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Selective expression of Narp in primary nociceptive neurons: Role in microglia/macrophage activation following nerve injury

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ABSTRACT

Neuronal activity regulated pentraxin (Narp) is a secreted protein implicated in regulating synaptic plasticity via its association with the extracellular surface of AMPA receptors. We found robust Narp immunostaining in dorsal root ganglia (DRG) that is largely restricted to small diameter neurons, and in the superficial layers of the dorsal horn of the spinal cord. In double staining studies of DRG, we found that Narp is expressed in both IB4- and CGRP-positive neurons, markers of distinct populations of nociceptive neurons. Although a panel of standard pain behavioral assays were unaffected by Narp deletion, we found that Narp knockout mice displayed an exaggerated microglia/macrophage response in the dorsal horn of the spinal cord to sciatic nerve transection 3 days after surgery compared with wild type mice. As other members of the pentraxin family have been implicated in regulating innate immunity, these findings suggest that Narp, and perhaps other neuronal pentraxins, also regulate inflammation in the nervous system.

1. Introduction

Members of the pentraxin family of proteins are expressed in a variety of tissues and mediate innate immunity and inflammation (Garlanda et al., 2005; Bottazzi et al., 2006). All members of this family share a conserved pentraxin domain but can be divided into two subgroups called "short" and "long" pentraxins, with the latter containing N-terminal extensions prior to the pentraxin domain located in their C-terminal portion. The prototypic "short" pentraxin, C-reactive protein (CRP), is an acute phase reactant which precipitates from human serum upon the addition of the C-polysaccharide of pneumococcus (Tillet and Francis, 1930, Marnell et al., 2005). PTX3, a "long" pentraxin, was originally identified as a novel protein induced by a tumor necrosis factor in human fibroblasts and later found to be regulated by inflammatory cytokines in a number of cell types, including macrophages and dendritic cells (Lee et al., 1990; Lee et al., 1993). Three "long" pentraxins, Narp, NP1 and NPR, are highly enriched in the nervous system, where they are selectively expressed in neurons, and are therefore referred to as neuronal pentraxins. The neuronal pentraxins are secreted at synapses, cluster AMPA receptors by binding to their extracellular surface and have been implicated in synaptic plasticity and synaptogenesis (O'Brien et al., 1999; Xu et al., 2003; Cho et al., 2008). However, it is unclear if neuronal pentraxins also play a role in regulating immune function or if the function of this branch of the pentraxin family is restricted to regulating AMPA receptor trafficking.

Although AMPA receptors are expressed ubiquitously throughout the nervous system, individual neuronal pentraxins display highly heterogeneous patterns of expression. For example, Narp is localized selectively to dentate granule cells in the hippocampus (Reti et al., 2002b), to the anterodorsal nucleus in the thalamus (Reti et al., 2002a), and to a small subset of neurons in the hypothalamus, including orexin and vasopressin neurons (Reti et al., 2002c, 2008a). As part of a survey of Narp expression in the nervous system, we identified Narp expression in dorsal root ganglia (DRG), where it appeared to be expressed predominantly in small diameter neurons, characteristic of primary nociceptive neurons. These neurons send axons to the dorsal horn of the spinal cord where they transmit nociceptive signals by the release of glutamate which acts on AMPA and other glutamate receptor subtypes (Nagy et al., 2004). Enhanced AMPA receptor signaling in the spinal cord has been implicated in pain sensitization (hyperalgesia) and allodynia in which benign stimuli become painful (Tao, 2010).

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Accordingly, we evaluated the effect of deleting Narp on acute and chronic models of mechanical, thermal, and inflammatory pain. Then, we performed sciatic nerve transection, an injury known to induce robust glial activation in the spinal cord, to assess differences in inflammatory response between Narp knockout (KO) and control mice.

2. Materials and methods

2.1. Animals

Generation of Narp KO mice has been described elsewhere (Johnson et al., 2007; Reti et al., 2008b). All control and KO mice used in these studies were generated by heterozygote breeding pairs that had been backcrossed to C57BL/6 for five generations. Both male and female mice were used between 9 and 14 weeks of age. Mice were used for all experiments except the rhizotomy study which was conducted with Sprague–Dawley rats obtained from Charles River (Wilmington, MA, USA). Rodents were maintained on a 12-h light/dark cycle and were given access to food and water ad libitum. Experiments complied with the NIH guidelines for animal care and were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used and their suffering.

2.2. Antibodies

The following primary antibodies were used: rabbit anti-Narp antibody (O'Brien et al., 1999), guinea pig anti-CGRP (Peninsula Laboratories, San Carlos, CA), rabbit anti-Iba1 (Wako Chemicals USA Inc., Richmond, VA), rabbit anti-NF200 (Chemicon, Billerica, MA), mouse anti-peripherin (Chemicon), and mouse anti-BrdU (Sigma-Aldrich, St. Louis, MO). In addition, we used a fluorochrome-conjugated isolectin B4 lectin (IB4; Molecular Probes, Carlsbad, CA) which binds non-peptidergic DRG neurons.

2.3. Immunoblot analysis

DRG were solubilized by sonication for 10 s in a solution of 0.1% Triton X-100 with protease inhibitors (aprotinin and leupeptin, each at 1 μg/ml, and 1 mM phenylmethylsulfonyl fluoride) in PBS (pH −7.4). Following sonication, samples were spun down at 14,000 ×g in a microcentrifuge for 5 min at 4 °C to remove insoluble material. Protein concentrations were determined using amido black colorimetric protein assay.

Solubilized DRG lysates were mixed with an equal volume of Laemmli Sample Buffer (Bio-Rad; Hercules, CA). Equal concentrations of protein were separated by SDS-PAGE (10% acrylamide) at 130 V for 60 min. Proteins were electrophoretically transferred to nitrocellulose paper. Blots were blocked with 3% nonfat milk in TBS and Tween-20 (TBS-T; 20 mM Tris (pH −7.7), 137 mM NaCl and 0.05% Tween-20) overnight at 4 °C. Narp antibody was diluted in blocking buffer (1:5000) and blots were incubated overnight at 4 °C. Blots were washed repeatedly (6 times) in TBS-T for 5 min. HRP-conjugated anti-rabbit secondary antibody was diluted in blocking buffer and blots were incubated overnight at 4 °C. Blots were washed in TBS-T for 5 min, 3 times, and then in TBS for 5 min, 3 times. Immunoblots were processed for protein visualization using enhanced chemiluminescence (Amersham Pharmacia Biotech Inc.; Piscataway, NJ) and exposed to Biomax-MR film (Kodak; Rochester, NY).

2.4. Tissue section staining

Mice were anesthetized with chloral hydrate (400 mg/kg, i.p., Sigma Aldrich) and perfused via the left ventricle with 4% paraformaldehyde. The vertebral column containing the spinal cord and DRG was carefully dissected from each mouse and incubated overnight at 4 °C in 4% paraformaldehyde before being switched to 25% sucrose made in PBS and allowed to incubate for an additional 2 days. Tissue sections (20 μm) were cut on a sliding microtome, placed on frosted glass microslides, air dried, and washed in PBS. Sections were incubated in 10 mM sodium citrate (pH 8.5) in 80 °C water bath for 30 min. They were then cooled to room temperature and washed with PBS. Sections were blocked in 3% BSA and 0.3% Triton-X 100 made in PBS for 1 h before incubation overnight with primary antibodies at the following dilutions: Narp (1:5000), CGRP (1:500), Iba1 (1:500), peripherin (1:1000), and NF200 (1:1000). Fluorochrome-conjugated IB4 lectin was used at 1:500. After washing in PBS, the sections incubated in primary antibodies were incubated with either a fluorochrome-labeled (cy3; 1:200) or biotinylated secondary antibodies (1:500; Jackson; West Grove, Pa) against the species in which the primary antibodies were generated. Slides processed with antibody complexes containing fluorochrome-labeled secondary antibodies or IB4 lectin were washed in PBS and cover-slipped. Antibody complexes containing biotinylated secondary antibodies were further processed with avidin-linked biotinylated peroxidase and exposed to tyramide for fluorescent visualization. For quantification of double-labeling with Narp and either CGRP, IB4, peripherin or NF200, we used 3–6 mice for each marker and ascertained the mean percentage of Narp positive cells that stain for the other marker based on 4 representative DRG sections from each mouse.

2.5. Electron microscopy

Immunogold studies of Narp localization were performed as described previously (Reti et al., 2008a). Briefly, mice were perfused with 4% paraformaldehyde with 0.5% glutaraldehyde and sections of tissue were frozen in liquid propane in a Leica CPC cryopreparation chamber and then freeze-substituted into Lowicryl HM-20 in a Leica AFS. Ultrathin sections on grids were incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris-buffered saline plus 0.1% Triton X-100 (TBST), followed by 10% normal goat serum (NGS) in TBST. Both sides of the sections were then incubated with rabbit anti-Narp (1/50–1/67) and guinea pig anti-CGRP (1/100) antibodies mixed together in 1% NGS/TBST overnight at 4 °C. After several washing and blocking steps, the secondary immunogold antibodies (goat F(ab)2 anti-rabbit IgG:5 nm gold and goat anti-guinea pig IgG:15 nm gold; BB International Gold, distributed by Ted Pella, Redding, CA, USA) in 1% NGS/TBST plus 0.5% PEG (20,000 MW) were applied to both sides of the sections for 1 h at room temperature. Finally, sections were stained with uranyl acetate and lead citrate. Figures were processed in Adobe Photoshop with minimal use of levels; brightness and contrast were employed uniformly over the images. Omission of primary antibodies yielded negligible staining.

2.6. Hot-water tail immersion test

Noxious thermal stimulation was produced by immersing the tip of the mouse tail into a hot-water bath. The hot-water bath was adjusted to maintain a temperature of 50.0 °C ± 0.2. After allowing acclimation in a clean cage identical to the home cage for a period of 30 min, the posterior one-third of the tail of each animal was immersed in the water bath until rapid removal of the tail was observed, signifying a pain response. The cut-off time was 15 s to avoid tissue damage.

2.7. Hargreaves model of thermal hyperalgesia

Thermal hyperalgesia was measured in a manner similar to that described by Hargreaves et al. (1988). After allowing acclimation atop a glass surface underneath 5 cm × 15 cm plexiglass containers, the plantar surface of the hind paw of each mouse was exposed to a high-intensity projector light source before and after injection of 3 μl, 6 μl, and 12 μl of 1:1 solution of Complete Freund's Adjuvant (CFA) and normal saline. The light source was directed onto the plantar surface of the hindpaw