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DE NOVO EXPRESSION OF NAV1.7 IN INJURED PUTATIVE PROPRIOCEPTIVE AFFERENTS: MULTIPLE TETRODOTOXIN-SENSITIVE SODIUM CHANNELS ARE RETAINED IN THE RAT DORSAL ROOT AFTER SPINAL NERVE LIGATION

T. FUKUOKA,* K. MIYOSHI AND K. NOGUCHI

Department of Anatomy & Neuroscience, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

Abstract—Tetrodotoxin-sensitive (TTX-s) spontaneous activity is recorded from the dorsal roots after peripheral nerve injury. Primary sensory neurons in the dorsal root ganglion (DRG) express multiple TTX-s voltage-gated sodium channel α-subunits (Navs). Since Nav1.3 increases, whereas all other Navs decrease, in the DRG neurons after peripheral nerve lesion, Nav1.3 is proposed to be critical for the generation of these spontaneous discharges and the contributions of other Navs have been ignored. Here, we re-evaluate the changes in expression of three other TTX-s Navs, Nav1.1, Nav1.6 and Nav1.7, in the injured 5th lumbar (L5) primary afferent components following L5 spinal nerve ligation (SNL) using *in situ* hybridization histochemistry and immunohistochemistry. While the overall signal intensities for these Nav mRNAs decreased, many injured DRG neurons still expressed these transcripts at clearly detectable levels. All these Nav proteins accumulated at the proximal stump of the ligated L5 spinal nerve. The immunostaining patterns of Nav1.6 and Nav1.7 associated with the nodes of Ranvier were maintained in the ipsilateral L5 dorsal root. Interestingly, putative proprioceptive neurons characterized by α3 Na^+/K^+ ATPase-immunostaining specifically lacked Nav1.7 mRNA in naïve DRG but displayed de novo expression of this transcript following SNL. Nav1.7-immunoreactive fibers were significantly increased in the ipsilateral gracile nucleus where central axonal branches of the injured A-fiber afferents terminated. These data indicate that multiple TTX-s channel subunits could contribute to the generation and propagation of the spontaneous discharges in the injured primary afferents. Specifically, Nav1.7 may cause some functional changes in sensory processing in the gracile nucleus after peripheral nerve injury.

Key words: voltage-gated sodium channel α-subunits, primary afferent neurons, α3 Na^+/K^+ ATPase, spinal nerve ligation, dorsal root ganglion, mRNA.

INTRODUCTION

Peripheral nerve injury often causes spontaneous ectopic discharges in primary afferents originating at injury sites and/or related dorsal root ganglia (DRGs). While multiple cation channels might be involved in this hyperexcitability, since these discharges are highly sensitive to tetrodotoxin (TTX) (Matzner and Devor, 1994; Omana-Zapata et al., 1997; Liu et al., 2001), it is believed that voltage-gated sodium channels, especially TTX-sensitive (TTX-s) subtypes, likely play a major role in ectopic action potential generation and propagation (Devor et al., 1992; Chung and Chung, 2004). Each voltage-gated sodium channel is composed of an α-subunit (Nav) and one or more accessory β-subunits. Among the nine cloned α-subunits (Goldin et al., 2000), TTX-s Nav1.1, Nav1.6, Nav1.7, as well as tetrodotoxin-resistant (TTX-r) Nav1.8 and Nav1.9 are normally distributed in different subpopulations of the primary afferent neurons in the DRG (Fukuoka et al., 2008). Following peripheral nerve injury, the expression of these Nav mRNAs are down-regulated (Dib-Hajj et al., 1996, 1998; Kim et al., 2001, 2002). However, another TTX-s subtype, Nav1.3, which is normally expressed at just beyond the detectable level dramatically increases in injured DRG neurons (Waxman et al., 1994; Kim et al., 2001). Therefore, only Nav1.3 has historically been implicated in the spontaneous ectopic discharges recorded from injured primary afferents (Chung and Chung, 2004). However, ectopic discharges also can be recorded from damaged nerves of Nav1.3 knockout mice (Nassar et al., 2006), suggesting the contribution of other TTX-s subtypes to the generation and propagation of spontaneous discharges.

The 5th lumbar (L5) spinal nerve ligation (SNL) is one of the most widely used rat neuropathic pain models (Kim and Chung, 1992). In this model, spontaneous ectopic discharges are recorded from afferent A-fibers (Aβ and

*Corresponding author. Address: Department of Anatomy & Neuroscience, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan. Tel: +81-798-45-6416; fax: +81-798-45-6417.
E-mail address: tfukuoka@hyo-med.ac.jp (T. Fukuoka).

Abbreviations: DR, dorsal root; DRG, dorsal root ganglion; dSpN, spinal nerve distal to the ligation site; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FA, formaldehyde; GN, gracile nucleus; ISHH, *in situ* hybridization histochemistry; L5, 5th lumbar; LTMs, low-threshold mechanoreceptors; Navs, voltage-gated sodium channel α-subunits; PB, phosphate buffer; PFA, paraformaldehyde; RT-PCR, reverse transcription polymerase chain reaction; S/N, signal/noise; SNL, spinal nerve ligation; SSC, standard saline citrate; TBS, Tris-buffered saline; TrkA, tropomyosin receptor kinase A; TrkC, tropomyosin receptor kinase C; TTX, tetrodotoxin; TTX-r, tetrodotoxin-resistant; TTX-s, tetrodotoxin-sensitive.

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Aδ), but not C-fibers, of the ipsilateral L5 dorsal root (DR) (Boucher et al., 2000; Liu et al., 2000a,b, 2001; Ma et al., 2003; Sukhotinsky et al., 2004), and these continuous inputs to the central nervous system are thought to be a key driver of abnormal sensations in this model (Chung and Chung, 2004; Devor, 2009).

This study was undertaken to find out whether three other TTX-s subtypes, Nav1.1, Nav1.6, and Nav1.7, were maintained at transcript and protein levels in the injured L5 primary afferents after L5 SNL. We also examined the possibility that any of these Navs were up-regulated in certain neuronal population after nerve injury.

EXPERIMENTAL PROCEDURES

Animals and treatments

Adult male Sprague-Dawley rats (230–250 g) were used (Nippon Dobutsu Co., Nishinomiya, Japan). These animals were housed in an air-conditioned room (23 ± 1 °C) in a 12-h light/dark cycle. Food and water were given *ad libitum*. All surgical procedures were done on rats that were deeply anesthetized with sodium pentobarbital (50–60 mg/kg, i.p.). Additional doses of the anesthetics were given as needed. All animal experimental procedures conformed to the regulations of the Hyogo College of Medicine Committee on Animal Research and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study on Pain. All efforts were made to minimize the number of animals used and their suffering.

L5 SNL on the left side was performed as described previously (Fukuoka et al., 2001). Briefly, under adequate anesthesia, the low back skin was cut and the muscles were separated from the spinal process and vertebral arch on the left side at the L6 level. After removal of the left L6 transverse spinal process, the L5 spinal nerve was identified and ligated tightly with a 4-0 silk suture. Sham control rats underwent the same surgery on the left side without ligation.

In situ hybridization histochemistry (ISHH)

For the time course study, rats were sacrificed at 1, 3, 7, 28 days after L5 SNL and 3 days after sham surgery (n = 4 each). The other four rats were used as naïve controls. These rats were deeply anesthetized with sodium pentobarbital (75 mg/kg body weight, i.p.) and killed by decapitation. The left (ipsilateral) L5 DRGs were dissected out, rapidly frozen in powdered dry ice, and cut on a cryostat at an 8-μm thickness. Sections were thaw-mounted onto MAS-coated glass slides (Matsunami, Osaka, Japan). All slides contained the ipsilateral L5 sections obtained from 24 animals sacrificed at different periods (naïve, sham, 1, 3, 7, and 28 days after SNL, n = 4, each) in order to compare the results under the same histochemical condition. For the colocalization study, serial 5-μm-thick sections were cut from the left (ipsilateral) L5 DRGs that were obtained from the 12 rats (naïve, sham, and 3 days after SNL, n = 4, each). This later time point was chosen because injured DRG neurons down-regulate the expression of tropomyosin receptor kinase C (TrkC) and α3 Na^+/K^+ ATPase after SNL and the percentages of positive neurons for these markers decrease at longer time points. Sections were stored at −80 °C until use.

The tissue sections were treated with 10 μg/ml protease K in 50 mM Tris and 5 mM EDTA (pH 8.0) for 3 min, postfixed in 4% formaldehyde (FA) in 0.1 M phosphate buffer (PB), acetylated with acetic anhydride in 0.1 M triethanolamine, rinsed with PB, and dehydrated through an ascending ethanol series. Partial cDNAs of rat Nav1.1, Nav1.3, Nav1.6, Nav1.7, and TrkC were synthesized by reverse transcription polymerase chain reaction (RT-PCR) from rat DRG-derived total RNA, cloned into p-GEM T-easy vector (Promega, Madison, WI, USA), and sequenced, as described previously (Fukuoka et al., 2008). These vectors were digested by a restriction enzyme *SpeI* or *NcoI* (Takara Bio, Otsu, Japan) in adequate buffer, and alpha ^{35}S UTP-labeled antisense and sense cRNA probes were synthesized using T7 or SP6 RNA polymerase (Promega). These probes were mixed at 10^4 cpm/μl in hybridization buffer [50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl (pH 7.5), 5 mM EDTA (pH 8.0), 10% dextran sulfate, 1 × Denhardt's solution, 0.2% sarcosyl, 250 μg/ml yeast tRNA, 400 μg/ml salmon testis DNA, and 20 mM dithiothreitol (DTT: pH 8.0)], applied at a 200 μl in volume to the section, and then incubated at 55 °C overnight in a humidified box. Following hybridization, these sections received a 30-min wash at 60 °C in 2 × standard saline citrate (SSC: 1 × SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0) containing 5 mM DTT, a 30-min wash in 50% formamide, 2 × SSC containing 5 mM DTT, treated with 1 μg/ml RNase A (Roche, Mannheim, Germany) in RNase buffer [0.5 M NaCl, 10 mM Tris–HCl, and 1 mM EDTA (pH 7.5)] for 30 min at 37 °C, washed in 50% formamide, 2 × SSC containing 5 mM DTT for 30 min at 60 °C, rinsed in 1 × SSC and 0.1 × SSC for 10 min at room temperature each, dehydrated through an ascending ethanol series, and air dried. For autoradiography, these sections were dipped in NTB emulsion (Eastman Kodak, Rochester, NY, USA) diluted 2:3 with distilled water at 45 °C and exposed for 2–4 weeks at 4 °C. Following development in D-19 (Eastman Kodak) and fixation in 24% sodium thiosulfate, the sections were counterstained with hematoxylin–eosin, dehydrated in ascending ethanol series, cleared in xylene, and coverslipped.

Characterization of the primary antibodies

The rabbit polyclonal anti-Nav1.1 antibody (ASC-001, Alomone, Jerusalem, Israel) is raised against amino acids 465–481 of rat Nav1.1 (Accession# P04774) and detects a 250-kDa band on Western blots of rat brain membrane (manufacturer's data sheet). The rabbit polyclonal anti-Nav1.6 antibody (ASC-009, Alomone) is raised against amino acids 1041–1061 of rat Nav1.6 (Accession# O88420) and recognizes a main band about 250 kDa as well as a much less band around 75 kDa (manufacturer's data sheet). The