The mammalian sodium channel BNC1 is required for normal touch sensation

Margaret P. Price*,†, Gary R. Lewin*,‡, Sabrina L. McIvor*, Chun Cheng*, Jinghui Xie*, Christopher J. Benson*, Ron F. Hrstka, Howard Hughes Medical Institute, Departments of *Internal Medicine, ‡Anesthesia, §Obstetrics and Gynecology, and ¶Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, USA

§Growth Factors and Regeneration Group, Department of Neuroscience, Max-Delbrück-Center for Molecular Medicine, Robert-Rösle-Straße 10, D-13302 Berlin-Buch, Germany

* These authors contributed equally to this work

Of the vertebrate senses, touch is the least understood at the molecular level. The ion channels that form the core of the mechanosensory complex and confer touch sensitivity remain unknown1–3. However, the similarity of the brain sodium channel 1 (BNC1)4–6 to nematode proteins involved in mechanotransduction indicated that it might be a part of such a mechanosensor7,8. Here we show that disrupting the mouse BNC1 gene markedly reduces the sensitivity of a specific component of mechanosensation: low-threshold rapidly adapting mechanoreceptors. In rodent hairy skin these mechanoreceptors are excited by hair movement5. Consistent with this function, we found BNC1 in the lanceolate nerve endings that lie adjacent to and surround the hair follicle5. Although this has been proposed to have a role in pH sensing9,10, the acid-evoked current in cultured sensory neurons and the response of acid-stimulated nociceptors were normal in BNC1 null mice. These data identify the BNC1 channel as essential for the normal detection of light touch and indicate that BNC1 may be a central component of a mechanosensory complex.

Stimulation of sensory receptors in the skin generates a variety of sensations, including touch and pain11. However, these are the least understood vertebrate senses at the cellular and molecular level. Specifically, the ion channel receptors that convert mechanical stimuli into electrical ones in vertebrate nerve endings remain unknown1. A clue to their identity came from the discovery of MEC-4 and MEC-10 in a genetic screen of touch-insensitive Caenorhabditis elegans mutants12. MEC-4 and MEC-10 belong to the DEG/ENaC family of proteins, which associate as homo- and heteromultimers to form voltage-insensitive Na+ channels13. BNC1 (ref. 4) (also called MDEG (ref. 5), BNaC1 (ref. 6) and ASIC2 (ref. 7)) is one of several mammalian DEG/ENaC channels. The

References

fact that mutation of a specific residue in the nematode channels\textsuperscript{13,14} and in BNC\textsuperscript{1} (ref. 5) generates a constitutively active channel indicated that they might have functional similarity. Therefore, we hypothesized that BNC\textsuperscript{1} forms part of the mechanosensory complex in mammals. BNC\textsuperscript{1} might also be involved in acid-induced nociception\textsuperscript{10,11}; this hypothesis is based on the finding that when BNC\textsuperscript{1} is expressed in heterologous systems it is activated by acidic extracellular pH. To test these hypotheses, we disrupted the mouse BNC\textsuperscript{1} gene.

We inserted a neo cassette to replace two exons encoding the second transmembrane domain (M2), the most highly conserved region of DEG/ENaC proteins and one that contributes to the channel pore\textsuperscript{12}. BNC\textsuperscript{1} has two splice variants, BNC\textsuperscript{1a} and 1b (also called MDEG\textsuperscript{1} and MDEG\textsuperscript{2}), which have different amino termini and first transmembrane domains\textsuperscript{4,5,15}. An earlier report indicated that BNC\textsuperscript{1b} is expressed in dorsal root ganglion (DRG)\textsuperscript{15}; our data indicate that BNC\textsuperscript{1b} is the predominant transcript, although by polymerase chain reaction with reverse transcription (RT-PCR) we detected both 1a and 1b transcripts in wild-type DRG (data not shown). Our targeting strategy ensured disruption of both splice variants (Fig. 1a). BNC\textsuperscript{1–/–} heterozygous and wild-type mice (Fig. 1b) were produced in the expected 1:2:1 Mendelian ratio, BNC\textsuperscript{1–/–} mice had normal appearance, growth, size, temperature, fertility and life span, and showed no obvious behavioural abnormalities. Using RT-PCR with primers from the targeted region and northern analysis using a probe common to both splice variants, we failed to detect transcripts in the brain of BNC\textsuperscript{1} null animals (Fig. 1c, d).

In DRG, most large sensory neurons are low-threshold mechanoreceptors that detect innocuous stimuli such as light touch, whereas most small neurons are nociceptive, detecting noxious mechanical, thermal or chemical stimuli\textsuperscript{2,3}. In situ hybridization and immunostaining detected transcripts and protein, respectively, in both large and small DRG neurons of wild-type animals but not.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{example.png}
\caption{Mechanosensitivity of wild-type and BNC\textsuperscript{1} null mice. a, Example of RA mechanoreceptor from wild-type and BNC\textsuperscript{1} null mouse. b, Stimulus–response function of RA mechanoreceptor from wild-type (closed squares, \(n = 48\)) and null mice (open circles, \(n = 27\)), showing flattening of stimulus–response function of BNC\textsuperscript{1} null mice (\(P < 0.005\) two-way analysis of variance (ANOVA)). At each stimulus point above 5 \(\mu\)m, response in BNC\textsuperscript{1} null mice was significantly different from wild type, \(P < 0.05\), unpaired \(t\)-test. c, Stimulus–response function of SA mechanoreceptor fibres. Null mice showed significant decrease in sensitivity of SA mechanoreceptors (\(n = 55\)) compared with wild type (\(n = 87\)) (\(P < 0.05\) two-way ANOVA). d–f, Stimulus–response function from slowly conducting D-hair afferents and myelinated and non-myelinated nociceptors (\(n = 18–43\) for each fibre type and genotype). Frequency of firing was calculated over the entire 10 s of stimulus except in the case of D-hair afferents where only the first 2 s were used. g, Median force required to activate low threshold mechanoreceptors. VFT is von Frey threshold.}
\end{figure}
in null animals (Fig. 1c, f). In cultured DRG neurons both large and small neurons stained, but the largest neurons tended to show the most intense staining (Fig. 1g). Expression of BNC1 in small and large DRG sensory neurons is consistent with a role in both mechanosensation and acid-evoked nociception.

To learn whether BNC1 has a role in mechanosensation, we examined the response of single sensory neurons to mechanical stimuli using an in vitro skin-nerve preparation16. Myelinated and unmyelinated single fibres were classified into rapidly adapting (RA) and slowly adapting (SA) low-threshold mechanoreceptors, D-hair receptors (D-hair), A-fibre mechano-nociceptors (AM) and C-fibre mechano-nociceptors26. We investigated the relationship between the strength of the stimulus and the neural response using a standard ascending series of mechanical stimuli. Increasing stimuli produced increasing numbers of action potentials in wild-type RA mechanoreceptors (Fig. 2a, b). In BNC1 null mice, we found a reduced sensitivity of the low-threshold RA mechanoreceptors; their stimulus-response function showed a reduced discharge frequency and flattening in the absence of the BNC1 channel. Thus, wild-type afferent neurons displayed their highest dynamic sensitivity between 5 and 20 μm displacement, whereas different from null mice showed a much reduced response in this range. We also found a smaller but significant shift in the stimulus-response function of SA mechanoreceptors (Fig. 2c). The proportion of fast conducting A-fibres classified as SA and RA was identical in BNC1+/+ (33% RA, 67% SA, n = 82) and BNC1+/− mice (34% and 66%, n = 132). The stimulus-response functions of the rapidly adapting D-hair mechanoreceptors and the AM and C-fibre mechano-nociceptors were not affected by the absence of BNC1 (Fig. 2d–f). Using von Frey hairs, we found that the minimal force required to elicit action potentials was not different for RA mechanoreceptors, SA mechanoreceptors or other fibre types in BNC1 null mice (Fig. 2g and data not shown); however, this method may not be sensitive enough to detect a small shift in threshold, as it is limited by the number of hairs used in the lower range.

Sensory mechanotransduction is a two-step process19. First, mechanically gated ion channels open, generating a graded receptor potential. Second, the membrane depolarization initiates action potentials. We tested whether the absence of the BNC1 channel might affect the second process. We current-clamped cultured large-diameter sensory neurons and studied cells with narrow spikes characteristic of low-threshold mechanoreceptors30. The amount of current injection required to initiate action potentials was identical in BNC1+/+ and BNC1−/− neurons (Fig. 3a, b). Thus the reduced mechanosensitivity of low-threshold mechanoreceptors in BNC1 null mutants probably reflects a requirement for the channel in the generation of the mechanically gated receptor potential. However, the size of sensory nerve endings in skin precludes voltage-clamp recordings of receptor potentials in situ.

We also tested the role of BNC1 in acid-evoked responses. Acidic pH activates heterologously expressed BNC1α and the related neuronal channels, acid-sensing ion channel (ASIC) and dorsal root acid-sensing ion channel (DRASIC)10. Earlier studies showed acid-evoked Na+ currents in cultured large-diameter sensory neurons20. Therefore, we tested the contribution of BNC1 to these currents. Whole-cell currents from large wild-type and null DRG neurons showed similar H+-gated transient and sustained currents (Fig. 3c). The pH sensitivity was the same, and the currents were blocked by amiloride (Fig. 3d, e). A similar fraction of wild-type (59%, n = 29) and null neurons (55%, n = 33) showed H+-gated currents. In addition, small-diameter neurons (<25 μm) showed the same inward current induced by pH5 in BNC1+/+ and BNC1−/− neurons (7/16+/+ neurons, 210 ± 48 pA; 5/14−/− neurons, 238 ± 101 pA). These data indicate that BNC1 makes little contribution to H+-gated currents in sensory DRG neurons.

Earlier studies have shown that acid applied to the receptive terminals of many polymodal nociceptors, or C-mechano-heat (C-MH) receptors, evokes a sustained discharge23. We found that the proportion of C-fibres classified as C-MH and C-mechano-nociceptors was not different between wild-type (64%, n = 39) and BNC1 null mice (54%, n = 45, p > 0.2 χ² test). In both types of mouse, acid application (pH 5.0) induced a sustained discharge in

**Figure 3** Response of sensory neurons to current injection, acidic pH and heat.

**a.** Examples of current-evoked action potentials from large-diameter sensory neurons.

**b.** Amount of current injection needed for large-diameter neurons (wild type 35 ± 1.8 μm and null 33.5 ± 1.7 μm), mean ± s.e.m., n = 5–8 per genotype.

**c.** pH-dependent whole-cell currents. Voltage was −70 mV; downward deflection indicates inward current. Cells were 35.7 ± 0.5 μm for wild-type and 35.8 μm for BNC1−/− mice.

**d.** Effect of pH on transient current, n = 4–6 cells obtained from at least two mice.

**e.** Effect of amiloride on current activated by pH5; n = 3–5 at each point.

**f.** Response of single C-fibre nociceptors to pH5. Data are from C-MH fibres (n = 9); the only fibres found to be consistently excited by low pH.

**g.** Response of C-MH fibres to a brief noxious heat stimulus. Skin temperature was raised transiently to over 50°C and then rapidly fell (n = 24–25 each genotype).
around 35% of C-MH fibres. The time course and magnitude of response in C-MH fibres were identical (Fig. 3f). Consistent with earlier reports\(^5\), we saw no response to the pH 5 stimulus in C-M fibres or low-threshold mechanoreceptors from mice of either genotype. We also found that the response to a standardized noxious heat stimulus was the same in wild-type and null mice (Fig. 3g). These data indicate that sustained excitation of polymodal C-fibres by acidic pH does not require the BNC1 channel.

Rapidly adapting mechanoreceptors are stimulated by hair movement\(^6\); thus we hypothesized that BNC1 would be localized in nerve endings around hairs (Fig. 4, inset)\(^7\). We found BNC1 expressed in the palisades of lanceolate nerve endings. These fibres surround the hair follicle, running longitudinally to the shaft at and below the level of the sebaceous glands (Fig. 4a). Thus, they are well positioned to detect movement of the hair shaft\(^8\). In contrast, immunostaining was weaker and less consistent in the pilo-Ruffini endings spiralling around the lanceolate nerve endings. Lanceolate endings did not stain in BNC1 null mice. The number and appearance of lanceolate fibres was similar in BNC1\(^\text{+/+}\) and BNC1\(^\text{-/-}\) animals as evaluated by blinded observers (Fig. 4b, c and Table 1).

The role of BNC1 in mechanosensation indicates evolutionary conservation of function within the DEG/ENaC channel family. A new Drosophila channel, NompC, unrelated to the DEG/ENaC family, has been described as necessary for mechanotransduction in ciliated mechanosensory neurons\(^23\). It is unclear whether mechanotransduction in sensory hair cells requires channels from the DEG/ENaC family; of interest, the related αENaC channel is not required for normal hair cell mechanotransduction\(^24\).

Studies in humans and nonhuman primates indicate that the dynamic sensitivity of both RA and SA mechanoreceptors is critical for the perception and discrimination of touch sensation, especially that associated with hair movement and skin indentation\(^2\). Our data show that BNC1 is specifically required for the sensitivity of these low-threshold mechanoreceptors. We speculate that mechanical deformation of sensory terminations, for example in hair follicles, may activate a channel complex that includes BNC1, thereby eliciting a depolarizing action current that triggers action potentials. It is also clear, however, that RA and SA mechanoreceptors retain some mechanosensitivity in the absence of BNC1. Perhaps the mechanosensory channel complex is a heteromultimer composed of BNC1 and other DEG/ENaC subunits, and the absence of BNC1 precludes a normal graded response to mechanical stimuli. Of interest, the related DEG/ENaC channels β- and γENaC have been localized in rats to lanceolate nerve endings of the vibrissal follicle and Merkel cell and lamellated corpuses in the foot pad\(^25,28\). Moreover, it seems likely that additional proteins tether BNC1 to the extracellular matrix and intracellular cytoskeleton, thereby transmitting applied stresses to the channel\(^2,5,8\). Our identification of a molecular component for a specific cutaneous sensory modality in vertebrates is an essential first step toward a molecular description of touch.

### Methods

#### Construction of targeting vector and generation of BNC1 mutant mice

We disrupted the BNC1 gene by homologous recombination using the P1339 LoxP-PGKNeoneo vector (a gift from T. J. Ley, Washington University) and standard methods\(^2\). We characterized mice generated from two different embryonic stem cell lines. For RT-PCR, we extracted mouse brain total RNA and used the following primers directed to the targeted region: BNC1 5\(\text{TTG CAG GAC AAG 3}\) and 5\(\text{CTG GAT GAT GG 3}\). Openlab software (Improvision) was used for quantification of the mRNA.

#### Immunocytochemistry and in situ hybridization

We used a rabbit polyclonal anti-BNC1 antibody against amino acids 186–421 (numbering as in ref. 4) to recognize both BNC1a and BNC1b. DRG (10 μm) and hair skin (50 μm) frozen sections were prepared as described\(^27\). Sections were incubated with affinity-purified BNC1 antibody and Rhodamine Red X-conjugated donkey anti-rabbit IgG. In hairy skin, sections were also stained with a nerve cocktail consisting of hair anti-neurofilament and sheep anti-65 kDa. We used a Biorad MRC1024 laser-scanning confocal microscope to examine samples.

For DRG neurons in culture, we used a rabbit polyclonal antibody (raised against amino acids 382–396, as in ref. 4) as described\(^29\). Openlab software (Improvement) was used for quantification of the immunofluorescence. Ten individual cultures from five animals per genotype were done in parallel. Staining intensity of BNC1\(^\text{-/-}\) cultures was comparable to that of peptide controls. The mean fluorescence value for relative intensity was corrected for this background staining. For each cell, mean diameter was calculated and the proportion of cells positive for BNC1 was calculated for each bin.

#### Culture of DRG neurons

DRG from 2–3-month-old mice were dissociated and cultured as described for rat\(^30\) with the modification that ganglia were digested with papain (10 U/ml\(^2\)) and collagenase/dispase solution (1.67 μg/ml\(^2\)) separately for 15 min at 37°C. For the study of small-diameter neurons, we prepared cultures of adult DRG neurons as described\(^4\). No nerve growth factor or other neurotrophins were added to the medium.

---

**Figure 4** Immunostaining of BNC1 around guard hair follicles. a, Oblique sections through hair follicle of a wild-type mouse; micrographs were obtained by confocal microscopy with stacking of 12 images, each 0.8 μm thick. Green indicates staining of BNC1 and red nerves. b–c, Complementary images from BNC1\(^\text{+/+}\) and BNC1\(^\text{-/-}\) hair follicles. Inset, neural network innervating guard hair follicle. h, hair follicle shaft; g, sebaceous gland. Sebaceous glands stain nonspecifically with primary and secondary antibodies.

**Table 1** Evaluation of lanceolate fibres of hair follicles

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Hair diameter (μm)</th>
<th>Number of lanceolate fibres per 16 μm</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNC1(^\text{+/+})</td>
<td>20</td>
<td>10.1 ± 0.9</td>
<td>6.5 ± 1.2</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>BNC1(^\text{-/-})</td>
<td>23</td>
<td>10.6 ± 0.9</td>
<td>7.2 ± 1.5</td>
<td>1.0 ± 0.8</td>
</tr>
</tbody>
</table>

Diameters of hairs and number of lanceolate fibres in 16 μm were counted on guard hair follicles. Data are mean ± s.d. Appearance of fibres was graded from 2 (good morphology with regular distribution and density) to 0 (morphology irregular, fibres not distributed evenly, and low density). Observers were blinded to genotype.
Whole-cell patch-clamp recording

DRG neurons were bathed in extracellular solution containing, in mM: 128 NaCl, 5 MgCl2, 1.8 CaCl2, 5.4 KCl, 5.6 glucose, 20 HEPES, pH 7.4. Acidic solutions were buffered by 10 mM MES and 10 mM HEPES. Pipettes contained, in mM: 100 KCl, 30 NaCl, 2 MgCl2, 10 EGTA, 20 HEPES and 1 ATP. All cells exhibited depolarization-activated currents. Methods to assess pH-gated currents in small diameter neurons and membrane properties of isolated mechanoreceptors were as described24. Under current-clamp conditions action potentials were evoked with current injection. Neurons with broad action potentials displaying a hump on the falling phase (a marker of nociceptor type neurons) were tested for pH 5-induced currents. The amount of current required to initiate action potentials in large diameter neurons with narrow spikes was also measured. Cells were used from at least four mice per genotype in each experimental series.

Single fibre recording

We used an in vitro skin/nerve preparation to record from functionally single primary afferents in micro-dissected teased filaments of the superficial nerve as described27,28. A standard ascending series of displacement stimuli were applied to the receptive field at 30-s intervals. Each displacement was maintained for 10 s, and neurons that maintained a discharge throughout the stimulus were characterized as SA mechanoreceptors. Those neurons that responded only at the beginning and end of the 10-s stimulus were classified as RA mechanoreceptors. Each stimulus–response function started at threshold as the probe was adjusted so that the first 5 μm displacement evoked spikes. A group of C-fibre nociceptor neurons (axonal conduction velocity <1.0 m s−1) was characterized with the same stimulus series (n = 46). C-fibres were not divided into C-MA and C-fibres29,30. All groups have essentially identical mechanosensory transduction channels, pH-gated currents, and sensitization to mechanical stimulation of nociceptors in rat skin, bladder pressures. Immunohistochemical studies localize P2X3 to innervation density. Thus, P2X3 is critical for peripheral pain responses and afferent pathways controlling urinary bladder innervation. Therefore, P2X3-deficient mice exhibit a profound urinary bladder hyporeflexia, characterized by decreased voiding frequency and increased bladder capacity, but normal bladder pressures. Immunohistochemical studies localize P2X3 to nerve fibres innervating the urinary bladder of wild-type mice, and show that loss of P2X3 does not alter sensory nerve innervation density. Thus, P2X3 is critical for peripheral pain responses and afferent pathways controlling urinary bladder volume reflexes. Antagonists to P2X3 may therefore have therapeutic potential in the treatment of disorders of urine storage and voiding such as overactive bladder.

The in-channel subunit P2X3 is one of seven known subunits that form homomeric and heteromeric receptors for ATP. Uniquely, P2X3 is expressed by a subgroup of small sensory neurons of the dorsal root and cranial ganglia. ATP and α,β-Me-ATP (a
We carried out PFGE essentially as described and included forming units (p.f.u.) per cell and collected at the indicated hour post-infection (h.p.i.).

Antibodies
We visualized viral replication centres by staining for DBP with either a mouse monoclonal antibody (B-86) or rabbit polyclonal antiserum. Antibodies were purchased from Novus (NB1, ATML) and Genetex (Mre11-12D7, Rad50-13B3), Santa Cruz (haemagglutinin (A), Hu6, DNA-PKcs, NBS1-S343) and Roche (6d5). Antiserum to E606, pTP, PML, RPA70 and DBP were gifts from P. Branton, J. Schack, T. Sternsdorf, T. Melendez, A. Levine and P. van der Vliet, respectively. All secondary antibodies were from Jackson Laboratories.

Viruses, infections, PFGE and western blotting
The E4 mutant viruses have been described and were propagated and titred by plaque assays on C6/36 (Aedes aegypti) or Vero-derived E4-complementing cell line 162 (Wild-type Ad5 type 5 Ad5 and Ad110 were propagated in human 293 cells. We purified all viruses by two sequential rounds of ultracentrifugation in CaCl2 gradients and stored them in 40% glycerol at −20°C. Hela cells were infected with a multiplicity of infection (MOI) of 25 plaque-forming units (p.f.u.) per cell and collected at the indicated hour post-infection (h.p.i.). Other cell lines were infected with MOIs of 25–100 and collected between 48 and 72 h.p.i. We carried out PFGE essentially as described and included DNA size markers. Western blotting was done as described.

Immunofluorescence
Cells were grown on glass coverslips in 24-well dishes and infected with wild-type or mutant viruses at an MOI of 25 p.f.u. per cell. After 8–18 h, the cells were washed with PBS and fixed at −20°C for 10 min with ice-cold methanol/acetic acid (1:1). We carried out immunofluorescence essentially as described. In all cases, control staining experiments showed no crossreaction between the fluorophores, and images obtained by staining with individual antibodies were the same as those shown for double-labeling. We stained nuclear DNA with 4',6-diamidino-2-phenylindole (DAPI) and mounted coverslips using Fluormount-G (Southern Biotechnology Associates) or Vectashield (Vector Labs). Immunoreactivity was visualized by using a Nikon microscope in conjunction with a CCD camera (Cooke Sensicam), or by deconvolution using a DeltaVision microscopy system with software from the supplier (Applied Precision). Images were obtained in double or triple excitation mode and processed using Slidebook and Adobe Photoshop.

Methods
Cell lines
All cell lines were obtained from the American Type Culture Collection or the Coriell Institute apart from ATLD1 and ATLD3 cells (a gift from J. Petreni), ATM cells (a gift from Y. Shiolo and J. Engelhardt), and cells with mutant ligase IV (180BRM; a gift from H. Wang and G. Iliakis). To complement cells, the complementary DNAs for Mre11 and NBS1 were amplified by PCR and cloned under control of the CMV promoter in a modified version of the pCLNC retrovirus vector. Retroviruses were generated by transfecting gag/pol packaging cell lines, together with a plasmid expressing the VSV-G envelope protein. ATLD or NBS cells were infected with retrovirus supernatants and selected in 900 μg/ml G418. We assessed expression of Mre11 and NBS1 by immunoblotting and used pools of respective clones for complementation experiments. The mre11-3 mutant contains the HD129/130LV mutation generated by site-directed mutagenesis using QuickChange technology.

Vaccines, infections, PFGE and western blotting