Sigma, St. Louis, MO, USA), 1 mM PMSF, 20 mM Tris, 2 mM EGTA, 2 mM EDTA, 6 mM β-mercaptoethanol, and 10% Triton, and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was transferred to a fresh tube and stored at −20 °C until ready for use.

Immunoblotting

Proteins for Western blotting analysis were quantified using the BioRad reagent. 20 μg of solubilized proteins were loaded per lane on sodium dodecyl sulfate gels and separated by SDS-PAGE. The separated proteins were then transferred to immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). Membranes were blocked in 5% nonfat milk/TBST for 1 h at room temperature on a shaker, and then probed with primary antibody against FMRP (Darnell et al., 2005), Ago2 (Ikeda et al., 2006), P-100 (Keenan et al., 2000), Dcp1 (Lykke-Andersen, 2002; from Abnova; Walnut, CA, USA), Dcp2 (Lykke-Andersen, 2002; Wang et al., 2002), Rap55 (Yang et al., 2006), Ro52, and GWB IC-6 (Pauley et al., 2006) in 5% nonfat milk/PBST at 4 °C overnight. For negative control groups, membranes were incubated with preimmune serum. The membranes were washed three times with TBS, and then incubated with secondary antibody in 5% milk/TBST for 2 h at room temperature. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, bound antibodies were detected using a chemiluminescence detection system (ECL plus Western blotting detection reagent, Amersham; Arlington Heights, IL, USA). Densitometry was performed using Kodak 1D Image Analysis software. Band values were normalized to alpha-tubulin, to obtain the relative densitometric intensity. One-way ANOVA were performed on injured, naive versus intact relative densitometric intensity for each antibody tested.

List of antibodies

The following antibodies were used for immunodetection procedures: Rabbit polyclonal anti-Ago2-specific antibody (kindly donated by Tom Hobman, University of Alberta, Canada). Guinea-pig polyclonal anti-P100 antibody (kindly donated by Tom Keenan, VA Polytechinic Institute and State University, Blacksburg, VA, USA). Mouse monoclonal anti-FMRP antibody was purchased from Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Western blot analysis of P-body components in the sciatic nerve used rabbit polyclonal primary antibody against Dcp1, Dcp2 from Jens Lykke-Anderson (University of Colorado, CO, USA), rabbit polyclonal anti-Dcp2 primary antibody from M. Kilejian (Rutgers University, NJ, USA), Mouse anti-human Dcp1A monoclonal antibody from Abnova, human anti-Rap55 antibody (Donald Bloch, Harvard University, MA, USA) or antibody against GWB IC6 from Marvin Fritzler (University of Calgary, Alberta, Canada), or Rabbit anti-Ro52 polyclonal antibody from Millipore Corporation (Bedford, MA, USA). Mouse monoclonal neuron-specific beta III tubulin antibody (TUJ1) was obtained from Covance Research Products, Inc. (Denver, PA, USA). The specificities of the antibodies, provided by individual investigators, have been validated by original research groups and the corresponding articles have been published elsewhere. For secondary antibodies, we used HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG