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THE SENSORY MECHANOTRANSDUCTION ION CHANNEL ASIC2 (ACID SENSITIVE ION CHANNEL 2) IS REGULATED BY NEUROTROPHIN AVAILABILITY

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Abstract—Almost all sensory neurons of the dorsal root ganglia have a mechanosensitive receptive field in the periphery. We have shown that the sensitivity to mechanical stimuli of a subset of sensory neurons that are slowly adapting mechanoreceptors (SAM) is strongly dependent on the availability of brain-derived neurotrophic factor (BDNF). Here we have investigated whether the ASIC2 sodium channel, recently shown by us to be necessary for normal SAM sensitivity, might be regulated by BDNF and thus partially account for the down-regulation of SAM sensitivity seen in BDNF deficient mice. We show that the mRNA for ASIC2 channels is reduced in the DRG of BDNF deficient mice indicating that BDNF might maintain its expression *in vivo*. We also made short-term cultures of sensory neurons from adult BDNF deficient mice and used a specific antibody to detect the presence of ASIC2 channels in different classes of sensory neurons. We observed that the channel protein was dramatically down-regulated selectively in medium and large diameter neurons and this expression could be rescued in a dose and time dependent manner by addition of BDNF to the culture (10–100 ng/ml). Drugs that block new transcription or protein synthesis also prevented the rescue effects of BDNF. We observed that ASIC2 channels were down-regulated in sensory neurons taken from neurotrophin-4 and neurotrophin-3 deficient mice; these effects might be due to a selective loss of neurons that normally express large amounts of ASIC2 channels. In summary, our data identify the ASIC2 channel as a target of BDNF signaling *in vivo* and suggest that the functional down-regulation of sensory mechanotransduction in BDNF deficient mice is in part due to loss of ASIC2 expression.

Key words: mouse, BDNF, NT-4, NT-3, sensory neurons, mechanosensation.

Members of the neurotrophin family of which nerve growth factor (NGF) is the prototypical member are survival factors for functionally distinct sensory neuron types during development (Lewin, 1996; Lewin and Barde, 1996; Huang and Reichardt, 2001). In the adult NGF has also been shown to regulate the noxious heat sensitivity of nociceptive sensory neurons both under normal circumstances and after peripheral inflammation (Lewin and Mendell, 1994; Lewin et al., 1994; Bennett et al., 1998; Koltzenburg et al., 1999; Shu and Mendell, 1999a,b; Stucky and Lewin, 1999; Chuang et al., 2001; Galoyan et al., 2003). More recently characterized neurotrophins such as BDNF can also regulate the functional properties of sensory neurons. It was shown that BDNF is necessary *in vivo* to maintain normal mechanosensitivity of slowly adapting mechanoreceptors (SAM) that innervate Merkel cells in the skin (Carroll et al., 1998). Thus we reasoned that BDNF signaling via activation of its tyrosine kinase receptor trkB must specifically control the expression of molecules necessary for the mechanosensory function of SAM (Carroll et al., 1998).

The molecular basis of sensory neuron mechanosensitivity is however still poorly understood. Recently, vertebrate homologues of ion channels first identified as being necessary for touch sensation in the nematode worm *Caenorhabditis elegans* have been described (Garcia-Anoveros and Corey, 1997; Tavernarakis and Driscoll, 1997; Ernstrom and Chalfie, 2002). These channels belong to the Deg/ENaC family and have been proposed to act as acid sensors in nociceptive sensory neurons (Waldmann and Lazdunski, 1998; Alvarez de la Rosa et al., 2000) or as participants in sensory mechanotransduction (Lewin and Stucky, 2000; Lewin and Moshourab, 2004). The acid sensitive ion channel two (ASIC2; Waldmann and Lazdunski, 1998), also known as brain sodium channel 1 (Price et al., 1996), Mdeg1 (Waldmann et al., 1996), and BNaC1 (Garcia-Anoveros et al., 1997), is a member of the Deg/ENaC superfamily with high homology to the *C. elegans* proteins MEC-4 and MEC-10 (Ernstrom and Chalfie, 2002). Our studies using ASIC2 deficient mice have shown that the presence of this ion channel subunit in the sensory terminals of both rapidly and SAM is necessary for their normal mechanosensitivity (Price et al., 2000).

In the present study we have asked if the expression of ASIC2 channel protein in adult sensory neurons is controlled by neurotrophins. We have used short-term cultures of adult sensory neurons taken from mice deficient in the neurotrophins BDNF, neurotrophin-4 (NT-4) and neurotrophin-3 (NT-3) and performed quantitative immunofluores-

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Abbreviations: ASIC2, acid sensitive ion channel 2; BDNF, brain-derived neurotrophic factor; IB_4, isolectin-B_4; NT, neurotrophin; RIS, relative intensity of staining; SAM, slowly adapting mechanoreceptor.

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cence experiments with antibodies specific for the ASIC2 channel. Our data indicate that the ASIC2 channel transcript and protein is regulated in subsets of neurons through NT receptor signaling. The loss of low threshold mechanoreceptive sensory neurons in NT-4 and NT-3 knockout mice (Airaksinen et al., 1996; Minichiello et al., 1998; Stucky et al., 1998, 2002) may account for some of the reduction in ASIC2 protein that we observed in sensory neurons from these mice. We conclude that the mechanosensory deficits observed in BDNF deficient mice can be explained in part by NT regulation of the ASIC2 channel particularly in large diameter sensory neurons *in vivo*.

EXPERIMENTAL PROCEDURES

Knockout mice

For the experiments described in this paper we used mice heterozygous or homozygous for mutations in the brain-derived neurotrophic factor (BDNF), NT-4 and NT-3 genes. In the case of BDNF and NT-3 controls were age matched wild-type littermates. Mice with a null mutation of the NT-4 gene are viable and fertile and in this case we used an appropriate control strain obtained from the Jackson Laboratories (Bar Harbor, ME, USA; 129S3; catalogue number 002448). The generation of the BDNF mice was originally described in Korte et al. (1995) and further characterization of these mice has been described in several recent papers (Carroll et al., 1998; Heppenstall and Lewin, 2001). NT-4 and NT-3 mutant mice were obtained from the Jackson Laboratories and the generation of these mice was originally described by Ernfors et al. (1994) and Liu et al. (1995). All genotyping was carried out using standard PCR protocols from genomic DNA isolated from tail biopsies.

Neuronal cultures

Cultures of DRG neurons were prepared as described previously with slight modifications (Mannsfeldt et al., 1999). DRGs of all spinal levels from adult mice age 8–10 weeks, except in the case of NT4/5 −/− 5-week-old animals, were removed and collected in sterile PBS. DRGs were incubated in 1 mg/ml collagenase i.v. (Sigma, Germany) for 30 min at 37 °C and then in 0.1% trypsin (Sigma) for 30 min at 37 °C. After removal of trypsin, serum-containing medium (10% heat-inactivated horse serum [Biochrom, Germany], 20 mM glutamine, 0.8% sucrose, 100 units penicillin/100 μg streptomycin) was added to the DRGs. Tissue was dissociated by trituration with a fire-polished bore of a siliconized Pasteur pipette and cells were plated on polyornithine/laminin-coated coverslips (polyornithine; Sigma; 500 μg/ml; and laminin; Gibco, Germany; 20 μg/ml) at a density of 1000–2000 cells per coverslip. Cells were incubated in serum-containing medium that normally contained no NT at 37 °C, 5% CO_2 for approximately 16 h (overnight). For some experiments BDNF (recombinant human BDNF; kind gift from Regeneron, USA) was added to the cultures and analysis was carried out at 4 or 24 h.

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde in PBS for 10 min and then washed with PBS. The antibody ASIC2_EC 1.0 recognizes an extracellular epitope; it was accordingly not necessary to permeabilize the cells to obtain specific staining. The ASIC2b_IC 1.0 recognizes an intracellular epitope; with this antibody fixed cells were permeabilized with 0.05% Triton X-100 for 5 min before the primary antibody was applied. Non-specific binding was blocked by incubating the cell culture in 3% normal goat serum in PBS for 30 min. Both primary antibodies were diluted in the same blocking buffer each at a concentration of 1:100 and the antibody was left on the cells overnight at 4 °C. Primary antibody binding was detected with a Cy3-coupled secondary antibody (goat anti rabbit; Dianova, Hamburg, Germany). Cell surface binding of the plant isolectin-B_4 from *Griffonia simplicifolia* (IB_4) was detected in using FITC-labeled IB_4 (Sigma); this was applied to the cells (10 μg/ml) in 0.1 M PBS for 1 h at room temperature and the cells were then rinsed, mounted and labeled cells observed directly with the fluorescence microscope.

Soma size analysis

Openlab software (Improvision, UK) was used for the quantification of the immunofluorescence. For the analysis, images were collected with 20-fold magnification objective, about two to six cells per captured field, amounting to about 50 cells per cell culture. The perimeter of each neuron was traced and the mean soma diameter was calculated. Between four and six individual cultures from animals of each genotype or pharmacological experiment were stained with the antibody; control cultures were always made and analyzed in parallel. The mean intensities of staining observed between individual cultures of a given genotype or drug application varied very little. The highest intensity measured in each experiment of a wild-type animal was set as 100% and the data thus normalized. The normalized data were pooled for each genotype. This normalized intensity was denoted the relative intensity of staining (RIS value). Background staining was determined for both polyclonal antibodies ASIC2_EC 1.0 and ASIC2b_IC 1.0 separately both by staining sensory neuron cultures from ASIC2 null animals (ASIC2 knockout mice were a kind gift from Dr. Maggie Price) and in the presence of the immunizing peptide. The mean intensity of background staining found using ASIC2 null cultures or the blocking peptide was very similar and the values obtained plus 1.5 times the standard deviation was found to be equal to a maximum of 40% of the largest fluorescence intensity measured in wild-type neurons. This background value was subtracted from the relative intensity measured in all experiments. For this reason the actual maximum value for the RIS was 60%. This correction ensured that the immunostaining detected in sensory neurons was specific for the ASIC2 channel.

Real-time PCR and dot blotting

RNA isolation for real-time PCR. Qiagen RNeasy mini kit (Cat. No. 79254) was used and a protocol with an on column DNAse I (Qiagen, Germany, Cat. No. 74104) treatment. One microgram to 2 μg of total RNA was used as a template for the reverse transcription in a 50 μl volume. For the reverse transcription Superscript II RNAse H- was used with random hexamer primers. Template for the real-time PCR was a 1:40, 1:100 or 1:200 dilution of the first strand reaction mix. Primer concentration in the final mix is 0.9 micromolar each and the probe concentration is 0.25 micromolar. The primer probe mix was obtained from Applied Biosystems (Darmstadt, Germany) for ASIC2b (assay ID: Mm00475687_m1) and the reference gene prenylcysteine oxidase (assay ID: Mm00482162_m1). The prenylcysteine oxidase gene showed no change in three independent Affymetrix gene chip experiments (Anirudhan and Lewin, unpublished observations). FAM was used as the fluorescent dye in the 5′ position and a NFQ at 3′ position. ASIC2a primers (CGGACCCCACCGTGCT and TGCTTCGGTTTGTAGTGTTTGAA) and the probe (AGGC CCTCCGACAGAAGGCCAA) were ordered separately (Biotez, Berlin, Germany) and Tamra was at the 3′ position as the fluorescent quencher. Reaction conditions were as follows: one step of 50 °C for 2 min, one 95 °C for 10 min, and 40 cycles of 95 °C for 15 s denaturation and 59 °C for 1 min annealing and extension. Reactions (20 μl volume) were performed in 96 well plates (Micro Amp; Applied Biosystems; Cat No. N801-0560) covered with optical adhesive covers (Applied Biosystems; Cat No. 4313663). The

Instrument used was ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Reaction volume contained 10 μl 2× Real-time PCR Master mix (TaqMan Universal PCR Master Mix; Applied Biosystems; Cat. No. 4318157), 1 μl 20× primer probe mix, 4 μl DEPC-treated water and 5 μl template. All the reactions were done in triplicate. The threshold cycle number for product detection (Δ CT value) for the ASIC and control gene was used to calculate relative expression levels of ASIC2a and 2b.

Dot blotting. The entire coding region of human ASIC2b was PCR amplified and the product was dotted (total 5 μg) onto a nylon membrane (Amersham Pharmacia) using a slot blotting manifold (Schleicher and Schuell). For probe generation, total RNA from DRG from all spinal levels of BDNF WT and heterozygote mice (three of each group) was extracted using the Trizol reagent (Gibco). Generation of dsDNA was done by performing a polydT primed first strand reaction followed by second strand synthesis. The DNA then was labeled with Digoxigenin-11-dUTP using the random priming method. Labeled DNA (20 ng/ml) was diluted in a standard hybridization buffer (without formamide) and hybridized onto the DNA-dotted membrane at 67 °C overnight. The hybridization intensity was quantified by chemiluminescence detection using the Chemidoc system (Biorad, Uppsala, Sweden). The levels were normalized against GAPDH staining. Two independent hybridizations were performed. For a detailed description of the labeling, hybridization and detection see the DIG system users guide from Roche (Germany).

Cloning and cell culture

Using a human ASIC2b or rat ASIC2a cDNA template (kind gifts Maggie Price and Michel Lazdunski respectively) Flag-tagged ASIC2a and ASIC2b cDNAs were generated by performing a PCR with primers containing a Flag peptide coding sequence in frame with N-terminal sequences of the ASIC2 cDNA sequence. The digested PCR product was then cloned into the pTracer CMV2 (Invitrogen) or pcDNA expression vector. These constructs were checked by sequencing and were then used to transfect HEK 293 cells. Several cell clones that showed Zeocin or G418 antibiotic resistance over several weeks and stably expressed the ASIC2a and ASIC2b-Flag-tagged proteins were isolated. Expression of the protein in individual clones was confirmed by Western blot analysis using both anti-Flag and anti-ASIC2 antibody (EC 1.0).

RESULTS

Localization of ASIC2 channels in sensory neurons

We used two rabbit polyclonal antibodies to detect the presence of the ASIC2 ion channel in subsets of cultured adult mouse sensory neurons. The ASIC2 channel protein has two transmembrane domains, denoted M1 and M2, and a large extracellular loop between these two domains. Two splice variants of the ASIC2 channel exist. ASIC2a (also termed MDEG1, BNaC1α, and ASIC2A) differs from ASIC2b (Mdeg2, ASIC2B, BNaC1β) in the first 185 amino acids, a region that includes the cytoplasmic N-terminus, the first transmembrane domain M1, and 135 residues in the extracellular loop (Lingueglia et al., 1997; Waldmann and Lazdunski, 1998; Garcia-Anoveros et al., 2001). One of the polyclonal antibodies we generated called ASIC2_EC 1.0, recognizes an epitope in the ectodomain loop identical in both splice variants (Price et al., 2000). We generated several embryonic kidney 293 cell lines (HEK 293 cell) that permanently express a Flag epitope-tagged version of ASIC2a or ASIC2b. Using protein extracts from these cells we could show that the ASIC2_EC 1.0 antibody recognizes a single protein band of the correct size in Western blots from HEK 293 cells that stably express a ASIC2a or ASIC2b cDNA (Fig. 1A). A band of the same size could always be detected using the monoclonal antibody M2 (Brizzard et al., 1994) directed against the Flag epitope of the recombinantly expressed channels (Fig. 1A). The second antibody (ASIC2b_IC 1.0) was directed against the unique N-terminal region of the ASIC2b splice variant (peptides 1–17). In a previous study we could show using the ASIC2_EC 1.0 antibody that ASIC2 channels (potentially both splice variants) are present in around 70% of sensory neurons but the protein is enriched in larger sensory neurons (Price et al., 2000). Here we performed indirect immunofluorescence experiments with both the ASIC2b_IC 1.0 and ASIC2_EC 1.0 antibodies on fixed cultures of adult mouse sensory neurons (normally after 12–16 h in culture). The intensity of ASIC2 specific immu-

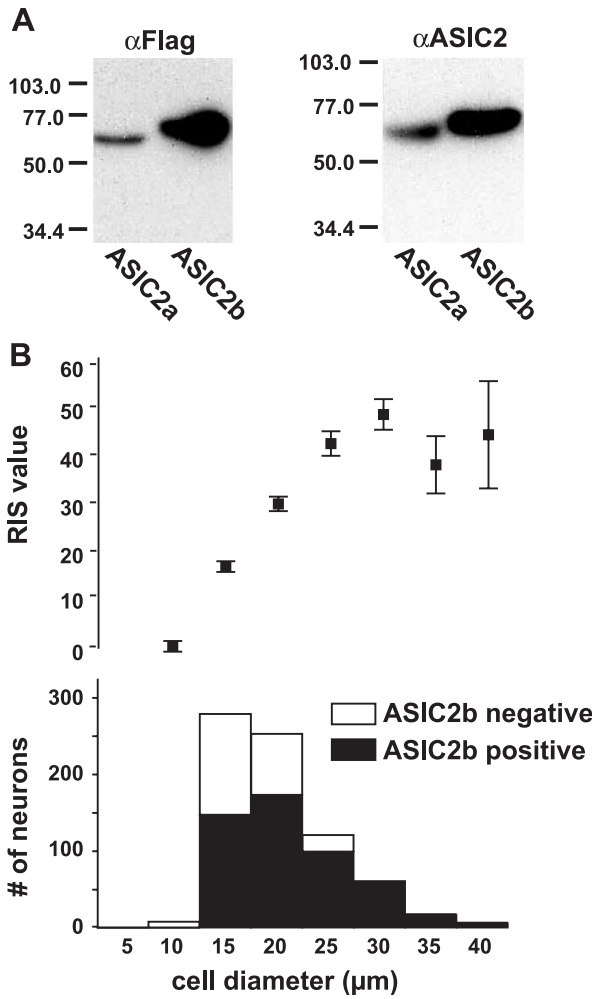

Fig. 1. Expression of recombinant flag-tagged ASIC2a and ASIC2b in HEK 293 cells. (A) Protein bands were detected using the monoclonal antibody M2 against the Flag-epitope (left) or the polyclonal antibody ASIC2_EC 1.0 (right); molecular weight marker in kDa. (B) Distribution of ASIC2b positive and negative wild-type sensory neurons by soma size (bottom). RIS (top) of positively labeled sensory neurons of each diameter bin.

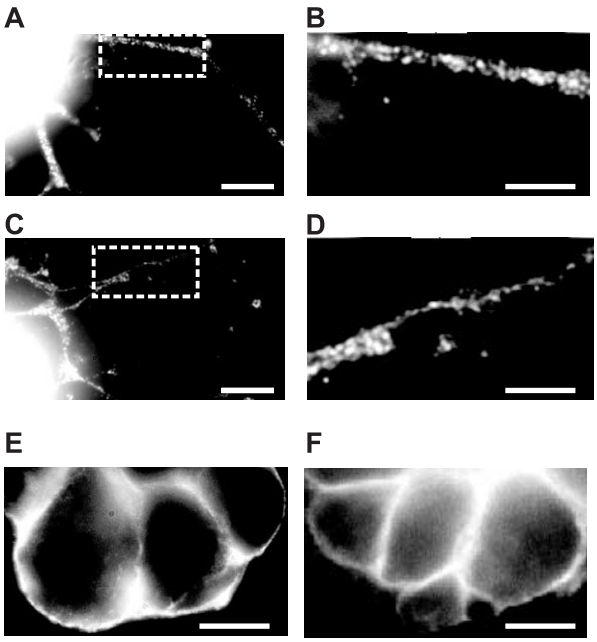

Fig. 2. Fluorescent images of the subcellular localization of ASIC2 in the plasma membrane of sensory neurons (A–D) and in heterologously expressing HEK 293 cells (E, F). Examples of neuronal staining using the antibody ASIC2_EC 1.0 are shown in A, B. Staining obtained with our antibody directed against ASIC2b (ASIC2b_IC 1.0) are shown in C, D. The boxed area in photomicrographs A and C is shown at a higher magnification in B and D, respectively to illustrate the punctate nature of the staining on the neurites of cultured sensory neurons. (E, F) Immunolocalization of ASIC2 in HEK 293 cells using the M2 anti-Flag antibody; scale bar=10 μm in A and C; scale bar=5 μm in B, D–F. Note that at similar magnification the distribution of the ion channel in HEK 293 cell membranes is very different from that in sensory neurons.

nofluorescence for each neuron was measured as RIS (see Experimental Procedures for details) and only cells with RIS values above that seen in cultures taken from ASIC2 knockout mice were counted as positive. In the present study we found that specific immunofluorescence was also detectable in cultured sensory neurons using the antibody directed specifically against the ASIC2b splice variant of the channel (Fig. 1B). The cell size distribution was then plotted and the proportion of positive neurons per bin represented in a stacked histogram (Fig. 1B). In addition the RIS value for positive cells in each bin was also plotted (Fig. 1B). This analysis revealed that most cells (>95%) above 25 μm in diameter were positive for the ASIC2b splice variant and the intensity of staining increased as the cell size increased (Fig. 1B). This pattern of immunofluorescence staining was practically identical to that previously shown using the ASIC2_EC 1.0 antibody that recognizes both variants of the channel (Price et al., 2000).

Using both the ASIC2_EC 1.0 and ASIC2b_IC 1.0 antibodies directed against the ASIC2 channel proteins we noticed that at high magnification neuronal staining in the plasma membrane was not uniform. Instead the pattern of staining was highly punctate on the cell soma and on the neurites (Fig. 2A–D). The fluorescent spots were observed using the ASIC2_EC 1.0 antibody in fixed cells without the use of detergent to permeabilize the plasma membrane. Because this antibody detects ASIC2 protein without needing to penetrate the cell membrane it is likely that the spots represent clustering of ion channels in the membrane and not in vesicles within the neurites. The size of the spots was hard to estimate accurately using light level microscopy but they were likely to average much less than 0.2 μm in diameter (Fig. 2B, D). We used the same procedures to detect epitope tagged ASIC2 channel proteins heterologously expressed in HEK 293 cells. Here staining of cells expressing either variant of ASIC2 revealed evenly distributed membrane-associated fluorescence (Fig. 2E, F). For the remainder of the experiments described in this paper we used the ASIC2_EC 1.0 antibody to detect the ASIC2 channel protein.

Colocalization of ASIC2 with markers of nociceptive sensory neurons

We observed that ASIC2 immunofluorescence is present in around 60% of small diameter neurons (Fig. 1; Price et al., 2000). This population, which is thought to comprise mostly nociceptors, can be divided into two populations separated by their binding of the plant IB_4 from *G. simplicifolia* (Snider and McMahon, 1998; Stucky and Lewin, 1999). Here we asked if the ASIC2-positive neurons could be an identical population to either the IB_4-positive or IB_4-negative population of small diameter sensory neurons. We observed that in mouse sensory neuron cultures grown for 16 h the same proportion (approximately 65%) of IB_4-positive and IB_4-negative sensory neurons (cell diameters <25 μm) were also positive for ASIC2 (670 cells measured from four cultures; Fig. 3). However, the RIS values of IB_4-positive neurons was nearly twice as high as that observed in IB_4-negative ($P<0.001$, unpaired *t*-test) small diameter neurons (Fig. 3B). These data suggest that functional differences between IB_4-positive and IB_4-negative nociceptive sensory neurons might be reflected by differential expression of ASIC2 channels. Moreover, since IB_4-positive neurons are nociceptors (Stucky and Lewin, 1999; Dirajlal et al., 2003) it was interesting that the RIS values in IB_4-positive neurons was still lower than the mean RIS values found in presumptive non-nociceptors, i.e. large diameter IB_4-negative neurons (see example in Fig. 3A).

Regulation of ASIC2 channel expression by BDNF

It has been estimated from several studies that between 6% and 33% of sensory neurons express full-length trkB receptors, the major receptor for BDNF and NT-4 (Mu et al., 1993; McMahon et al., 1994; Karchewski et al., 1999; Stucky et al., 2002). Sensory neurons expressing trkB have been reported to be of medium to large size (McMahon et al., 1994; Karchewski et al., 1999) but a much larger proportion of sensory neurons normally express ASIC2 channels (Fig. 2). Thus if BDNF regulates the expression of ASIC2 channels this should only happen in a subset of sensory neurons that possess trkB receptors. We therefore decided to see if a small reduction in ASIC2 mRNA

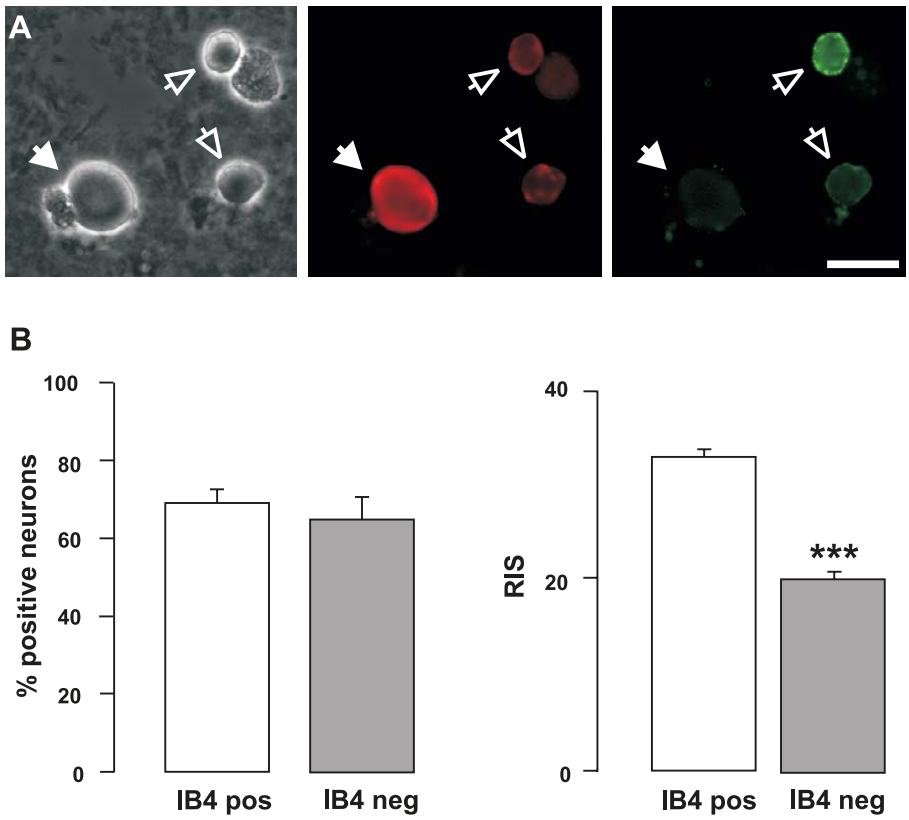

Fig. 3. ASIC2 channels are localized with IB_4-positive and negative sensory neurons. (A) Phase-contrast (left) and fluorescent (middle and right) images of a field of sensory neurons labeled with ASIC2_EC 1.0 (middle) and IB_4-FITC (right); scale bar=30 μm. Solid arrow, ASIC2 positive, IB_4-negative sensory neuron; hollow arrow, ASIC2 negative, IB_4-positive neuron. (B) Proportion of IB_4-positive and IB_4-negative nociceptors positively labeled for ASIC2 (left) and their RIS value (right). Note in this example (A, middle) that the large sensory neuron that is not positive for the nociceptor marker IB_4 exhibits much brighter ASIC2 immunofluorescence than adjacent small neurons.

could be detected in DRGs taken from adult BDNF compared with wild-type controls. To address this issue we used two different techniques. In the first experiment we isolated mRNA from the DRGs taken from age matched wild-type and BDNF+/−mice. Digoxigenin-labeled DNA was generated from the DRG-derived mRNA and hybridized with membranes spotted with an ASIC2 cDNA and a control GAPDH cDNA. Quantification of the hybridization signal from two independent experiments indicated that the amount of ASIC2 mRNA was reduced by 30% and 24% compared with the control GAPDH mRNA level in DRGs prepared from BDNF deficient mice. In the second experiment we used real time PCR to try and detect expression differences in ASIC2a and ASIC2b transcripts between wild-type and BDNF deficient DRG (Fig. 4A). Four independent experiments were carried out using ASIC2a and ASIC2b specific primers and expression calculated as a percentage of a control gene (prenylcysteine oxidase) that we found not to be down-regulated in the DRG of BDNF deficient mice. In this experiment a significant reduction in ASIC2a mRNA was observed in BDNF deficient DRG (mean reduction 24.4%, $P>0.05$, paired *t*-test; Fig. 4A). The ASIC2b mRNA level also appeared to be reduced (mean reduction 20%, $P=0.06$, paired *t*-test) but this did not reach statistical significance (Fig. 4A). The reduced expression of the ASIC2 gene in ganglia from BDNF+/−mice is consistent with the regulation of the ASIC2 mRNA transcripts via trkB signaling in just a subset of sensory neurons that normally express the ASIC2 channel protein.

We decided to use immunostaining of ASIC2 in sensory neuron cultures to examine more closely which subset of neurons shows NT-regulated ASIC2 protein expression. Our antibody did not work so reliably in tissue sections of the DRG as background fluorescence obscured specific staining in sub-populations of neurons. To look for changes in the basal expression of the channel we have therefore compared the ASIC2 immunofluorescence in parallel sensory neuron cultures taken from BDNF+/− mice and their wild-type littermates. We reasoned that if BDNF is responsible for maintaining normal expression of the channel then the proportion or intensity of immunostaining should differ among specific cell populations taken from mice where BDNF availability is reduced. We have therefore chosen to measure ASIC2 immunofluorescence using the ASIC2_EC 1.0 antibody in cultured neurons grouped into three size categories: small (<15 μm), medium (15 to ≤25 μm) and large (>25 μm) diameter. In all experiments two parameters were tested: the percentage of cells positive for ASIC2 per size category and their mean RIS value.

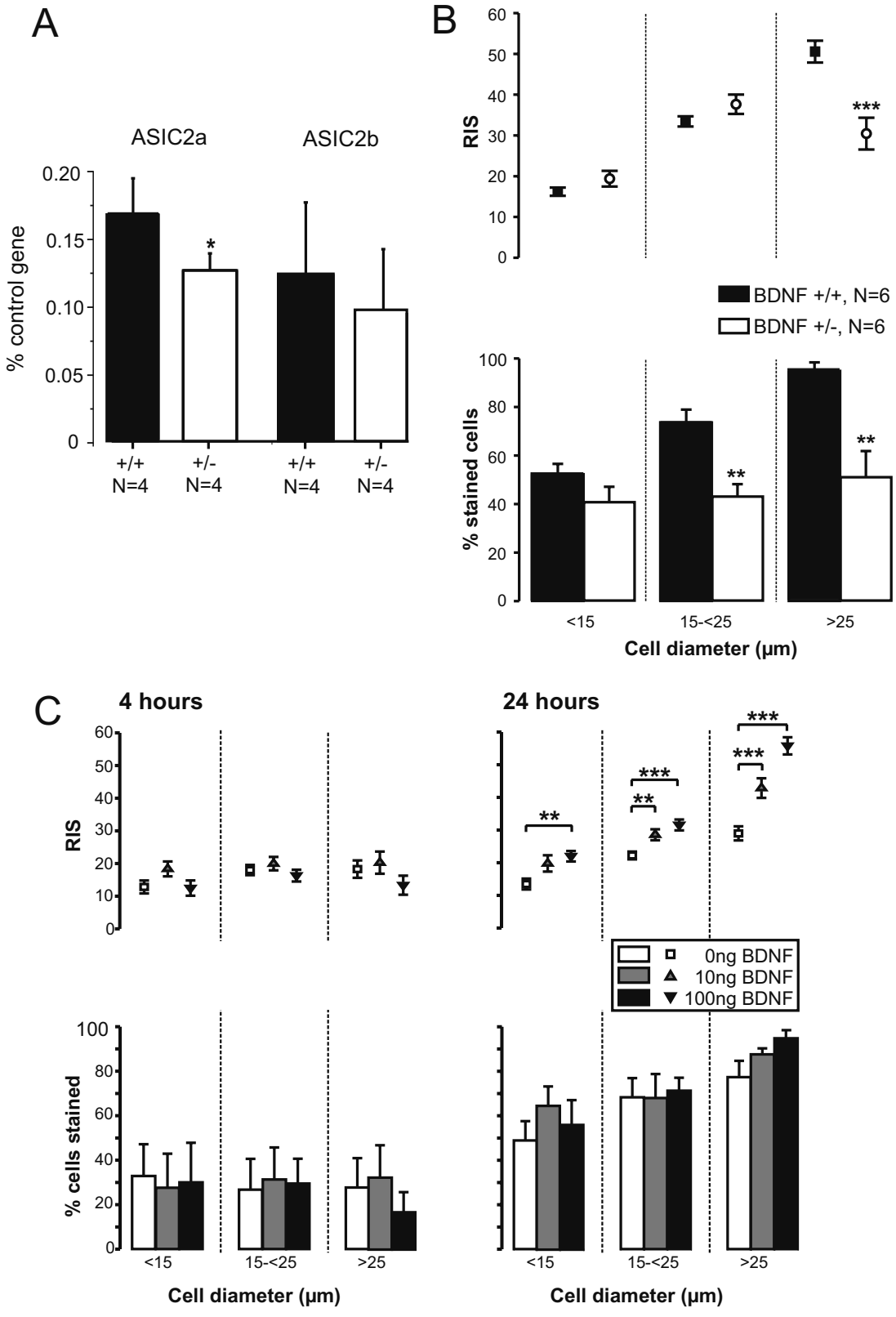
A
ASIC2a
ASIC2b
% control gene
0.20
0.15
0.10
0.05
0
*
+/+ N=4
+/- N=4
+/+ N=4
+/- N=4
B
RIS
60
50
40
30
20
10
0
***
BDNF +/+, N=6
BDNF +/-, N=6
% stained cells
100
80
60
40
20
0
**
**
<15
15-<25
>25
Cell diameter (µm)
C
4 hours
24 hours
RIS
**
**
***
***
***
0ng BDNF
10ng BDNF
100ng BDNF
% cells stained
<15
15-<25
>25
Cell diameter (µm)
Cell diameter (µm)

Fig. 4.

In primary neuronal cultures from wild-type mice 12–16 h after plating we consistently found that around 50% of small neurons, 75% of medium diameter and almost 100% of large diameter neurons were positive for ASIC2 (Fig. 4B). The mean RIS value also increased with the neuronal diameter (Fig. 4B). These data collected independently from our previous studies (Price et al., 2000) confirm again that most large cells express ASIC2 and their ASIC2 specific fluorescence intensity, measured as RIS, is on average more than three times brighter than in small sensory neurons (Fig. 3 and Fig. 4B). We then compared parallel cultures of neurons obtained from BDNF+/−mice with cultures from their wild-type littermates. Here a significantly lower percentage of large and medium sensory neurons were scored positive for ASIC2 compared with wild-type measured at 12–16 h: 51.0±11% of large neurons in BDNF±compared with 94.7±3.5% in wild-type neurons, $P<0.005$; *t*-test and 43.2±5% of medium sized neurons in BDNF± compared with 73.5±5.3% in wild-type, $P<0.01$; *t*-test. No significant difference was observed in the percentage of ASIC2-positive small diameter sensory neurons in wild-type and BDNF+/−cultures (Fig. 4B). Furthermore, the RIS value for large diameter sensory neurons but not that of medium and small diameter neurons differed significantly between the two genotypes: RIS value was 30.4±3.9 in BDNF+/−cultures compared with 50.3±2.7 in wild-type cultures ($P<0.0001$; *t*-test). These data indicate that *in vivo* deprivation of BDNF can lead to a highly selective reduction in the expression in ASIC2 channels by large diameter sensory neurons measured *in vitro*.

Our previous studies indicated that *in vivo* treatment of BDNF deficient mice with recombinant BDNF could rescue mechanosensory deficits observed in these mice (Carroll et al., 1998). We have therefore asked whether the loss of ASIC2 immunostaining could also be rescued *in vitro* by supplementary BDNF. We compared sensory neuron cultures fromBDNF±mice grown for 4 and 24 h in the presence of 0, 10 or 100 ng/ml of BDNF in the medium. The proportion of cells positive for ASIC2 for each cell size group after 4 h in culture was somewhat lower than that found at 12–16 h in the experiments described above (Fig. 4C). In addition the RIS value was low for medium and large diameter neurons (16–18 RIS units compared with 30–35 in 12–16 h cultures; Fig. 4C). However, even in the absence of added BDNF the proportion of positive neurons increased in each of the three size categories at 24 h. The increase was greatest for cells of medium and large size, although these differences did not reach statistical significance. Interestingly, even in the absence of BDNF the RIS increased in medium and large sized neurons over time: medium diameter neurons from 18.1±1.6 at 4 h to 24.5±2.2 at 24 h, and large diameter neurons from 18.3±2.7 at 4 h to 33.2±3.1 at 24 h ($P<0.05$, and $P<0.001$ respectively, Student's *t*-test). The effects of 10 and 100 ng/ml of BDNF were however evident in that after 24 h the intensity of ASIC2 staining was greatly increased compared with non-BDNF treated cultures. The effects of BDNF on the intensity of neuronal staining (RIS value) were dramatic and dose dependent and was most marked for medium and large diameter neurons (Fig. 4C). Thus addition of 100 ng/ml BNDF to the medium induced a pattern of ASIC2 immunofluorescence indistinguishable from the data obtained from wild-type cultures (compare Fig. 4B and C). The RIS for neurons in the three neuronal diameter categories was significantly higher than for neurons grown in the absence of added BDNF ($P<0.005$ for all comparisons). Incubating cell cultures from BDNF+/− animals in medium containing the lower 10 ng/ml dose of BDNF resulted in intermediate expression of ASIC2. Only medium and large diameter sensory neurons had significantly increased RIS values when compared with non-BDNF treated cultures (Fig. 4C). We also carried out experiments on normal wild-type sensory neurons ($n=4$ cultures and mice) treated with increasing concentrations of BDNF (0, 100 and 500 ng/ml). Compared with medium and large cells in control untreated cultures (RIS 33.6±4.0), neurons treated with 100 or 500 ng of BDNF were much more intensely stained (RIS 51.2±4.4 and 48.3±4.3, respectively $P<0.01$ Student's *t*-test). This substantial (150%) increase in staining intensity in the presence of BDNF again suggested that BDNF is able to directly regulate the expression of ASIC2 in sensory neurons of the DRG. In these experiments 100 ng/ml appeared to have a saturating effect on the intensity of ASIC2 staining.

However, the mechanism by which BDNF regulates the ASIC2 specific immunofluorescence still remains open from the above experiments. Thus BDNF might conceivably influence ASIC2 immunofluorescence by regulating the intracellular distribution or turnover of ASIC2 ion channels. To address this question we incubated sensory neurons fromBDNF±mice in the presence of a maximal dose of BDNF (100 ng/ml) for 12 h and in parallel cultures added either a blocker of transcription or a blocker of translation (cycloheximide and actinomycin, each at 10 μg/ml, respectively). In the presence of both cycloheximide and actinomycin the rescue of ASIC2 immunofluorescence to wild-type levels by BDNF ($n=6$ experiments) was completely

Fig. 4. ASIC2 is down-regulated after BDNF reduction. In panel A the results of real-time PCR experiments to detect ASIC2a and ASIC2b in the DRG taken from wild-type and BDNF+/−mice are shown. Expression was plotted as percentage of a control gene (a housekeeping gene called prenylcysteine oxidase) and the graph represents the means of four separate experiments (* indicates $P<0.05$, Student's *t*-test). (B) Histogram of the proportion of small, medium and large diameter sensory neurons positive for ASIC2 (bottom) and their RIS values (top) in neuronal cultures from BDNF+/−animals and wild-type littermates. In panel C the rescue of ASIC2 protein expression by BDNF is shown. Sensory neurons from BDNF+/−mice cultured for 4 h (left) and 24 h (right) in the presence of 0, 10 and 100 ng/ml of BDNF added in the culture medium. Histograms of the proportion of sensory neurons in each diameter bin positively labeled for ASIC2 (bottom) and their RIS values (top). No increase of ASIC2 protein expression was noted after 4 h of growing neurons in the presence of 100 ng/ml BDNF (left). After 24 h neuronal cultures grown in the presence of 100 ng/ml BDNF express the largest amounts of ASIC2 when compared with cells cultured in 0 ng/ml BDNF containing medium (right). The amount of ASIC2 measured in cultures grown in the presence of 10 ng/ml BDNF was intermediate.

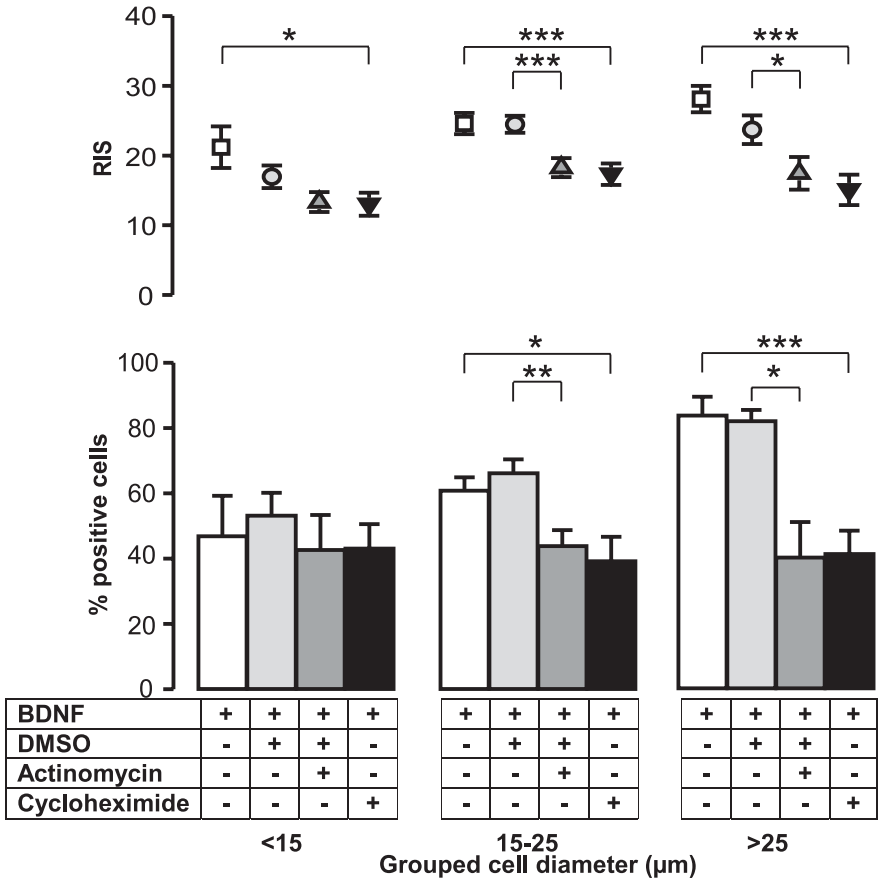

Fig. 5. New ASIC2 expression requires new transcription and translation. Applying 10 μg/ml transcription and translation blockers, cycloheximide and actinomycin respectively could inhibit ASIC2 expression in sensory neurons from BDNF+/−mice cultured in the presence of 100 ng/ml BDNF for 12 h. The application of actinomycin was controlled for by growing sensory neurons in the presence of 0.05% DMSO alone. The histogram (bottom) shows the proportion of neurons positive for ASIC2 in each diameter bin and the plot their RIS values (top).

blocked (Fig. 5). Both these drugs were effective at blocking both the increase in the proportion of large cells counted as positive for ASIC2 and in preventing the large increases in ASIC2 fluorescence intensity (RIS values) in medium and large diameter neurons. To dissolve actinomycin in the culture medium DMSO (0.05%) must be added but addition of DMSO without the drug did not block the effects of BDNF on ASIC2 fluorescence (Fig. 5). Thus we can conclude that new transcription and new protein synthesis is necessary for the effects of BDNF on ASIC2 channel expression in sensory neurons.

Role of NT-4 in regulating ASIC2 channel expression in sensory neurons

NT-4 like BDNF also activates the trkB tyrosine kinase receptor. Neurons taken from control NT-4 wild-type mice (129S3; catalogue number 002448; Jackson Laboratories, USA) displayed qualitatively the same pattern of ASIC2-immunofluorescence as has been observed in cultures from BDNF wild-type mice (compare with Fig. 4B). In primary neuron cultures from adult NT-4 −/− mice (>7 weeks) there was a large drop in the proportion of medium and large diameter sensory neurons positive for ASIC2; only 7.9±15% of medium and 10.0±17% of large diameter neurons were positive for ASIC2 (statistically different from controls, $P<0.001$ for medium, $P<0.0001$ for large neurons; Student's *t*-test). The ASIC2-positive neurons from older NT-4 −/− mice also expressed significantly lower amounts of the protein as reflected in lower RIS values (see Fig. 6A). We have recently observed that D-hair receptor sensory neurons in NT-4 knockout mice only die later in older mice (>7 week; Stucky et al., 2002; Shin et al., 2003; Dubreuil et al., 2004). We thus performed experiments on cultures of sensory neurons taken from younger 5 week-old NT-4 −/− animals. Here in marked contrast to older NT-4 deficient mice the proportion of ASIC2 positive medium and large diameter neurons was not different from control cultures 73.8±7.3% and 91.2±3.6%, respectively when measured *in vitro*. The mean RIS values for each cell size bin also did not differ from controls (Fig. 6A).

NT-3

We have previously demonstrated that NT-3 is required for the survival of a substantial number of low threshold mechanoreceptors with cell bodies in the DRG of the mouse (Airaksinen et al., 1996). We also therefore made cultures of sensory neurons from adult NT-3 deficient mice and

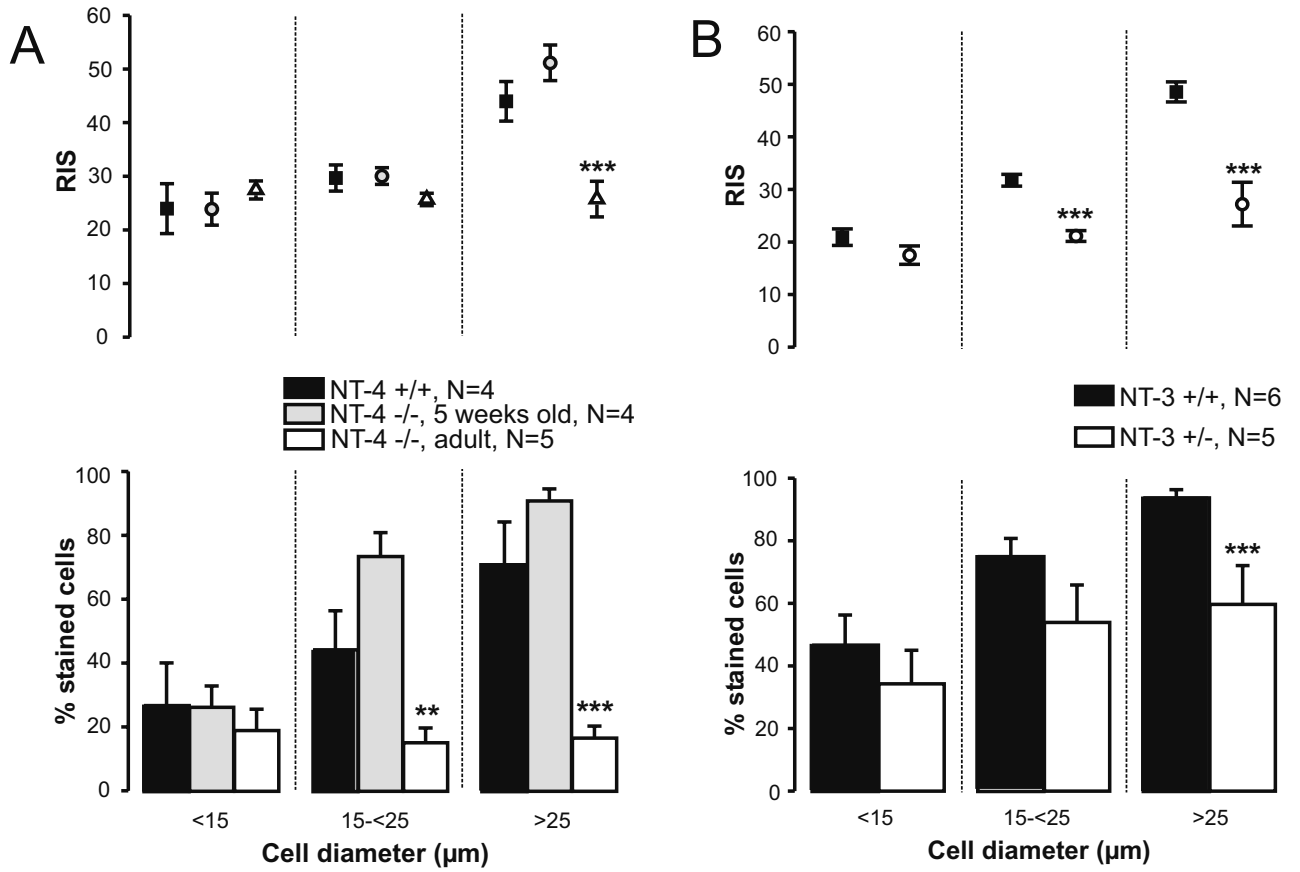

Fig. 6. Loss of NT-4 or NT-3 leads to a loss of ASIC2 immunostaining in cultured sensory neurons. (A) Histogram of the proportion of small, medium and large diameter sensory neurons positive for ASIC2 (bottom) and their RIS values (top) in neuronal cultures from adult NT-4 −/−, 5-week-old NT-4 −/− and wild-type mice. (B) Histogram of the proportion of neurons positively labeled for ASIC2 in each diameter bin (bottom) and their RIS values (top) in neuronal cultures from adult NT-3+/−animals and their wild-type littermates.

looked at the levels of ASIC2 protein *in vitro* with the same techniques. A significantly smaller proportion of large diameter sensory neurons from NT-3+/−mice, 60.0±12.2% were positive for ASIC2 compared with 93.8±2.6% of neurons from wild-type littermates ($P<0.05$; Student's *t*-test). The RIS values were also significantly lower in large diameter cells (NT3 ±=27.3±4.2, NT3 wt=48.4±1.9, $P<0.0001$; Student's *t*-test). The proportion of medium-sized neurons positive for ASIC2 was not significantly different from controls although the RIS value for the positive cells was significantly reduced in neurons from NT-3+/− mice (Fig. 6B). Overall the changes in ASIC2 expression in DRG neurons from NT-3 deficient mice were similar in magnitude to those observed in BDNF deficient mice but less than that observed in NT-4 knockout mice.

DISCUSSION

In this study we have shown that the sensory mechanotransduction channel subunit ASIC2 is highly expressed by large diameter sensory neurons (Price et al., 2000; Garcia-Anoveros et al., 2001; Alvarez de la Rosa et al., 2002). Using knockout mice deficient in neurotrophic factors we have been able to show that the expression of this channel in large- and medium-sized sensory neurons is regulated by the availability of NTs. The ASIC2 gene appears to be a novel target gene of BDNF signaling. The mRNA for ASIC2 is significantly reduced in BDNF+/−DRG compared with wild type; furthermore, addition of exogenous BDNF could rescue the reduced ASIC2 protein expression in cultured sensory neurons and this rescue was dependent on new transcription and protein synthesis. Our data suggest that the sensory mechanotransduction deficits observed in SAM in BDNF deficient mice (Carroll et al., 1998) can be in part explained by reduced expression of the ASIC2 channel protein.

It is now well established that the ASIC2 channel protein is expressed by low threshold mechanoreceptors in the DRG. Two lines of evidence support this conclusion. First using antibodies directed against the ASIC2 channel that recognizes both splice variants (Price et al., 2000) or just ASIC2a (Garcia-Anoveros et al., 2001), named in the latter study BNaC1α, the protein has been localized to the receptor endings of low threshold mechanoreceptors in the skin (Price et al., 2000; Garcia-Anoveros et al., 2001). Receptor endings found to stain positive for ASIC2 channels include palisade or lanceolate endings on hair follicles as well as in Merkel cell neurite complexes (Price et al., 2000; Garcia-Anoveros et al., 2001). In addition using

sensory neuron cultures where soma diameter can be unambiguously and accurately measured it was found that all large diameter neurons were positive for ASIC2 channels (Price et al., 2000). Furthermore, large cells exhibit up to three times more immunofluorescence than do small diameter sensory neurons (Price et al., 2000; Fig. 1 and example in Fig. 3). Here we confirm these findings with a new antibody directed specifically against the ASIC2b (BNaCβ) variant of the channel. When this antibody was used in acute cultured sensory neurons it reproduced an almost identical staining pattern to that seen with the antibody recognizing both splice variants (Price et al., 2000). The relative preponderance of ASIC2a and ASIC2b splice variants in sensory neurons at the single cell level remains an open issue. We have previously detected ASIC2b channel transcripts in sensory neurons using *in situ* hybridization, but we could not detect ASIC2a transcripts with the same methodology although the transcript could be amplified from DRG mRNA with RT-PCR (Price et al., 2000). In this study we found equivalent amounts of ASIC2a and 2b in the DRG using real time PCR (Fig. 4A). Garcia-Añoveras and colleagues (2001) were able to detect ASIC2a (named by them BNaCα) with *in situ* hybridization and immunocytochemistry. In yet another study it was found that ASIC2a and ASIC2b are co-localized in the same medium- and large-sized cells (Alvarez de la Rosa et al., 2002).

With both our polyclonal antibodies we noticed that the distribution of channels on the plasma membrane of sensory neurons in culture was highly punctate. Such a distribution of the immunostaining was not seen in HEK 293 cells expressing recombinant ASIC2 channels (Fig. 2). This suggests that some other proteins might function in neurons to cluster ASIC2 channels. The punctate distribution of ASIC2 channels on sensory neurons is reminiscent of that observed using an antiserum directed against another putative mechanotransduction gene the MEC-2 related protein stomatin (Mannsfeldt et al., 1999). Recombinant stomatin expressed in HEK cells itself forms punctate domains in the plasma membrane (Eilers et al., unpublished observations) and thus stomatin or stomatin-like proteins might function in sensory neurons to cluster ASIC2 ion channels. Interestingly, studies of the highly related MEC-4 mechanotransduction channel in *C. elegans* also indicate that it is normally distributed in a punctate fashion along mechanosensitive neurites *in vivo* (Chelur et al., 2002). Studies of the ASIC2 or stomatin protein in sensory nerve endings in the skin have not at the light level revealed appreciable clustering of ion channels (Fricke et al., 2000; Price et al., 2000; Garcia-Anoveros et al., 2001) but it is possible that such resolution is not easy to achieve in tissue sections where the detectable antigen abundance is low.

ASIC2 specific immunofluorescence in large and medium diameter sensory neurons was of lower intensity and often undetectable in neurons obtained from BDNF heterozygote mice. We have taken this as evidence that BDNF availability *in vivo* regulates the expression of the channel in cells expressing the BDNF receptor trkB. The fact that significant changes in expression were only observed in large and medium diameter neurons is consistent with the fact that trkB positive neurons are predominantly of large and medium diameter (Mu et al., 1993; McMahon et al., 1994; Karchewski et al., 1999; Stucky et al., 2002). Interestingly, when we added BDNF to cultured neurons taken from BDNF deficient mice we could observe a time- and dose-dependent rescue of ASIC2 channel expression to levels seen in wild-type cultures. Even in the absence of added BDNF ASIC2 channel expression increased in cultures between 4 and 24 h after plating (Fig. 5). We do not know what factor in the culture medium might mediate such an effect but it is known that BDNF and NT-4 are synthesized by adult sensory neurons (Schecterson and Bothwell, 1992; Wetmore and Olson, 1995; Apfel et al., 1996; Heppenstall and Lewin, 2001). It is thus tempting to speculate that secretion of BDNF might act in autocrine or paracrine fashion to regulate ASIC2 channel expression under these conditions (Acheson and Lindsay, 1996). Using blockers of new transcription and protein synthesis we could show that the rescue effect of BDNF on ASIC2 channel expression could be prevented. These experiments show that transcription of the ASIC2 gene is under the control of BDNF signaling. By performing dot blots from DRG mRNA derived from BDNF deficient mice we also observed that the transcript was less abundant in ganglia taken from BDNF deficient mice. More quantitative experiments using real time PCR on ASIC2a and 2b mRNA derived from BDNF mutant ganglia indicated the same reduction in ASIC message as indicated from dot blots (Fig. 4A). Many neurons that express ASIC2 channels do not possess trkB receptors, e.g. small diameter IB_4-positive neurons (Wright and Snider, 1995; Apfel et al., 1996; Snider and McMahon, 1998) and we suppose that other factors besides BDNF might regulate ASIC2 protein expression in these neurons. Interestingly, ASIC2 positive small diameter neurons were equally likely to be IB_4-positive or negative although fluorescence intensity was higher in IB_4-positive neurons (Fig. 3). The function of the ASIC2 channel in small diameter neurons is however still unclear as in our previous study with ASIC2 knockout mice no deficits in C-fiber mechanosensitivity or proton sensitivity was found (Price et al., 2000).

In BDNF+/− mice we have previously observed no substantial loss of sensory neurons of any particular physiological type (Carroll et al., 1998; Heppenstall and Lewin, 2001). Thus we have attributed the loss of ASIC2 channel expression in sensory neurons from BDNF deficient mice as due to a down-regulation of expression in individual neurons. In contrast mice deficient in NT-4 and NT-3 do lose substantial numbers of sensory neurons with large myelinated axons and medium to large soma diameters. A schematic diagram of sensory neurons losses and deficits in BDNF, NT-4 and NT-3 deficient mice is shown in Fig. 7. In NT-4 knockout mice D-hair mechanoreceptors almost completely disappear and this loss is dependent on trkB receptor signaling (Minichiello et al., 1998; Stucky et al., 2002). In the present experiments we observed a very substantial loss of ASIC2 staining in sensory neurons

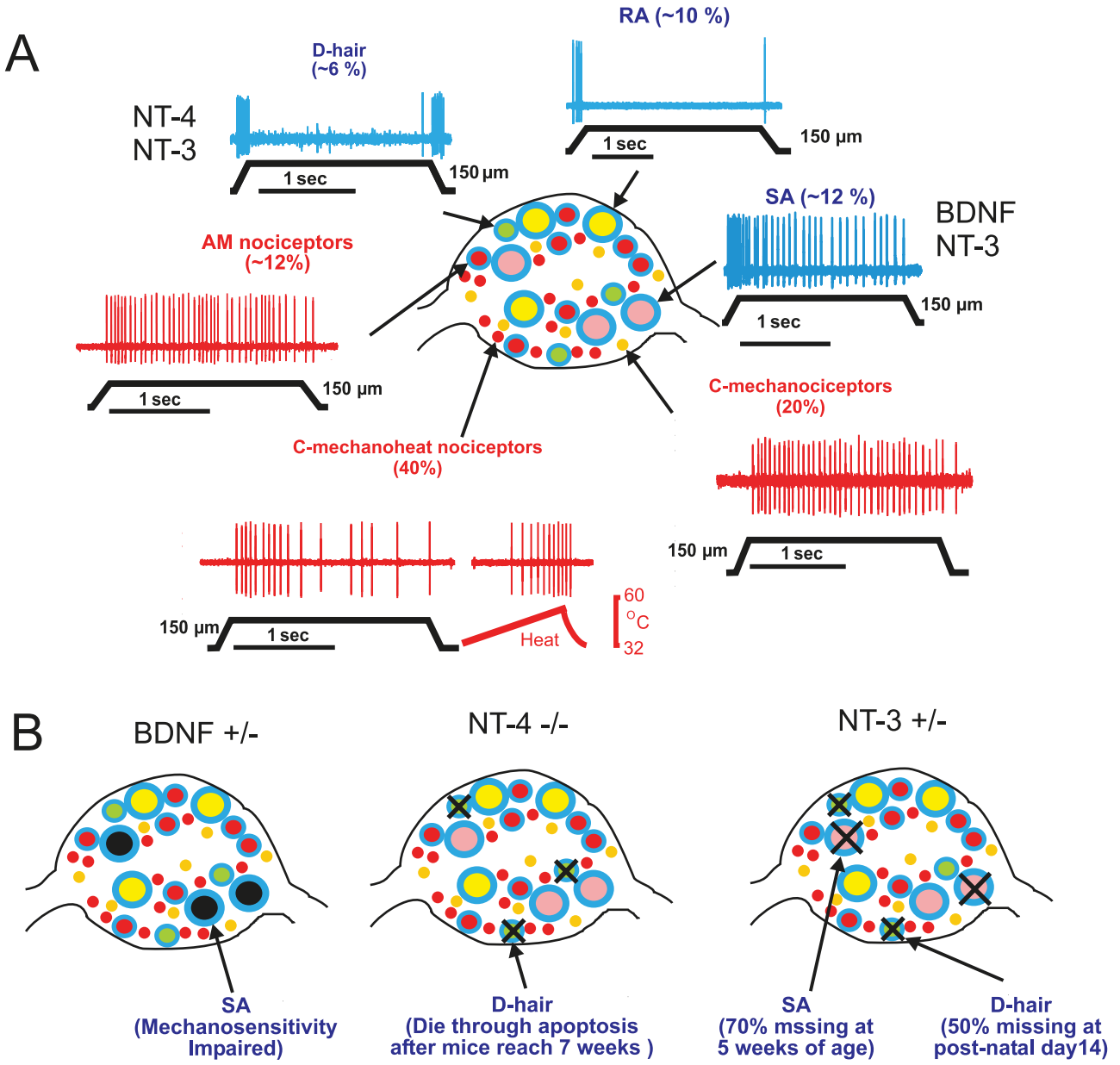

Fig. 7. Summary of functional losses in the dorsal root ganglion of BDNF, NT-4 and NT-3 mutant mice. (A) Typical response properties of mouse mechanoreceptors from the saphenous nerve to a standardized 2 s ramp and hold indentation stimulus of 150 µm are shown. In the center a schematic diagram of the dorsal root ganglia with color-coded sensory neurons depicts the approximate cell size and myelination state of the different mechanoreceptors (thick cell wall indicates myelinated neurons). The mechanoreceptors can be divided into two major groups; those depicted in blue are low threshold mechanoreceptors that all robustly respond to the ramp phase of the stimulus skin. Mechanoreceptors depicted in red are nociceptive and respond primarily to the static phase of the stimulus. The approximate incidence (% of total cutaneous sensory neurons) is indicated next to its name. The mechanoreceptor types that are influenced *in vivo* by NT deficiency are SAM and D-hair receptors. In B the precise deficits in cutaneous mechanoreceptors that have been described in the BDNF ±, NT-4 −/− and NT-3±are illustrated. No substantial loss of large diameter cutaneous was noted inBDNF±mice (left) but cell loss, indicated by crossed-out cells, has been described fro NT-4 (middle) and NT-3 (right) deficient mice.

taken from NT-4 knockout mice that cannot be explained solely by the loss of a small population of D-hair mechanoreceptors (Fig. 6). It therefore appears likely that NT-4 as well as BDNF is required to maintain normal ASIC2 channel expression in mechanoreceptive sensory neurons. The effects of NT-4 deprivation were strongly dependent on the age of the mice examined as mice younger than 7 weeks old displayed no loss of ASIC2 immunofluorescence compared with controls (Fig. 6). This finding correlates with the fact that D-hair mechanoreceptors only become dependent on NT-4 after 7 weeks of age (Stucky et al., 2002). In contrast to BDNF deficient mice it was previously found that the sensitivity of slowly and rapidly adapting cutaneous mechanoreceptors is unaffected in the absence of

NT-4 (Carroll et al., 1998; Stucky et al., 1998). Thus down-regulation of the ASIC2 channel protein in the absence of NT-4 is not sufficient to affect function to the same extent as in ASIC2 knockout mice (Price et al., 2000).

In NT-3 deficient mice it is known that both SAM and D-hair mechanoreceptors are substantially depleted from cutaneous nerves (Airaksinen et al., 1996). In addition a substantial loss of muscle proprioceptors is also observed in NT-3 heterozygote mice (Kucera et al., 1995). The loss of ASIC2 immunostaining in medium and large diameter sensory neurons is consistent with the interpretation that the mechanoreceptive neurons that die normally express larger amounts of the ASIC2 channel than the remaining neurons. It is however still possible that NT-3 like BDNF can directly regulate transcription of the ASIC2 channel gene via activation of trk receptors.

In summary, we have provided evidence that BDNF can regulate the expression of the ASIC2 gene in subsets of medium and large diameter sensory neurons that are mechanoreceptors. The functional phenotypes of mechanoreceptors in ASIC2 knockout mice are substantially similar to that observed in BDNF deficient mice. Thus it is logical to assume that the insensitivity of SAM to low threshold mechanical stimuli that we described in BDNF deficient mice can be partially explained by BDNF signaling regulating the ASIC2 gene. However, there must remain still more BDNF-regulated sensory mechanotransduction genes to be discovered in order to fully explain the sensory phenotype of BDNF deficient mice.

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