

A T-type calcium channel required for normal function of a mammalian mechanoreceptor

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The dorsal root ganglia (DRG) contain a variety of mechanoreceptors, but no molecular markers uniquely identify specific mechanoreceptor subtypes. We have used DNA microarrays and subtracted cDNA libraries to isolate genes that are specifically expressed by one type of mouse mechanoreceptor. The T-type calcium channel $Ca_v3.2$ was exclusively expressed in the DRG by D-hair receptors, a very sensitive mechanoreceptor. Pharmacological blockade of T-type calcium channels indicated that this channel may be essential for normal D-hair receptor excitability including mechanosensitivity. This is the first evidence that a calcium channel is required for normal function of a vertebrate mechanoreceptor.

Somatic sensory mechanoreceptors that transduce sensations of cutaneous touch and pain have their cell somas in the DRG. Despite the importance of cutaneous sensibility, the molecular and physiological mechanisms that underlie mechanoreceptor function are still poorly understood¹. The skin is innervated by diverse mechanoreceptor types that vary widely in their response thresholds, dynamic sensitivities and adaptation properties^{2–7}. Different mechanoreceptor types have highly specific connectivities in the spinal cord and brain stem^{8,9}. Consistent with this, psychophysical studies have shown that different qualities of tactile sensation are produced by the activation of different types of cutaneous mechanoreceptors¹⁰.

Degenerin-like sodium channels, which are insensitive to changes in membrane voltage¹¹, seem to participate in the transduction of mechanical stimuli by specific types of vertebrate mechanoreceptors^{12,13}. For example, in mice, rapidly adapting mechanoreceptors have a reduced sensitivity to indentation stimuli when the gene encoding the brain sodium channel or acid sensitive ion channel 2 (BNC1/ASIC2) is inactivated¹². Such channels might therefore be good markers of mechanoreceptor types. BNC1/ASIC2 channels are, however, expressed in a very large subpopulation of sensory neurons (~70%)^{12,14}, which is not coincident with the smaller subset of mechanoreceptors affected in *BNC1/ASIC2* knockout mice.

Alternative candidates for mechanoreceptor-specific genes have arisen from the discovery of genes whose expression is restricted to the DRG^{15–17}. One such gene encodes the noxious-heat and capsaicin gated channel VR1 (*TRP-V1*)¹⁸. The TRP-V1 channel is a very good marker of noxious heat-sensitive nociceptive neurons¹⁹. Of all the DRG-specific genes, however, none is expressed uniquely by a physiologically defined mechanoreceptor type. Thus molecular markers that reliably distinguish between different types of mechanoreceptors have yet to be identified.

The D-hair receptor is a mechanoreceptor that has the lowest mechanical threshold and the highest dynamic sensitivity of all vertebrate mechanoreceptors^{3–7}. We have designed a genetic strategy to isolate genes that are specifically expressed by D-hair receptors. We used

the neurotrophin 4 knockout mouse (*NT-4^{-/-}*; also known as *Ntf5*)²⁰, in which the DRG is devoid of D-hair receptor neurons in the mature animal^{21,22}, to screen for differentially expressed transcripts. Using murine oligonucleotide microarrays together with subtractive cDNA libraries, we identified a T-type calcium channel gene, *Ca_v3.2* (refs. 23,24), that is expressed within the DRG only by D-hair receptor neurons. Furthermore, selective pharmacological blockade of T-type calcium channels indicates that *Ca_v3.2* may be required for normal D-hair receptor mechanosensitivity.

RESULTS

Identification of D-hair receptor specific genes

To identify D-hair-specific genes, we took advantage of *NT-4^{-/-}* mice because, as adults, they almost completely lack D-hair receptors but have no other sensory deficits^{21,22}. Moreover, D-hair receptors are present in young *NT-4^{-/-}* mice but die off between 7 and 10 weeks²². We looked for genes whose expression is reduced in the DRG in parallel with the loss of D-hair receptors. Such genes should fulfill another criteria: Because D-hair receptors make up only a very small proportion of all DRG neurons (~6%), putative D-hair receptor genes should be expressed specifically in a small number of DRG neurons that disappear in older *NT-4^{-/-}* mice. We used Affymetrix microarrays and subtractive cDNA libraries to screen for the maximal number of candidate genes^{25,26}. Murine microarrays were hybridized with labeled cRNA from DRG taken from wild-type, young *NT-4^{-/-}* (4–5 weeks old) and old *NT-4^{-/-}* mice. Approximately 35% (~12,600) of all genes were present in both populations. We found 189 genes whose expression was consistently reduced with an appropriate temporal profile (see **Supplementary Table 1** online).

Because D-hair receptors are relatively rare, the microarray experiment might not reliably detect changes in transcripts that are specifically expressed by D-hair receptors. We therefore strengthened our screen by using the suppression subtractive hybridization technique²⁶ to generate a subtracted cDNA library of transcripts that are downregulated in the DRG of older *NT-4^{-/-}* mice (**Supplementary Fig. 1**

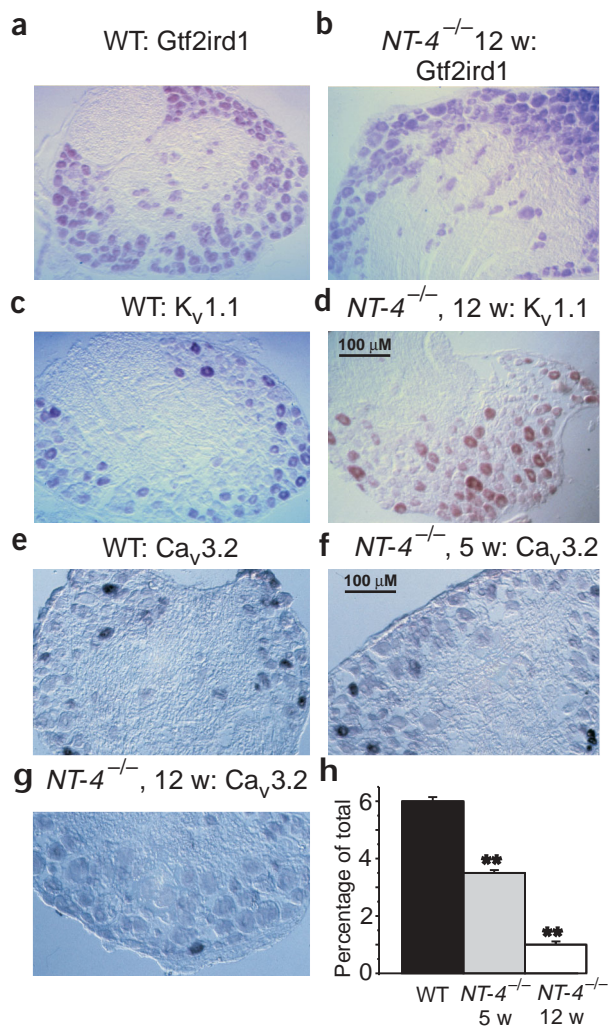


Figure 1 Gene expression in D-hair receptors from DRG of wild-type (WT) and *NT-4*^{-/-} mice. **(a)** A digoxigenin-labeled probe for the gene encoding transcription factor *Gtf2ird1* labeled all DRG neurons. **(b)** The pattern of expression was not different in 12-week-old (12 w) *NT-4*^{-/-} mice. **(c,d)** A probe directed against *K_v1.1* shows strong expression in a subset of medium- and large-diameter neurons in WT and *NT-4*^{-/-} mice. *Ca_v3.2* is expressed in a subset of medium-sized DRG neurons in **(e)** WT mice, and the number of *Ca_v3.2*-positive neurons is slightly reduced in **(f)** 5-week-old (5 w) *NT-4*^{-/-} mice and is dramatically reduced in **(g)** old (12 w) *NT-4*^{-/-} mice. **(h)** *Ca_v3.2*-positive neurons in WT, young and old *NT-4*^{-/-} mice (3 animals per group). Significant differences (***P* < 0.001, unpaired *t*-test) are indicated. Positive neurons were counted as a proportion of the unstained neurons with a clear nucleus as viewed under phase contrast.

in medium and large neurons in wild-type DRG (Fig. 1c), which is consistent with a recent report²⁸. We did not, however, notice any major change in the number of neurons that were positive for *K_v1.1* in *NT-4*^{-/-} mice as compared with wild type (Fig. 1d). Real-time PCR experiments showed that *K_v1.1* mRNA was considerably downregulated in older *NT-4*^{-/-} DRG (more than fourfold) but not in the 5-week-old *NT-4*^{-/-} DRG (Table 1 and Fig. 2). This is good evidence that D-hair receptors express *K_v1.1* at high levels but that its expression is not restricted to this receptor type.

Only two of the genes studied with *in situ* hybridization exactly matched our expression criteria for D-hair specificity. One of these was the gene encoding receptor tyrosine kinase *TrkB* (also known as *Ntrk2*), the cellular receptor for NT-4. Cells that are strongly positive for *TrkB* largely disappear with a time course that is coincident with the loss of D-hair receptors²². The second gene encoded the T-type calcium channel *Ca_v3.2* (refs. 23,24), a low voltage-activated calcium channel (Fig. 1e–g). Counts of the number of cells that were positive for *Ca_v3.2* mRNA clearly indicated an age-dependent loss of these neurons (Fig. 1h). Quantitative real-time PCR confirmed the age dependence of the changes in mRNA for *K_v1.1*, *TrkB* and *Ca_v3.2* (Fig. 2).

We also examined the expression of *Ca_v3.1* and *Ca_v3.3*, the other two known calcium channel genes that generate a T-type current when expressed²⁴. *Ca_v3.1* mRNA did not change under any conditions; *Ca_v3.3* transcripts were reduced, albeit with a time course that did not match the loss of D-hair receptors (Fig. 2). *Ca_v3.2* mRNA showed the greatest decrease in adult *NT-4*^{-/-} DRG when compared with wild-type and young *NT-4*^{-/-} DRG, confirming the results of expression profiling and *in situ* hybridization studies (Fig. 2). Other D-hair specific genes might have been missed for technical reasons. Nevertheless, our screening strategy was successful in showing that genes with a D-hair-specific expression pattern do exist.

Function of *Ca_v3.2* in mechanotransduction

Whole-cell patch-clamp studies of isolated DRG neurons have demonstrated that a subpopulation of medium-sized neurons displays unusually large T-type currents^{29,30}. Because D-hair receptors are lost in *NT-4*^{-/-} mice and because our data show that neurons that express high amounts of *Ca_v3.2* are lost, it is possible that medium-sized neurons with giant T-type currents may be D-hair receptors. One distinguishing feature of low-threshold mechanoreceptive sensory neurons is that they have a very narrow action potential, whereas nociceptive neurons have broader action potentials that display a prominent hump on the falling phase^{7,31,32}. Because it is difficult to measure pharmacologically isolated calcium currents and to obtain records of action potentials from the same sensory neuron using whole-cell patch-clamp techniques, we instead determined whether the voltage for half-maximal activation of voltage-gated inward currents shifts to

online). The DNA microarray data indicated that differentially expressed transcripts are rare in the DRG of *NT-4*^{-/-} mice (~189 of 12,600 or 1.5%); consistent with this, we found that a large part of the subtractive cDNA library contained non-differentially expressed transcripts (data not shown). To identify the transcripts present, we hybridized labeled cRNA generated from the subtracted library on the microarray chips and found that approximately 1,000 genes were present. We reasoned that the 28 transcripts found in both the microarray experiment and the subtractive library would be much less likely to represent background non-regulated genes. A flow chart of the screen and the further characterization of the 28 candidate genes are summarized (Supplementary Fig. 1 online).

We used the polymerase chain reaction after reverse transcription of RNA (RT-PCR) to clone DNA probes from DRG mRNA, which were used to generate digoxigenin-labeled cRNA probes for *in situ* hybridization experiments (Fig. 1 and Table 1). We could verify the downregulation of 12 of 18 (66%) of these genes in the *NT-4*^{-/-} DRG using quantitative real-time PCR (Table 1). For most of the 22 genes for which *in situ* hybridization experiments were carried out, expression was observed in all sensory neurons in the DRG with no apparent enrichment in any one subtype (for example, *Gtf2ird1*; Fig. 1a and Table 1). The expression pattern of *Gtf2ird1* was also not obviously changed in the DRG of *NT-4*^{-/-} mice (Fig. 1b).

One of the regulated genes listed in Table 1 encodes the shaker-related potassium channel *K_v1.1* (ref. 27). This channel was expressed

a more positive value in the presence of 1 μM mibefradil, a selective T-type calcium channel antagonist³³. Indeed, a significant shift toward more positive voltages for activation was observed in a sample of medium-sized sensory neurons with narrow action potentials (<2 ms half-peak amplitude width). Only a small and nonsignificant shift was, however, observed in medium-sized sensory neurons with broader action potentials (Fig. 3a). This methodology is limited because D-hair receptors are not uniquely identifiable from their action potential configuration, as other low-threshold mechanoreceptors also have narrow action potentials^{7,31,32}.

To test more directly whether T-type calcium currents contribute to D-hair receptor function, we used an *in vitro* skin-nerve preparation because D-hair receptors can be unequivocally identified only by their conduction velocity and mechanical response properties^{6,12,13}. The presence of $\text{Ca}_v3.2$ might increase the electrical excitability of D-hair receptors, which is supported by our whole-cell patch-clamp recordings on isolated neurons (Fig. 3a). It is more difficult to test this directly at the receptor ending as intracellular voltage-clamp recordings are impossible. We therefore measured spike frequency adaptation, a technique whereby a microelectrode is used to deliver trains of suprathreshold electrical stimuli of increasing frequency to the receptor ending. We tested the spike frequency adaptation of D-hair receptors as well as A δ mechanonociceptor neurons (AM receptors). The latter class of sensory neurons has similar conduction velocities and cell soma diameters to D-hair receptors³² but very different receptor properties²⁻⁷. At low frequencies (<10 Hz), an action potential follows each electrical stimulus in both D-hair and AM receptors (Fig. 3b,c). As the frequency is increased, the percentage of failures steadily increases. When a large number of such recordings were made, it became clear that AM fibers were less able to follow high-frequency trains than were D-hair receptors. The median following frequency for the sample of AM fibers was just over 20 Hz as compared with 100 Hz for D-hair receptors (Fig. 3b,c). We observed a leftward shift in the spike frequency adaptation curve for D-hair receptors in the presence of 3.3 μM mibefradil, which corresponds to a median frequency shift from 100 to 40 Hz (Fig. 3b). In parallel experiments with AM receptors using 3.3 μM of mibefradil, no effect on the spike frequency adapta-

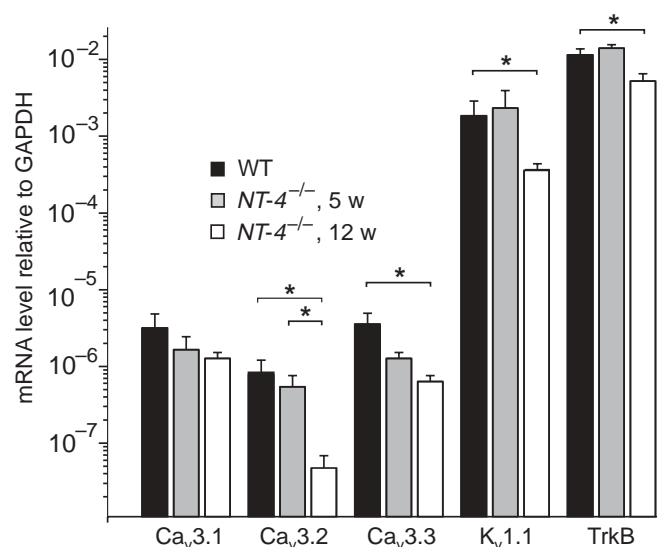


Figure 2 Real-time PCR validation of candidate genes. Specific primers against $\text{Ca}_v3.1$, $\text{Ca}_v3.2$, $\text{Ca}_v3.3$, $\text{K}_v1.1$ and TrkB were used to amplify products from cDNA derived from young (5 w) $\text{NT-4}^{-/-}$ and old (12 w) $\text{NT-4}^{-/-}$ mice. Experiments were done in triplicate and mRNA was isolated from two mice per experiment. Values were normalized to GAPDH mRNA levels. In all comparisons, $*P < 0.001$, Mann-Whitney U -test.

tion curve was observed (Fig. 3c). An even larger shift in the median following frequency of D-hair receptors (to ~20 Hz) was observed with higher doses of mibefradil (15 μM).

High concentrations of mibefradil (50 μM) completely abolished mechanosensitivity within minutes after application to a receptive field that had been isolated with a metal ring (data not shown). The block was in most cases reversible, as some mechanosensitivity returned after 30 min of mibefradil washout. The same doses of mibefradil applied to the nerve trunk leading to the receptive field had no effect on the mechanosensitivity of the recorded fibers (data not

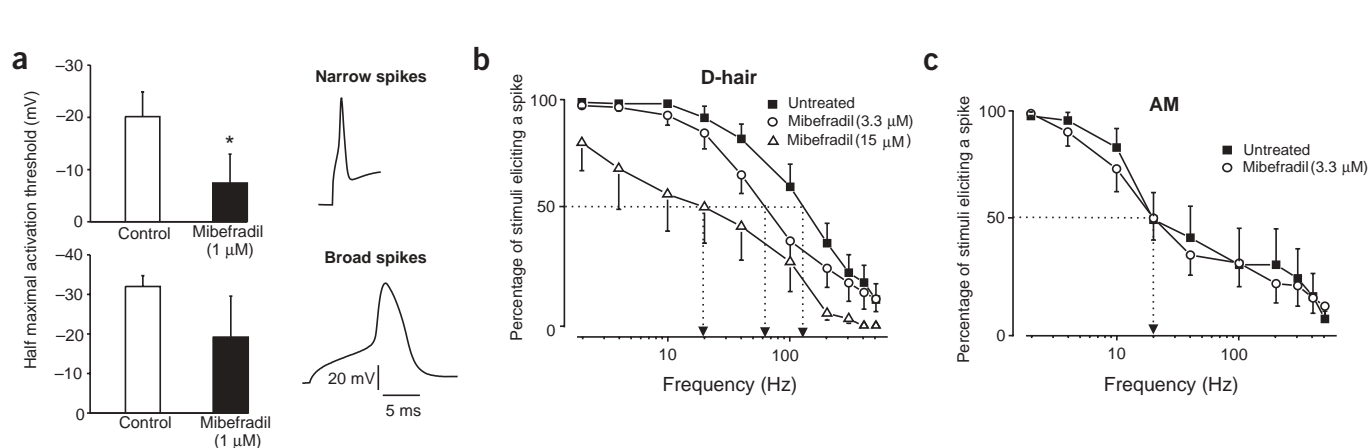


Figure 3 A T-type calcium antagonist selectively blocks electrical excitability of low-threshold sensory neurons. (a) Neurons with narrow action potentials (top) are more sensitive to the T-type calcium antagonist mibefradil (1 μM) and inward currents have a significantly increased half-maximal threshold in the presence of the antagonist ($n = 7$; $P < 0.01$). In contrast, currents from neurons with broad action potentials (bottom) are not significantly inhibited by 1 μM mibefradil ($n = 6$; $P = 0.18$). Measurements of spike frequency adaptation in a large sample of (b) D-hair receptors and (c) AM receptors ($n = 20$ –36 units per group), treated as shown. The percentage of stimuli after which the unit discharged one spike per stimulus is plotted as a function of the stimulus frequency (10 stimuli per frequency). The spike frequency adaptation curve for D-hair receptors is shifted leftward in the presence of 3.3 μM mibefradil ($P = 0.06$; repeated-measures ANOVA), whereas no change was observed in AM receptors. A higher dose of 15 μM mibefradil was used for a further group of D-hair mechanoreceptors (open triangles), and this shifted the spike frequency adaptation curve even further ($P < 0.05$).

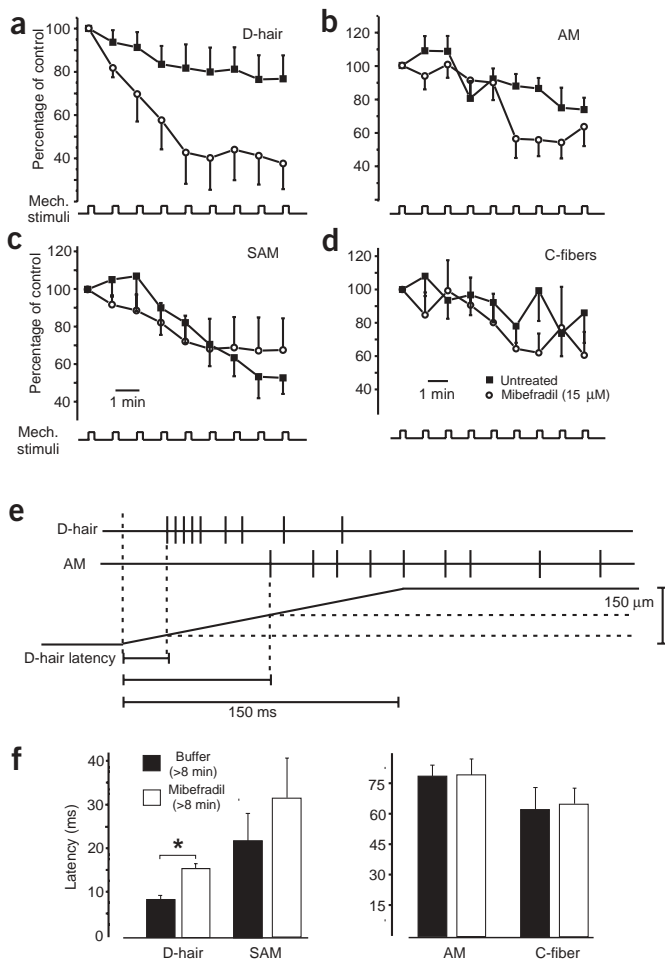


Figure 4 Effects of T-type calcium block on mechanoreceptor function. A repetitive mechanical stimulus was applied to (a) D-hair receptors (b) AM receptors (c) slowly adapting mechanoreceptors (SAM) receptors and (d) C-fiber nociceptors in the presence or absence of 15 μ M mibefradil ($n = 8-9$ fibers per group). The response of the units is normalized to the value before mibefradil or control buffer application. The D-hair receptors show a small decrease in response over time in the absence of mibefradil and a significant further decrease in the presence of the drug. The other receptors showed no significant reduction of response amplitude after drug application. (e) The relationship between the mechanical latency of the time to first spike after the mechanical probe starts to move. (f) D-hair receptors have a very short mechanical latency (~ 8 ms), whereas AM high-threshold mechanoreceptors and C-fiber polymodal nociceptors (the majority responded also to a noxious heat stimulus) have longer latencies (~ 80 ms). Mechanical latency of D-hair receptors increased significantly in mibefradil as compared with buffer (45% increase; $P < 0.01$, ANOVA). No significant effect of mibefradil treatment on the mechanical latency of the other tested receptors was observed.

DISCUSSION

Here we show that a genomic screening technique can be used to identify D-hair specific genes. Of the two genes identified, which encode the calcium channel $Ca_v3.2$ and the neurotrophin receptor $TrkB^{34}$, we focused our functional studies on the former. We had previously identified *TrkB* as having a D-hair-specific expression pattern, based on the hypothesis that NT-4-dependent D-hair receptors express high amounts of this receptor²². Of the regulated genes that were confirmed with real-time PCR (Table 1), some of the largest changes in expression were observed for $Ca_v3.2$ and *TrkB* (12-fold and 2.6-fold decreases, respectively). Many of the remaining regulated genes (5 of 12) are predicted transcription factors, making it likely that they are downstream targets of NT-4 signaling in sensory neurons. A cell-sorting approach was recently used to identify novel genes involved in touch reception in *Caenorhabditis elegans*³⁵. Our discovery here of a D-hair-specific membrane ion channel may make it possible to sort D-hair receptor neurons from other sensory neurons in the DRG. This may facilitate the discovery of new D-hair-specific genes. The heterogeneity of mechanoreceptor neurons observed in the dorsal root ganglia has in the past made it difficult to study the development and plasticity of individual receptor types without using electrophysiological techniques³⁶.

Physiological and pharmacological studies have demonstrated the existence of a variety of T-type calcium currents in isolated sensory neurons^{29,30,37}. Most investigators have favored the idea that such channels might be selectively expressed by nociceptive neurons. The presence of 'giant' T-type currents in a small subset of medium-sized sensory neurons^{29,30} correlates with the expected size range and incidence of D-hair receptor neurons³². In the present study, we show a correlation between a narrow action potential in isolated neurons and the susceptibility of voltage-gated inward currents in these cells and a selective T-type calcium channel antagonist mibefradil (Fig. 3a). This finding is consistent with the idea that D-hair receptor neurons express very large T-type currents by virtue of their high expression of $Ca_v3.2$. We tried to detect the expression of the other two T-type calcium channel genes $Ca_v3.1$ and $Ca_v3.3$ in the DRG with *in situ* hybridization but were unable to observe a specific signal. However, our real-time PCR experiments indicate that the expression of $Ca_v3.1$ was unchanged in the absence of D-hair receptors (Fig. 2), and thus this channel may not normally be expressed by these receptors. The $Ca_v3.3$ gene was significantly reduced in expression level in older *NT-4^{-/-}* mice as compared with wild type, and therefore this channel may be co-expressed with $Ca_v3.2$ in D-hair receptors. It is still possible

shown), indicating that the block was specific for the receptive terminal. Thus we wished to examine more quantitatively the effects of lower concentrations of mibefradil on mechanosensitivity. To do this, we used constant repetitively applied mechanical displacements (150 μ m, for 2 s/min) and measured the effect of mibefradil on mechanosensitivity. The mean responses of a group of control D-hair receptors to repeated mechanical stimuli declined only slightly in the absence of drug. There was, however, a rapid (< 90 s) and significant reduction in sensitivity (reduction to 40% of control) in the presence of 15 μ M mibefradil (Fig. 4a; $P < 0.05$; repeated-measures ANOVA). We used an identical protocol to test the effects of mibefradil on slowly adapting mechanoreceptors, AM fibers and C-fiber nociceptors. The same doses of mibefradil had no significant effect on the response of any of these other mechanoreceptor types ($P > 0.4$; Fig. 4b-d). For each stimulus, we calculated the latency from the onset of the displacement from the start position until the first spike (mechanical latency). Because the velocity of the displacement stimulus was constant (0.7 mm/s), an increase in mechanical latency reflects an increased mechanical threshold of the tested fiber (Fig. 4e). For D-hair receptors, mechanical latency almost doubled after 8 min of mibefradil application, as compared with buffer-treated control fibers ($P < 0.01$); no significant change in the mechanical latency of the other mechanoreceptor types was observed (Fig. 4f). Mechanical latencies before drug application were in all cases not significantly different between control and mibefradil-treated groups. Thus low doses of mibefradil can significantly and selectively inhibit the mechanosensitivity of D-hair receptors.

Table 1 Twelve downregulated genes in the adult *NT4^{-/-}* DRG (verified with real-time PCR)

Protein description and Ensembl database ID#	Mean fold change	Real-time PCR fold change	<i>in situ</i> hybridization
Vaccina-related kinase VRK3	-1.5	-1.4	all neurons
Transcription factor 12; ENSMUSG00000032228	-1.8	-1.3	all neurons
T-type calcium channel Ca_v3.2	-1.6	-12	medium-sized neurons
K _v 1.1	-2.5	-4.6	large- and medium-sized neurons
Mouse RAD52; ENSMUSG00000030166	-1.9	-1.5	all neurons
Putative 3' of Lim homeobox protein Lmx1b; ENSMUSG00000038765	-1.6	-1.2	all neurons
Similar to human STAR-related lipid transfer protein 8 (STARD8); ENSMUSG00000031216	-2.2	-1.4	all neurons
Contains BTB/POZ and Zn finger C2H2 domain, putative transcription factor; ENSMUSG00000028807	-2	-1.6	all neurons
Transcriptional enhancer factor TEF-5	-1.6	-1.2	all neurons
Transcription factor similar to Gtf2ird1	-1.7	-1.5	all neurons
Contains asp-protease site; ENSMUSG00000032930	-1.6	-1.5	all neurons
TrkB	-2	-2.6	medium-sized neurons

Bold genes that showed the expected expression pattern of a D-hair specific gene. Ensembl database (www.ensembl.org) identification number is given in cases where the gene is not extensively characterized. 'Mean fold change' refers to the Affymetrix data. For more detailed information on all 189 regulated genes, see **Supplementary Table 1** online.

that Ca_v3.2 is expressed at low levels in other mechanoreceptor types, but we could not find evidence for such expression.

By recording from sensory fibers innervating the skin⁶, we show that low concentrations of the T-type calcium channel antagonist mibefradil specifically reduce the mechanosensitivity of D-hair receptors. No significant effect on the mechanosensitivity of other receptors was observed, including C-fiber polymodal nociceptors (Fig. 4). Mibefradil blocks all three Ca_v3 T channels with equal potency (EC₅₀, 1–3 μM) but may also block L-type channels at higher concentrations³³. The specificity of the mibefradil effect, however, strongly supports our interpretation that D-hair receptors are susceptible to this drug because they express high levels of Ca_v3.2. T-type calcium channels including Ca_v3.2 have been proposed to control aspects of neuronal firing frequency^{38–40}. During the movement phase of our standard mechanical stimulus (150 μm at 0.7 mm/s), D-hair receptors fire at a high firing frequency (>50 Hz), which is required for the receptor to adequately code the stimulus. It is likely then that by reducing the maximal firing frequency, mibefradil treatment could lead to a reduction in mechanosensitivity.

It is also possible that Ca_v3.2 channels are directly gated by mechanical stimuli. Indeed there are reports that voltage-gated calcium channels may be mechanosensitive, although this effect was observed for N-type but not T-type channels⁴¹. Based on our expression and physiological data, however, we propose a simple model that explains the functional significance and the specificity of Ca_v3.2 channel expression in D-hair receptors. This calcium channel is normally activated near the resting membrane potential^{23,38,39}; furthermore, the simulation of high-frequency action potential trains in cells expressing recombinant Ca_v3.2 channels activates the current maximally during the first 100 ms (5–10 spikes) of the train³⁹. This is consistent with a maximal opening probability of this channel during the beginning of a rapid movement of the D-hair receptive field. The activation of Ca_v3.2 may result from the opening of mechanosensitive channels, which will thus shorten the time until enough voltage-dependent sodium channels open to initiate an action potential. This model explains why blockade of T-type channels can increase the mechanical threshold of D-hair receptors (Fig. 4f).

A lowering of the threshold for the generation of action potentials after odorant stimulation of olfactory sensory neurons has also been attributed to T-type calcium channels⁴². In addition, large T-type currents may facilitate high-frequency firing, which is particularly necessary for D-hair receptors to code a velocity stimulus. The rapid inactivation of calcium currents during a high-frequency spike train⁴¹ would ensure that voltage-dependent inward currents do not interfere with the very rapidly adapting response of this receptor. At the moment, no pharmacological tools are available to specifically target the Ca_v3.2 channel. Mouse knockout models may prove useful to address in detail how this channel contributes to D-hair receptor sensitivity, but it is still possible that the expression of Ca_v3.2 in other tissues including brain might preclude analysis of a sensory neuron phenotype⁴³.

We have demonstrated for the first time that a genome-wide screen can be used to identify a functionally relevant gene that is specific to one type of vertebrate mechanoreceptor. Our identification of Ca_v3.2 as a D-hair specific channel enabled us to demonstrate a role for a specific T-type calcium channel in mechanosensory transduction. Following this example, it is likely that other types of sensory receptors will also be distinguished by the specific expression of single genes. As demonstrated here, the function of such genes can provide insight into how receptor properties are specified at the molecular level.

METHODS

Expression analysis using Affymetrix gene chips. DRG from all spinal levels were dissected from 4- and 12-week-old *NT-4^{-/-}* and wild-type mice. Total RNA was isolated using Trizol reagent (Gibco) and purified with RNeasy microcolumns (Qiagen). Biotin-labeled cRNA was hybridized to Affymetrix Mouse Genome U74 version 2 set (MG U74vs2; 36,000 transcripts). Experiments were carried out independently in triplicate (6–10 mice per group). We used the MICROARRAY SUITE 5.0 (Affymetrix) for data analysis. Signal intensities were scaled using a target intensity value of 1,000.

Suppression subtractive hybridization. We used the PCR Smart and PCR Select kits (Clontech) for the amplification and subtraction procedure. We used 1 μg total RNA for the poly(dT)-based first-strand synthesis, followed by PCR amplification. The subtraction procedure was carried out according

to manufacturer's instructions. Briefly, common genes of both groups hybridize, whereas differentially expressed genes do not hybridize and are therefore capable of being amplified by PCR. The subtracted library was used as template for T7-driven *in vitro* transcription to generate biotin-labeled cRNA. Labeled cRNA was hybridized to MG U74vs2 arrays (Affymetrix) according to manufacturer's instructions. For analysis, we searched for genes that showed high hybridization signals in the subtracted but not in the unsubtracted control.

Real-time PCR. Total RNA was extracted from DRG of *NT-4* wild-type, *NT-4^{-/-}* (4–5 week) and *NT-4^{-/-}* (12 week) mice and used for cDNA synthesis. The primers and probes for all genes of interest were designed with PRIMER-EXPRESS software (Applied Biosystems). The PCR mix contained 1 μ l cDNA template; 1 \times Taqman buffer; 5 mM MgCl₂; 200 μ M each of dATP, dCTP and dGTP; 400 μ M dUTP; 1.25 U AmpliTaq Gold DNA polymerase; 0.25 U AmpErase UNG; primer and probe (300 nM each of primers and 200 nM probe for K_v1.1, TrkB and GAPDH, and 600 nM primers and 600 nM probe for the three T-type channels) in a total volume of 25 μ l. Standard reactions were carried out using an Applied Biosystems PRISM 7700 Sequence Detection System. All experiments were done in triplicate. Fold changes were calculated relative to the level of GAPDH mRNA.

In situ hybridization. We did *in situ* hybridization with 10- μ m cryosections of mouse DRG as described^{12,44}. Sections were hybridized with digoxigenin-labeled cRNA probes that had around 400 bp of sequence that was complementary to the coding region. Hybridization temperatures ranged between 52 °C and 58 °C.

Whole-cell patch-clamp recordings from isolated DRG neurons. Cultures of DRG neurons from adult mice were prepared as previously described^{12,45}. We added no nerve growth factor or other neurotrophins to the medium. Whole-cell recordings were made from DRG neurons using fire-polished glass electrodes with a resistance of 3–5 M Ω . Extracellular solution contained 154 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 5.6 mM KCl, 8 mM glucose and 10 mM HEPES (pH 7.4). Electrodes were filled with a solution containing 122 mM KCl, 10 mM Na⁺, 1 mM MgCl₂, 1 mM EGTA and 10 mM HEPES (pH 7.3). Whole-cell voltage currents were activated by pre-pulsing voltage-clamped cells to –120 mV for 150 ms and then depolarizing from –50 to +50 mV in 5-mV increments. The potential for half-maximal activation was determined from the current voltage (*I*–*V*) curves. Under current-clamp conditions, action potentials were evoked with current injection.

In vitro single-fiber recording. An *in vitro* skin–nerve preparation was used to record from functionally single primary afferents in micro-dissected teased filaments of the saphenous nerve as described previously^{6,12,13}. Using a probe fixed to a linear stepping motor under computer control (Nanomotor; Kleindiek Nanotechnik), standardized displacement stimuli could be applied to the receptive field at regular intervals. We collected raw electrophysiological data with a Powerlab 4.0 system (AD Instruments) and discriminated spikes off line with the spike histogram extension of the software. The delay between the start of the mechanical probe movement and the first spike, corrected for the conduction delay of the tested fiber, was designated as the mechanical latency. The C-fibers in these experiments were all tested for a response to noxious heat by applying a heated solution to the receptive field so that the skin temperature transiently exceeded 50°C. All animal experiments were carried out according to German national laws and guidelines.

Microarray data is available under accession code E-MEXP-10 at ArrayExpress (www.ebi.ac.uk/arrayexpress/).

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank A. Kanehl and H. Thränhardt for technical assistance. We thank the following individuals for help, advice and discussion: N. Hübner, F. Rathjen, T. Willnow, H. Schulz and C. Stucky. C.M.S. was supported by a Marie Curie fellowship from the European Union. This work was supported by the Deutsche Forschungsgemeinschaft and German National Genome Network grants to G.R.L. We are grateful to Roche for kindly donating mibefradil for these studies.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Neuroscience* website for details).

Received 27 March; accepted 12 May 2003

Published online 15 June 2003; doi:10.1038/nn1076

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