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IMMUNOHISTOCHEMICAL ANALYSIS OF ACID-SENSING ION CHANNEL 2 EXPRESSION IN RAT DORSAL ROOT GANGLION AND EFFECTS OF AXOTOMY

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Abstract—Several studies have suggested that acid-sensing ion channel 2 (ASIC2) plays a role in mechanoperception and acid sensing in the peripheral nervous system. We examined the expression and distribution of ASIC2 in the rat dorsal root ganglion, the co-localization of ASIC2 with tropomyosin-related kinase (trk) receptors, and the effects of axotomy on ASIC2 expression. ASIC2 immunoreactivity was observed in both neurons and satellite cells. ASIC2-positive neurons accounted for 16.5±2.4% of the total neurons in normal dorsal root ganglion. Most ASIC2-positive neurons were medium-to-large neurons and were labeled with neurofilament 200 kD (NF200). Within these neurons, ASIC2 was not evenly distributed throughout the cytoplasm, but rather was accumulated prominently in the cytoplasm adjacent to the axon hillock and axonal process. We next examined the co-localization of ASIC2 with trk receptors. trkA was expressed in few ASIC2-positive neurons, and trkB and trkC were observed in 85.2% and 53.4% of ASIC2-positive neurons, respectively, while only 6.9% of ASIC2-positive neurons were co-localized with trkC alone. Peripheral axotomy markedly reduced ASIC2 expression in the axotomized dorsal root ganglion neurons. On the other hand, intense ASIC2 staining was observed in satellite cells. These results show that ASIC2 is expressed in the distinct neurochemical population of sensory neurons as well as satellite cells, and that peripheral axotomy induced marked reductions in ASIC2 in neurons.

Key words: nerve injury, dorsal root ganglion, immunohistochemistry, acid sensing.

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Abbreviations: ASIC2, acid-sensing ion channel 2; BDNF, brain-derived neurotrophic factor; CHO, Chinese hamster ovary; DEG/ENaC, degenerin/epithelial sodium channel; DRG, dorsal root ganglion; EDTA, ethylenediaminetetraacetic acid; GFAP, glial fibrillary acid protein; IR, immunoreactivity/immunoreactive; NF200, neurofilament 200 kD; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; PB, phosphate buffer; PBS, phosphate-buffered saline; PBS-t, phosphate-buffered saline containing 0.2% Triton X-100; PCR, polymerase chain reaction; RT, room temperature; trk, tropomyosin-related kinase.

Cation channels of the degenerin/epithelial sodium channel family (DEG/ENaC) have two transmembrane domains and a large extracellular loop. They form homomeric or heteromeric channels that are sensitive to amiloride and are not gated by voltage (Kellenberger and Schild, 2002). One branch of the DEG/ENaC family is acid-sensing ion channels (ASICs) that are gated by extracellular protons. In mammals, six different proteins arise from four genes; ASIC1a (Waldmann et al., 1997a) and -1b (Chen et al., 1998) are spliced forms of *ASIC1*; ASIC2a (Garcia-Anoveros et al., 1997) and -2b (Lingueglia et al., 1997) are spliced forms of *ASIC2*; and ASIC3 (Waldmann et al., 1997b) and ASIC4 (Akopian et al., 2000) arise from *ASIC3* and *ASIC4*, respectively.

ASICs are almost ubiquitous in both the peripheral nervous system and CNS and have been suggested to play important roles in physiological/pathophysiological conditions. Among the ASICs, ASIC2 may serve modulatory roles in cutaneous and visceral mechanosensory functions in the peripheral nervous system (Price et al., 2000; Page et al., 2005). Although previous reports have shown that ASIC2 is primarily expressed by medium- to large-diameter dorsal root ganglion (DRG) neurons, which are thought to be mainly mechanosensitive, questions remain regarding distribution within DRG and cellular localization (Alvarez de la Rosa et al., 2002; Garcia-Anoveros et al., 2001).

The relationship between ASIC2 and other phenotypic markers is also poorly understood. Particular neurotrophins are required to maintain the phenotype of specific DRG neuronal populations in adult animals, in addition to their developmental role (Verge et al., 1996). Experimental data indicate that the neurotrophin family of neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), exert their effects on specific receptors. All neurotrophins have been shown to bind to the p75 neurotrophin receptor with a similar affinity (Radeke et al., 1987; Rodriguez-Tebar et al., 1990, 1992). In addition, tropomyosin-related kinase (trk) receptor exhibits greater ligand selectivity and higher binding affinity. NGF mediates its effects via trkA (Kaplan et al., 1991; Klein et al., 1991); BDNF and NT-4 via trkB (Soppet et al., 1991; Squinto et al., 1991), and NT-3 primarily through trkC (Lamballe et al., 1991) but also binds to a lesser degree to trkA and trkB (Cordon-Cardo et al., 1991; Soppet et al., 1991; Squinto et al., 1991). Each trk receptor is expressed in the distinct population of adult DRG neurons (McMahon et al., 1994; Kashiba et al., 1997; Wright and Snider, 1995). However,

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little is known about the relationship between ASIC2 and trk receptors.

When a peripheral sensory neuron is injured, profound cytochemical and functional changes occur in DRG neurons (Alvarez de la Rosa and Fitzgerald, 1999). Responses to nerve injury include dramatic changes in expression of peptides, ion channels and receptors in DRG. However, it is unknown whether peripheral nerve injury alters ASIC2 expression. In the present study, we examined the expression and distribution of ASIC2 in DRG, the co-localization of ASIC2 with trk receptors, and the effects of peripheral nerve injury on ASIC2 expression.

EXPERIMENTAL PROCEDURES

All animal experiments conformed to the regulations of the Sapporo Medical University Animal Care Committee and were carried out in accordance with NIH guidelines on animal care. All efforts were made to minimize the number of animals used and their suffering.

Animals

A total of 40 adult male Sprague–Dawley rats (weight, 150–200 g; Japan SLC, Hamamatsu, Japan) were used. All experimental procedures were performed in rats anesthetized with sodium pentobarbital (50 mg/kg body weight, given i.p.) or general anesthesia (isoflurane 3% in oxygen).

cDNA constructs

Total RNA was obtained from rat brain with Trizol reagent (Invitrogen, Grand Island, NY, USA). First-strand cDNA was synthesized using Oligo dT25 primer and reverse transcriptase (Superscript II, Invitrogen) for 1 h at 42 °C. Synthesized single-strand cDNA was added to a polymerase chain reaction (PCR) mixture containing dNTPs, *Taq* polymerase and primers (5′-TTGAATTCCCACCATGGACCTCAAGGAGAGCCCCAGTGA-3′ (forward)/5′-ATGCGGCAGTTACAGTTCTCCACGATGT-3′ (reverse) for ASIC2a nucleotides 154–1124; 5′-GACCTTCGTGGCCACACAAGAGCA-3′ (forward)/5′-TTGTCGACTCAGCAGGCAATCTCCTCCAGGGT-3′ (reverse) for ASIC2a nucleotides s 960–1692; 5′-ATGAATTCCCACCATGAGCCGGAGCGGCGGAG-3′ (forward)/5′-ACAGGAAAGAAGTCGAGTCCCATCTCTGAGG-3′ (reverse) for ASIC2b nucleotides 16–1070; 5′-AACGGGCTGGAGATCATGCTGGAC-3′ (forward)/5′-AAGTCGACTCAGCAGGCAATCTCCTCCAGGGT-3′ (reverse) for ASIC2b nucleotides 811–1707). Since the *Bal*I site is located in the overlapping region of the two amplified PCR products for ASIC2a and -2b, the PCR products were enzyme-digested with *Bal*I and each was ligated. Each sequenced product for ASIC2a or ASIC2b had an *EcoR* I site and KOZAK sequence added to the 5′-site and a *Sal*I site to the 3′-site and was ligated into a pEGFP-N1 vector (Clontech, Mountain View, CA, USA) at the *EcoR* I/*Sal*I sites. Ligated clones were transformed into DH5α *E. coli* competent cells, which were cultured overnight on LB/Kanamycin agar plates. The resulting plasmids were confirmed by PCR and restriction enzyme analysis.

Antibody generation

The ASIC2 splice variants, ASIC2a and ASIC2b, are identical in their C-terminal residues but differ in their N termini. We raised a polyclonal antibody, anti-ASIC2 antibody, in order to recognize rat ASIC2a and ASIC2b. First, peptide VPLQTALGTLEEIA was synthesized. This peptide corresponds to the C-terminal 14 amino residues common to ASIC2a and ASIC2b (accession no. U53211 and Y14635). Synthetic peptides coupled to keyhole limpet hemocyanin (Sigma, St. Louis, MO, USA) were emulsified with Freund's complete adjuvant (DIFCO, Detroit, MI, USA). For immunization, emulsions containing a given amount of antigen peptide were injected into guinea pigs at 2-week intervals. From antisera sampled at 2 weeks after the sixth injection, anti-ASIC2 antibody was affinity-purified by passing the purified IgGs through sepharose columns to which the unconjugated ASIC2 peptide had been immobilized.

Cell culture and transfection

Chinese hamster ovary (CHO) cells were cultured on Ham's F12 with 10% fetal bovine serum. For immunocytochemistry and Western blot, cells were seeded on glass coverslips coated with poly D-lysine (13 mm) and 10-cm^2 dishes, respectively. Cells were transiently transfected with plasmids containing the cDNAs of ASIC2a or ASIC2b or with pEGFP-N1 alone using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's protocol and were maintained for up to 24 h at 37 °C in a humidified atmosphere of 95% air/5% CO_2 in an incubator.

Western blots

Rats were deeply anesthetized with sodium pentobarbital and killed by decapitation. Lumbar DRGs were rapidly removed. DRGs and transfected CHO cells were homogenized in the presence of ice-cold lysis buffer containing 0.5% NP-40, 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and complete protease inhibitors (Roche Diagnostics, Basel, Switzerland). Crude homogenates were centrifuged at 15,000×*g* for 20 min at 4 °C. Supernatants of the homogenates were collected, and protein concentrations were determined by DC protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) with bovine serum albumin. Equal amounts of protein (20 μg) were resolved by sodium dodecylsulfonate–polyachrylamide gel electrophoresis (SDS-PAGE) (7.5%) and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were incubated with anti-ASIC2 antibody (0.055 μg/ml) in phosphate-buffered saline (PBS) containing 10% skim milk overnight at 4 °C. Concentrations of actin, a housekeeping protein, were also measured using rabbit anti-actin antibody (1:1000; Sigma). Immunoreactions were visualized using the ECL chemiluminescence detection system (Amersham). The reaction product was visualized on X-ray film (Figs. 1G and 2D) or using an image analyzer (Fig. 5E) (LAS-3000mini, Fuji Film, Tokyo, Japan).

RT-PCR of ASIC2 mRNA expression in DRG

For the RT-PCR, the rats were killed by decapitation under ketamine anesthesia, and DRGs and amygdala were removed and rapidly frozen with powdered dry ice and stored at −80 °C until ready for use. Total RNA was isolated with Trizol (Invitrogen) and 0.3 μg was used for cDNA synthesis with Super Script III reverse transcriptase and random hexamer primers (Invitrogen). The cDNA was used as a template for PCR amplification with Hot Start *Taq*DNA polymerase (Takara, Tokyo, Japan) and the following primers: 5′-TCAACCTACAGATTCCCGACCCG-3′ (forward)/5′-CGAGTCCCATCTCTGAGGACCGG-3′ (reverse) for ASIC2a (Waldmann et al., 1996); 5′-CTGCCTTCATGGACCGTTTG-3′ (forward)/5′-CGAGTCCCATCTCTGAGGACCGG-3′ (reverse) for ASIC2b (Lingueglia et al., 1997) and 5′-ACCACAGTCCATGCCATCAC-3′ (forward)/5′-TCCACCACCCTGTTGCTGTA-3′ (reverse) for GAPDH. Each PCR amplification was performed under the condition of 40 cycles of 30 s at 94 °C, 30 s at 59 °C and 45 s at 72 °C for ASIC2a, 40 cycles of 30 s at 94 °C, 30 s at 58 °C and 45 s at 72 °C for ASIC2b and 25 cycles of 30 s at 94 °C, 30 s at 58 °C and 45 s at 72 °C for GAPDH. Samples without the addition of reverse transcriptase or without the addition of RNA (negative controls) revealed no detectable product.

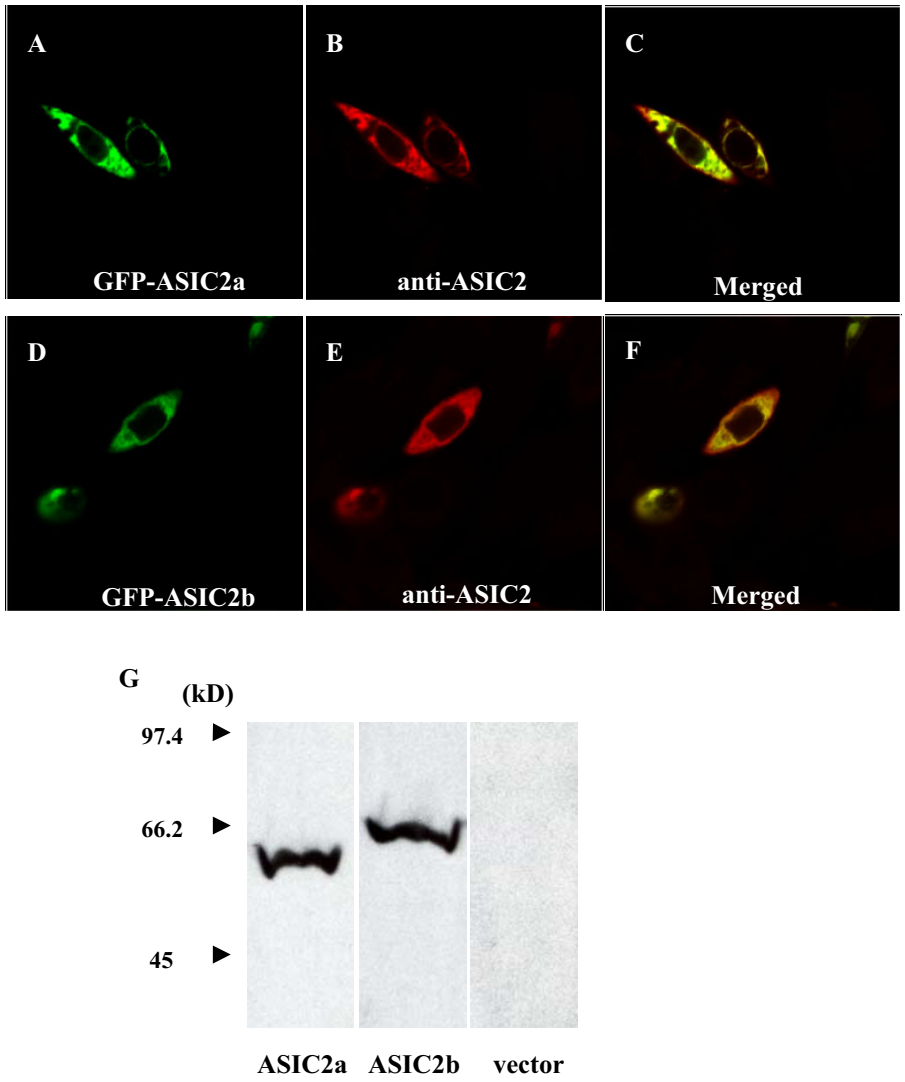


Fig. 1. (A–F) Antibody recognition of ASIC2a (A–C) and -2b (D–F) heterologously expressed in cultured CHO cells. (B, E) Immunochemical labeling with the anti-ASIC2 antibody (red) of cells expressing rat ASIC2a or -2b. (A, D) Cells expressed green fluorescent protein (GFP) as a marker of expression of ASIC2a or -2b protein. (G) Western blot detection of proteins from extracts of rat ASIC2a-, -2b- and vector alone-expressing cells.

Immunocytochemistry of transfected cells

Transfected CHO cells grown on coverslips were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min. Cells were then rinsed three times with PBS for 10 min each and were permeabilized with phosphate-buffered saline containing 0.2% Triton X-100 (PBS-t) for 10 min, followed by blocking with 10% normal goat serum for 30 min and incubation for 18 h at 4 °C with anti-ASIC2 antibody (0.11 μg/ml). Cells were incubated for 2 h at room temperature (RT) with anti-guinea-pig fluorescent secondary antibody (1:1000; Alexa Fluor 594, Molecular Probes; Eugene, OR, USA).

Immunohistochemistry of DRG

Rats were deeply anesthetized with sodium pentobarbital and transcardially perfused with 100 ml of saline followed by 300 ml paraformaldehyde (4% in 0.1 M PB). The left L5 DRG was dissected, post-fixed in 4% paraformaldehyde (5 h at 4 °C), and transferred to 25% sucrose (overnight at 4 °C). DRGs were then blocked in OCT embedding compound on dry ice and stored at −80 °C. Frozen sections (16 μm) of DRGs were thaw-mounted onto gelatin-coated slide glass. Sections were blocked with 10% normal goat serum in PBS-t for 30 min at RT and incubated for 18 h at 4 °C with anti-ASIC2 antibody (0.11 μg/ml). Sections were then incubated for 2 h at RT with Alexa Fluor 488-labeled anti-guinea-pig secondary antibody (1:500; Molecular Probes). For double immunofluorescence, after incubation with 10% normal goat serum in PBS-t, sections were incubated with a mixture of anti-ASIC2 antibody and mouse anti-neurofilament protein 200 kD (NF200) antibody (1:2000; Sigma), rabbit anti-trk B antibody, which showed no reactivity with truncated receptor gp95^{trkB} (manufacturer's technical information) (1:500; Santa Cruz) (Botchkarev et al., 1999), rabbit anti-trk A antibody (1:100; Santa Cruz), or glial fibrillary acid protein (GFAP) antibody (1:1000; Sigma), followed by a mixture of secondary antibodies (1:500; Alexa Fluor 488-labeled anti-guinea-pig antibody and 1:1000; Alexa Fluor 594-labeled anti-rabbit antibody, or 1:1000; Alexa Fluor 594-labeled anti-mouse antibody). In the case of double immunofluorescence for anti-ASIC2 antibody and anti-trk C antibody, sections were blocked with 10% normal donkey serum in PBS-t and incubated

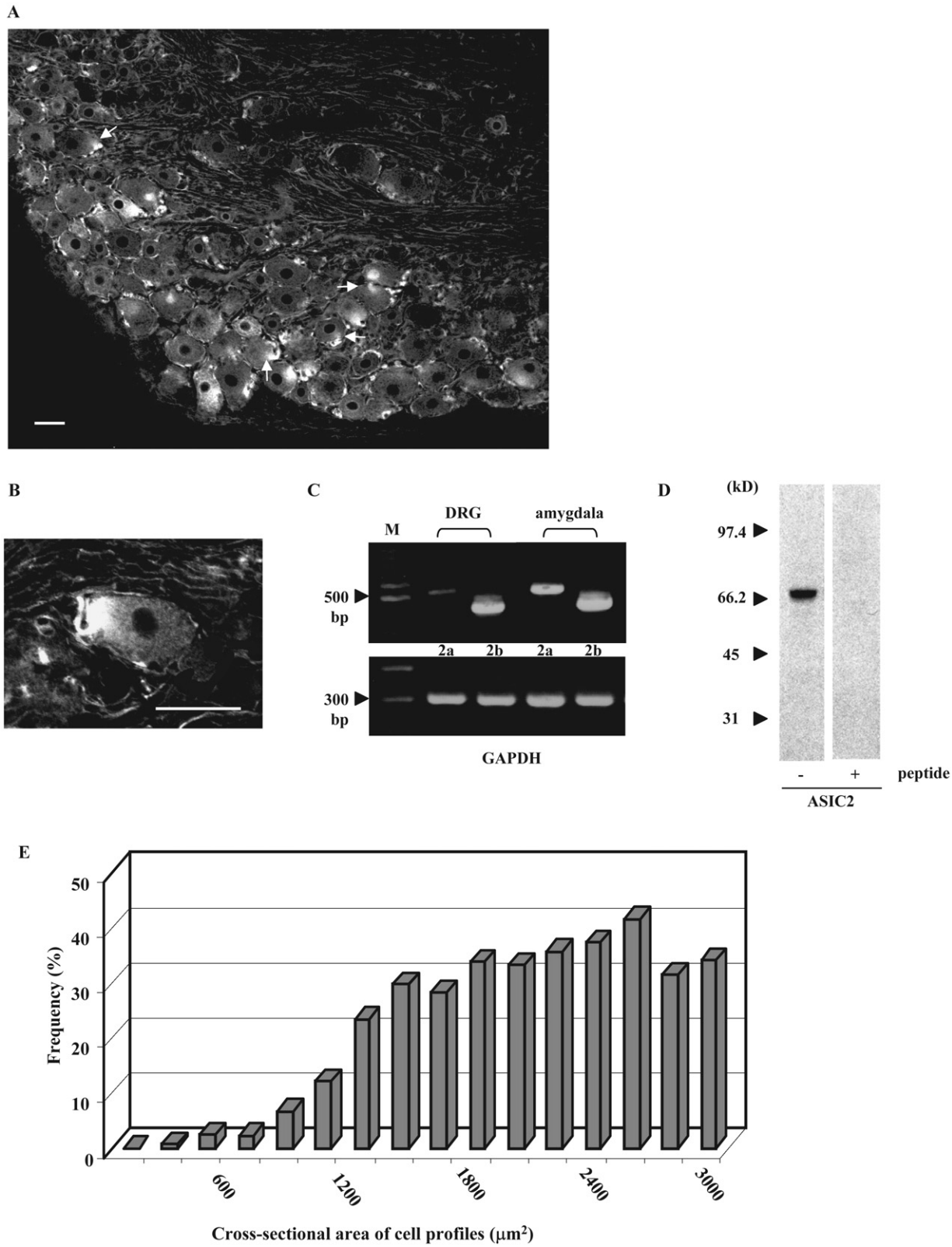


Fig. 2. Expression of ASIC2 in the rat DRG. (A) Immunohistochemical labeling of a rat DRG section with ASIC2 antibody. Arrows indicate subcellular accumulation of ASIC2 IR (white) within the cell body near the axon hillock and the proximal neuronal process. Scale bar=50μm. (B) A high magnification image. Note the intense ASIC2 IR near the axon hillock. (C) RT-PCR analysis of ASIC2a, -2b and GAPDH mRNAs in the rat DRG and amygdala. Both ASIC2a and -2b mRNAs were observed in amygdala. ASIC2a mRNA was present at a very low level in DRG compared with ASIC2b

with a mixture of anti-ASIC2 antibody and goat anti-trk C antibody (1:100; R&D System, Minneapolis, MN, USA) (Tamura et al., 2005), followed by a mixture of secondary antibodies (1:200; FITC-labeled anti-guinea-pig antibody from Jackson ImmunoResearch, West Grove, PA, USA and 1:1000; Alexa Fluor 594-labeled anti-goat antibody). For triple immunofluorescence, sections were blocked with 10% normal donkey serum and incubated with a mixture of anti-ASIC2 antibody, rabbit anti-trk B and goat anti-trk C antibody, followed by a mixture of secondary antibodies (1:200; FITC-labeled anti-guinea-pig antibody, 1:1000; Cy5-labeled anti-rabbit antibody from Jackson ImmunoResearch and 1:1000; Alexa Fluor 594-labeled anti-goat antibody). On Western blot analysis of DRG, the anti-trkB antibody recognized a single prominent band of ~150 kD. Using the anti-trkC antibody, a single prominent band of ~150 kD was also detected. (Fig. 5E). The predicted molecular weights of full-length trkB and trkC are 145 kD and 145 kD, respectively. These antibodies were thus considered suitable for immunohistochemical studies. Photographs were taken using a confocal laser scanning microscope (LSM510; Zeiss, Oberkochen, Germany). L5 DRGs from four perfused rats were analyzed for number and size distribution of ASIC2-immunoreactive (IR) cells. Analyses were performed at ×20 objective magnification. Four sections from each DRG were analyzed for number and size distribution of ASIC2-IR cells. At least 300 neuron profiles from each DRG section were examined. The proportion of ASIC2-IR neurons was determined by counting 1200–1600 L5 DRG neuronal profiles in each rat. Only neurons with clearly visible nuclei were counted. An average percentage of ASIC2-IR cells, relative to the total number of neurons, was obtained for each animal across the different DRG sections; then the mean±S.D. across animals was determined. For size-frequency histogram data, measurements of the area of positive neurons over selected tissue profiles were performed using a computerized image analysis system (Image-Pro Plus v.4.1, Media Cybernetics, Silver Spring, MD, USA) and only neurons with clearly visible nuclei were used for quantification. We classified the DRG neurons into small ($<600\ \mu m^2$), medium ($600–1200\ \mu m^2$) and large ($>1200\ \mu m^2$) neurons based on their cross-sectional area. In addition, to evaluating the colocalization of NFP, trks and GFAP with ASIC2, we counted the number of double or triple-positive cells among 200–500 ASIC2-positive cells with clearly visible nuclei. Because a stereological approach was not used in this study, quantification of the data may represent a biased estimate of the actual number of cells and neurons. All counting was performed by an assistant who was blinded to the treatment groups for the sections.

L5 nerve transaction

Changes in expression of ASIC2 were assessed in rats after L5 nerve ligation and transections. Under general anesthesia (isoflurane 3% in oxygen) and aseptic precautions, the left L5 transverse process was removed, and the L5 and L4 nerves were identified. The L5 nerve was tightly ligated with 5–0 silk and transected immediately distal to the ligature. The ligature was located approximately 3–4 mm proximal to the junction of the L4 nerve and 5–6 mm distal to the L5 DRG. The incision was closed in layers. Seven days after transection, rats were perfused and L5 DRG was processed.

RESULTS

Characterization of ASIC2 antibody

ASIC2 antibody intensely labeled CHO cells transiently transfected with either cDNA of ASIC2a or ASIC2b (Fig. 1A–F), but not with vector alone (data not shown). On Western blot analysis of cultured CHO cells transiently transfected with either ASIC2a or ASIC2b, anti-ASIC2 antibody recognized prominent bands of ~58 kD and ~66 kD, respectively (Fig. 1G). The ASIC2 antibody did not exhibit IR for cell lysates from CHO cells transiently transfected with vector alone. The predicted molecular weights of ASIC2a and ASIC2b are 57 kD and 63 kD, respectively. These antibodies were thus considered suitable for subsequent immunohistochemical studies of ASIC2.

Expression of ASIC2 in DRG

We performed RT-PCR with rat DRG total RNA. Although we detected both ASIC2a and -2b mRNAs in DRG, ASIC2a mRNA was present at a very low level compared with ASIC2b mRNA (Fig. 2C). Western blot analysis of DRG tissues showed that ASIC2 antibody recognized a single prominent band of ~66 kD (Fig. 2D), thus suggesting that ASIC2b, but not ASIC2a, is present in the DRG. This IR was completely abolished by preincubation with the peptide (10 nM) used for generation of ASIC2 antibody (Fig. 2D). Thus, ASIC2b is likely to be mainly expressed in the DRG. In normal DRG, ASIC2 antibody revealed positive IR in both neurons and satellite cells (Figs. 2A, 3A–C). When ASIC2 antibody was preincubated with the peptide (10 nM) used for generation of ASIC2 antibody, immunolabeling was completely abolished (data not shown). The ASIC2 IR neurons in L5 DRG of the normal rats were 16.5±2.4% of the total neurons (848 positive neurons of 5217 total neurons, derived from four sections/rat, $n=4$). Most ASIC2 IR neurons were medium-to-large neurons (Fig. 2A, 2E). ASIC2 was expressed in 5% of medium-sized neurons and in 37% of large neurons in normal rat DRG. About 30% of medium-to-large neurons were IR for ASIC2. Within these neurons, ASIC2 IR was not evenly distributed throughout the cytoplasm, but rather accumulated prominently in the cytoplasm adjacent to the axon hillock and axonal process (Fig. 2A, 2B). Nearly all ASIC2 IR neurons were labeled with NF200 (97.4%, 455 of 467 ASIC2-positive neurons counted) (Fig. 3D–F), indicating that ASIC2 expressed in the neuron with the myelinated axon. In addition to neuronal expression, ASIC2 IR was observed in the pericellular regions and overlapped with GFAP IR (Fig. 3A–C), thus suggesting that ASIC2 is also expressed in satellite cells. ASIC2 IR was observed in the satellite cells surrounding both medium-to-large neurons and small neurons.

mRNA in DRG. 2a And 2b indicate ASIC2a and ASIC2b, respectively. M, marker. (D) Western blot detection of ASIC2 from rat lumbar DRG protein extract with the anti-ASIC2 antibody. Using the anti-ASIC2 antibody, a single prominent band of ~66 kD was detected in extracts of DRG (peptide, −), whereas preabsorption with the peptide (10 nM) used for generation of ASIC2 antibody prevented the labeling (peptide, +). (E) Size-distribution histogram of ASIC2-IR neuronal profiles in the rat DRG. Each bar indicates the percentage of ASIC2-IR neurons relative to the total number of neurons in each cross-sectional area.

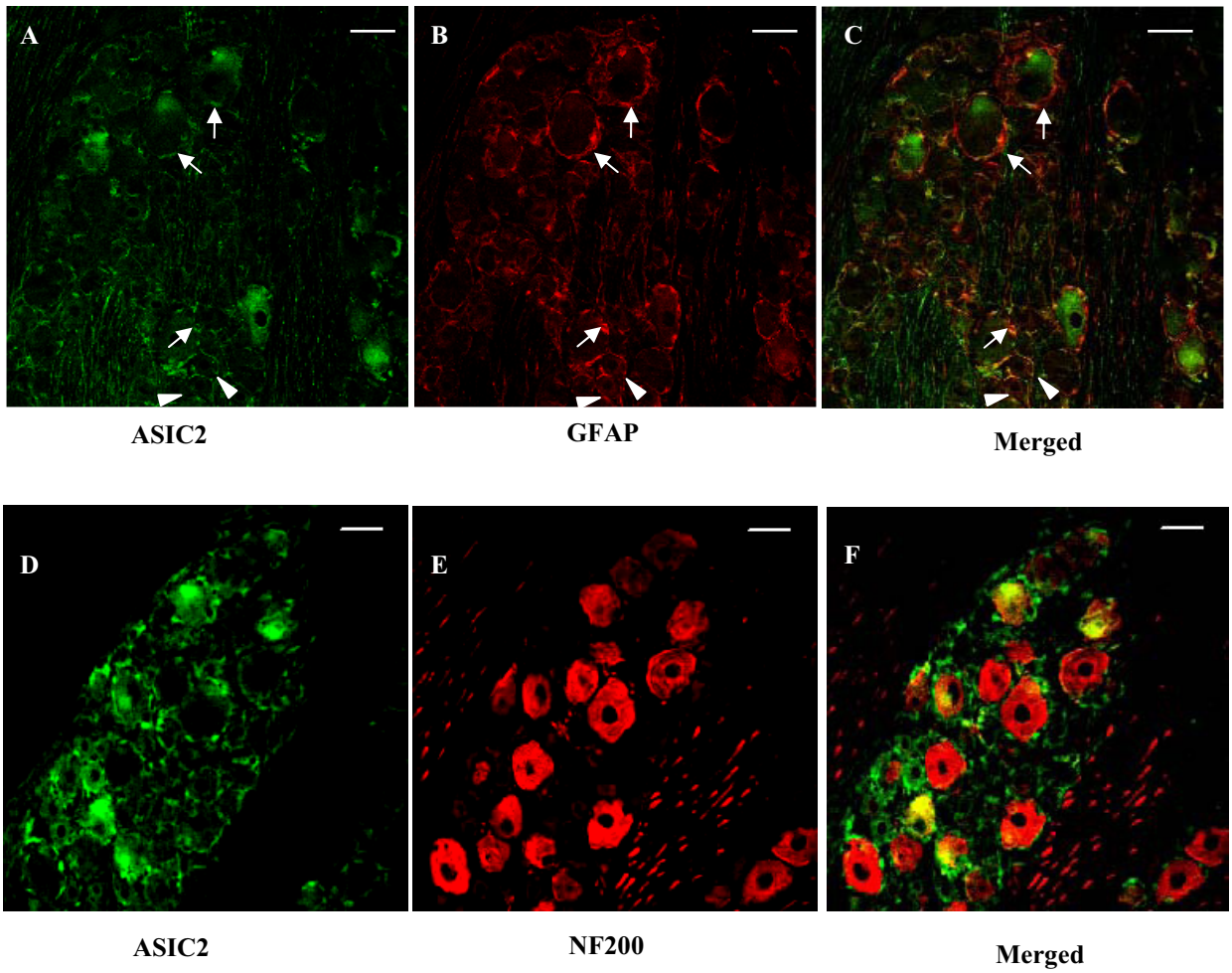


Fig. 3. Immunohistochemical co-localization of ASIC2 with GFAP (A–C) or NF200 (D–F). Arrows and arrowheads indicate co-localization of ASIC2 with GFAP in medium-to-large neurons and small neurons, respectively. Most ASIC2-positive neurons showed NF200 IR. In addition, ASIC2 IR was observed in GFAP-positive cells. Scale bar=50μm.

Co-localization of ASIC2 with neurotrophin-receptor in DRG neurons

We performed double immunofluorescence staining in order to investigate the co-localization of ASIC2 with neurotrophin receptor in DRG neurons. trkA IR was mainly found in small diameter cells and was seen in few ASIC2-positive neurons (1%, three of 302 ASIC2-positive neurons counted) (Fig. 4A–C). Both trkB and trkC were mainly found in medium-to-large cells (Fig. 4D–I), and were observed in 85.2% (419 of 492 ASIC2-positive neurons counted) and 53.4% of ASIC2-positive neurons (241 of 451 ASIC2-positive neurons counted), respectively. The percentage of cells co-localized with trk suggested that some ASIC2-positive neurons express both trkB and trkC. Co-existence of trkB and trkC in central neurons is a common phenomenon (Lindholm et al., 1996; Minichiello and Klein, 1996), and it has been reported that many adult cutaneous sensory neurons express both trkB and trkC (McMahon et al., 1994). We thus performed triple immunofluorescence staining (Fig. 5A–D). Accordingly, 49.2% of ASIC2-positive neurons (213 of 432 ASIC2-positive neurons counted) were found to be trkB and trkC positive. The percentage of ASIC2-positive neurons expressing trkC alone was 6.9% (30 of 432 ASIC2-positive neurons counted). In addition, the percentage of ASIC2-positive neurons expressing trkB alone was 35.9% (155 of 432 ASIC2-positive neurons counted). Therefore, most ASIC2-positive neurons express both trkB and C or trkB alone.

Changes in ASIC2 expression pattern in DRG after axotomy

Changes in the expression of ASIC2 in L5 DRG were examined after L5 nerve ligation and transection. Immunohistochemical analysis revealed a marked reduction in ASIC2 expression in axotomized L5 DRG neurons, compared with that in the contralateral DRG to transaction (Fig. 6A, 6B). Few neurons showed ASIC2 IR in the somata, the axon hillock and the axonal process, although it is possible that neurons expressed ASIC2 protein at levels below the detection limit. However, intense ASIC2 IR was observed in satellite cells (Fig. 6C–E). Although

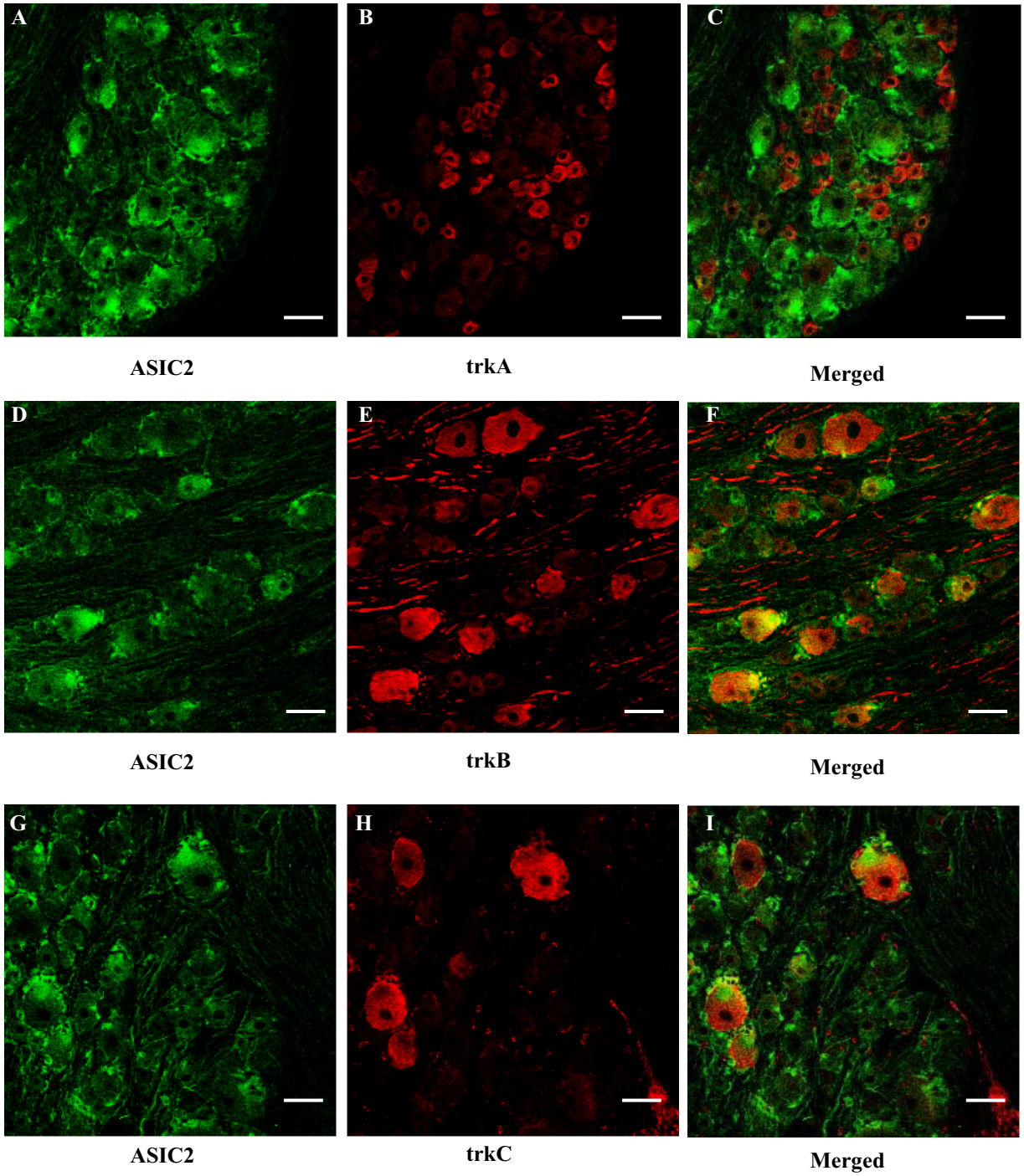
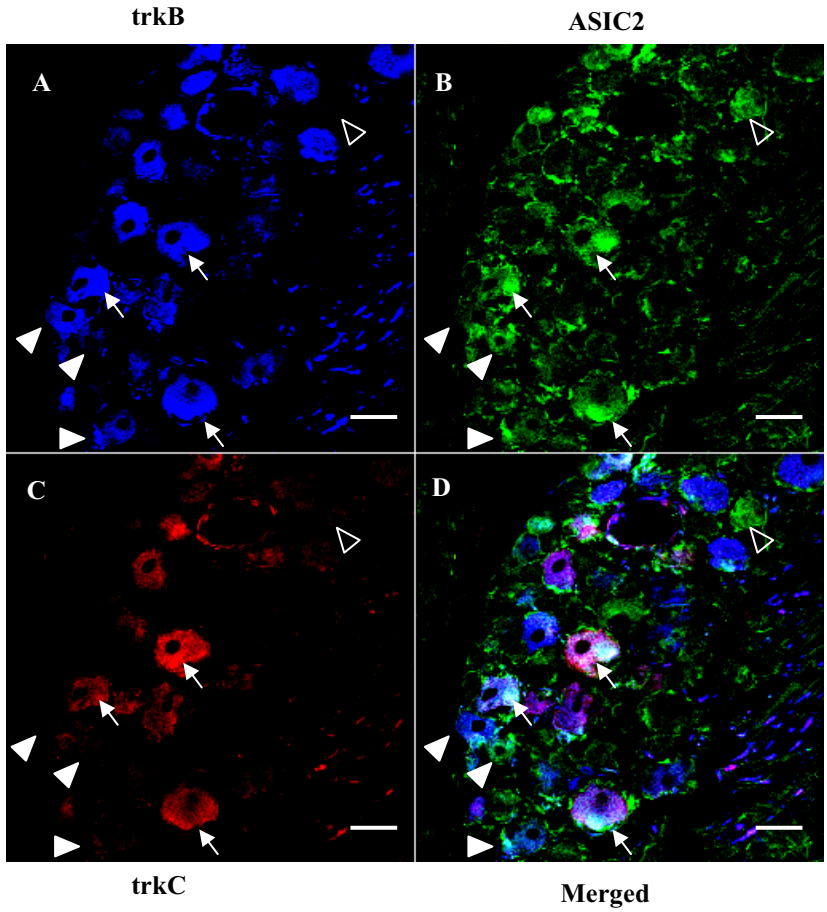


Fig. 4. Immunohistochemical co-localization of ASIC2 with trkA (A–C), trkB (D–F) or trkC (G–I). Most ASIC2-positive cells showed trkB or trkC IR but not trkA IR. Scale bar=50μm.

we did not perform quantitative analysis, it is likely that ASIC2 expression in the satellite cells was not altered by axotomy.

DISCUSSION

In the present study, we investigated the expression of ASIC2 in rat DRG. Our major findings are as follows: (1) ASIC2 is expressed in medium-to-large neurons, as well as satellite cells; (2) most ASIC2-positive neurons were also NF200 positive, indicating that ASIC2 is expressed in neurons with myelinated axons; (3) most ASIC2-positive neurons expressed trkB, and one half also expressed trk C; (4) peripheral axotomy induced marked reductions in ASIC2 expression in neurons, but not in satellite cells, in axotomized DRG.



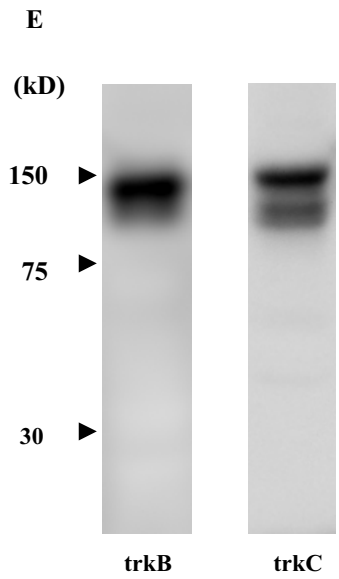


Fig. 5. Immunohistochemical co-localization of ASIC2 with trkB and trkC (A–D). Open arrowheads indicate cells with only ASIC2 IR. Closed arrowheads indicate cells with both ASIC2 and trkB immunoreactivities but not trkC IR, and closed arrows indicate cells with triple immunofluorescence staining for ASIC2, trkB and trkC. Scale bar=50μm. (E) Western blot detections of trkB and trkC from rat lumbar DRG protein extract with the anti-trkB antibody and trkC antibody. The anti-trkB antibody recognized a single prominent band of ~150 kD. Using the anti-trkC antibody, a single prominent band of ~150 kD was also detected.

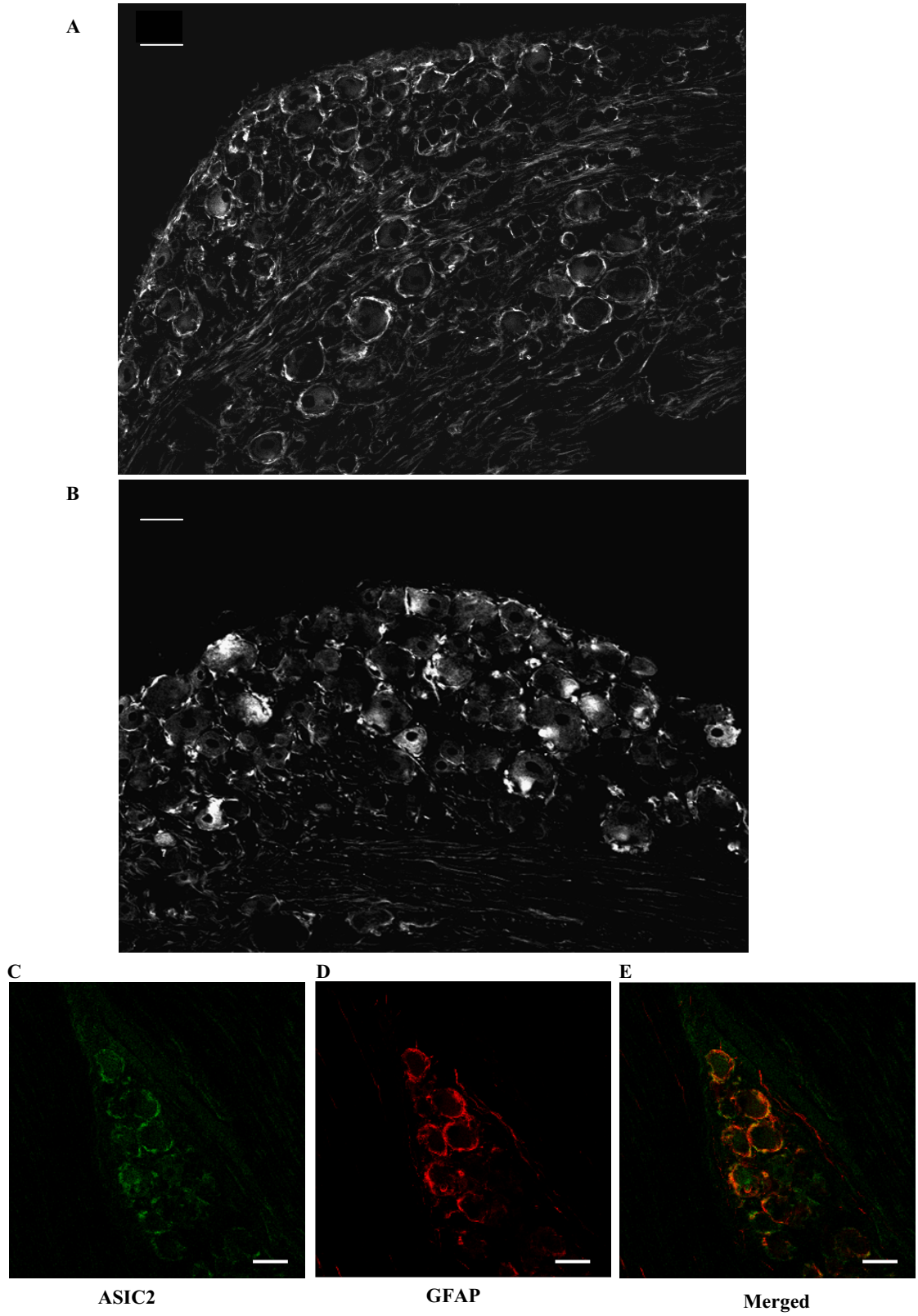


Fig. 6. Changes in ASIC2 expression at 7 days after transection. (A) and (B) are photomicrographs showing ASCI2 IR in the ipsilateral and the contralateral L5 DRGs to transection, respectively. (C–E) Immunohistochemical co-localization of ASIC2 (green) with GFAP (red). Scale bar=50μm.

Expression of ASIC2 in the DRG

In our study, the results of RT-PCR and Western blot analysis suggested that ASIC2b, but not ASIC2a, is primarily expressed in the DRG. Other investigators have also reported that ASIC2b is expressed predominantly in the DRG; ASIC2b mRNA expression was observed in the DRG, while ASIC2a mRNA was not detected or was present at low levels on RT-PCR or *in situ* hybridization (Lingueglia et al., 1997; Price et al., 2000; Voilley et al., 2001). Western blots analysis also indicated that ASIC2b was predominant, although both ASIC2a and ASIC2b were expressed in DRG (Alvarez de la Rosa et al., 2002). ASIC subtypes form homomeric or heteromeric channels. Electrophysiological studies have shown that, among ASIC homomeric or heteromeric channels, the heterologously expressed ASIC2b/ASIC3 channel generates a biphasic inward current that is similar to the naïve proton-activated current in DRG neurons (Lingueglia et al., 1997; Voilley et al., 2001). Taken together, these observations indicate that ASIC2b rather than ASIC2a is primarily expressed in DRG. On the other hand, it has been reported that ASIC2a is clearly expressed by medium-to-large neurons and is localized in the receptor endings of low threshold mechanoreceptors in the skin, suggesting that ASIC2a may participate in the transduction of touch and mechanical stimuli (Garcia-Anoveros et al., 2001). Although no studies have examined the expression and distribution of ASIC2b in DRG tissue using a specific anti-ASIC2b antibody, further studies examining the expression of both ASIC2a and ASIC2b in DRG are necessary.

Most ASIC2 IR neurons had medium-to-large diameters and were positive for NF200, which is similar to previous reports (Alvarez de la Rosa et al., 2002; Garcia-Anoveros et al., 2001), indicating that ASIC2 is expressed in neurons with myelinated axons. Two papers have addressed the subcellular neuronal localization of ASIC2 in DRG. Alvarez de la Rosa et al. (2002) reported that ASIC2 is localized in the plasma membrane and is distributed around the soma and along the axon. In contrast, Garcia-Anoveros et al. (2001) identified ASIC2a in the cytoplasm adjacent to the axon hillock, but not conspicuously in the plasma membrane. In this study, ASIC2 IR was not evenly distributed throughout the cytoplasm, but was primarily accumulated in the cytoplasm adjacent to the axon hillock and the axonal process, similar to the results of Garcia-Anoveros et al. (2001). This distinct subcellular localization suggests that ASIC2 is actively transported in an anterograde direction, toward the terminals.

In the present study, ASIC2 protein was expressed in neurons and in satellite cells in normal DRG. To our knowledge, no other papers have reported the non-neuronal expression of ASICs, although it has been reported that Müller cells in the retinal glia of rabbits express α-ENaC and β-ENaC, which are members of the DEG/ENaC family and exhibit amiloride-sensitive Na^+ current (Brockway et al., 2002).

Co-localization of ASIC2 with receptor tyrosine kinase

Our data showed that about 90% of ASIC2-positive neurons were co-localized with trkB. Although about 50% of ASIC2-positive neurons were trkC positive, most ASIC2-positive neurons were positive for both trkC and trkB, while few were only trkC positive. Recent studies support our results (McIlwrath et al., 2005). ASIC2 mRNA was significantly reduced in BDNF-deficient mice, and ASIC2 protein was also reduced in BDNF-, NT-4- and NT-3-deficient mice when compared with wild-type mice. In BDNF- and NT-4-deficient mice, there were large drops in the proportion of medium and large sensory neurons positive for ASIC2. A significantly smaller proportion of large sensory neurons in NT-3-deficient mice were positive for ASIC2 when compared with wild-type mice. In addition, the reduction of ASIC2 expression in BDNF-deficient mice was recovered by addition of BDNF. Because BDNF and NT-4 act via trkB (Soppet et al., 1991; Squinto et al., 1991), and NT-3 acts primarily on trkC (Lamballe et al., 1991), it appears that ASIC2 is expressed in trkB- and/or trkC-positive neurons. The trkB gene encodes at least two glycoproteins, $gp^{145trkB}$ (full-length trkB) containing the signal-transducing tyrosine kinase domain and $gp95^{trkB}$ (truncated trkB) lacking this domain, both of which bind BDNF and NT-4. Full-length trkB mediates the crucial effects of BDNF and NT-4, but the physiological roles of truncated trkB have not been fully established. The antibody used in this study is specific for full-length trkB, and thus ASIC2 is expressed in neurons reactive for full-length trkB. In addition, full-length trkB is expressed in neurons, but not in satellite cells in the peripheral nervous system (Frisen et al., 1993; Rose et al., 2003; Wetmore and Olson, 1995); satellite cells express truncated trkB, but not full-length trkB. Although it remains unclear whether the reduction of ASIC2 expression in NT-4- and NT-3-deficient mice is recovered by exogenous NT-4 and NT-3, respectively, the ASIC2 gene appears to be the target for BDNF/NT-4-full-length trkB and NT-3-trkC signaling in sensory neurons. However, ASIC2 protein was expressed in both neurons and satellite cells in normal DRG, while full-length trkB and trkC were seen in neurons, but not in satellite cells (Chen et al., 1996). Therefore, the mechanism of ASIC2 expression in satellite cells may differ from that in neurons.

Analyses of gene-deficient mice revealed that trkB- and trkC-dependent sensory neurons might be involved in tactile perception and proprioception, respectively (Klein et al., 1993, 1994). Taken together with our results, it could be assumed that ASIC2 in DRG neurons play a role in mechanoperception rather than nociception. Some studies using ASIC2-deficient mice also showed the reduction of the sensitivity of low-threshold rapidly adapting mechanoreceptors (Price et al., 2000) and altered mechanosensitivity of the digestive tract (Page et al., 2005). On the other hand, the other studies did not support a role of ASIC2 in mechanoperception (Drew et al., 2004; Roza et al., 2004).

In addition, it is unlikely that ASIC2 is involved in nociception (Price et al., 2000; Roza et al., 2004). Further studies will be necessary in order to clarify physiological function of ASIC2.

Effects of axotomy

In the present study, there was a marked reduction in ASIC2 expression in axotomized DRG neurons, while intense ASIC2 IR was observed in satellite cells. Two consequences of axotomy may trigger down-regulation of ASIC2 expression in DRG neurons. The first is loss of target-derived factors. BDNF, NT-4 and NT-3 are expressed in peripheral tissues. BDNF is produced by peripheral innervation targets such as visceral epithelia (Lommatzsch et al., 1999) and skin, including murine and rat hair follicles (Botchkarev et al., 1999, 2004; Peters et al., 2005; Bergman et al., 2000). ASIC2 IR fibers wrap around the hair follicles and encircle them with comb-like terminals (Price et al., 2000; Garcia-Anoveros et al., 2001). BDNF can be retrogradely transported by distinct populations of peripheral neurons (DiStefano et al., 1992). Furthermore, NT-4 and NT-3 are produced in peripheral tissues, including hair follicle, muscle and skin (Funakoshi et al., 1993; Timmusk et al., 1993; Arumae et al., 1993; Maisonpierre et al., 1990; Copray and Brouwer, 1994; Botchkarev et al., 2004). In addition, BDNF, NT-4 and NT-3 are also retrogradely transported (DiStefano et al., 1992; Tonra et al., 1998; Weible et al., 2004). This suggests that disconnection of the neuron from its source of target-derived neurotrophic factors results in down-regulation of ASIC2 in DRG neurons. This notion may be supported by examining whether exogenous neurotrophic factors prevent axotomy-induced down-regulation of neuronal ASIC2 expression *in vivo*.

A second consequence of axotomy is the induction of factors that could inhibit neuronal ASIC2 expression. For example, leukemia inhibitory factor (LIF) is induced following axotomy and is involved in increases in galanin in axotomized DRG neurons (Corness et al., 1996; Sun and Zigmond, 1996). To date, however, it has been unclear whether axotomy induces factors that inhibit neuronal ASIC2 expression.

CONCLUSION

In conclusion, the present study shows that ASIC2 is expressed in both the distinct neurochemical population of sensory neurons as well as satellite cells. Peripheral axotomy induced marked reductions in ASIC2 expression in neurons, and thus neuronal ASIC2 expression may be maintained by peripheral target-derived neurotrophic factors.

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