Sensory Neurons From Nf1 Haploinsufficient Mice Exhibit Increased Excitability

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Wang, Yue, G. D. Nicol, D. Wade Clapp, and Cynthia M. Hingtgen. Sensory neurons from Nf1 haploinsufficient mice exhibit increased excitability. J Neurophysiol 94: 3670–3676, 2005. First published August 10, 2005; doi:10.1152/jn.00489.2005. Neurofibromatosis type 1 (NF1) is a common genetic disorder characterized by tumor formation. People with Nf1 also can experience more intense painful responses to stimuli, such as minor trauma, than normal. NF1 results from a heterozygous mutation of the NFI gene, leading to decreased levels of neurofibromin, the protein product of the NFI gene. Neurofibromin is a guanosine triphosphatase activating protein (GAP) for Ras and accelerates the conversion of active Ras-GTP to inactive Ras-GDP; therefore mutation of the NFI gene frequently results in an increase in activity of the Ras transduction cascade. Using patch-clamp electrophysiological techniques, we examined the excitability of capsaicin-sensitive sensory neurons isolated from the dorsal root ganglia of adult mice with a heterozygous mutation of the NFI gene (Nfi+/−), analogous to the human mutation, in comparison to wild-type sensory neurons. Sensory neurons from adult Nfi+/− mice generated a more than twofold higher number of action potentials in response to a ramp of depolarizing current as wild-type neurons. Consistent with the greater number of action potentials, Nfi+/− neurons had lower firing thresholds, lower rheobase currents, and shorter firing latencies than wild-type neurons. Interestingly, nerve growth factor augmented the excitability of wild-type neurons in a concentration-related manner but did not further alter the excitability of the Nfi+/− sensory neurons. These data clearly suggest that GAPs, such as neurofibromin, can play a key role in the excitability of nociceptive sensory neurons. This increased excitability may explain the painful conditions experienced by people with NFI.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disease with an incidence of 1 in 3,500 people (Lakkis and Tennekoon 2000). It is characterized by formation of neurofibromas (complex tumors composed of axonal processes, Schwann cells, fibroblasts and mast cells) as well as malignant tumors such as neurofibrosarcomas, malignant astrocytomas, and myeloid leukemias. In addition to tumor formation, some people with NFI also experience a more intense painful response to stimuli, such as minor injuries, than normal (Creange et al. 1999; Riccardi and Eichner 1992; Wolkenstein et al. 2001). Although the mechanism by which the NF1 mutation causes these symptoms has not been elucidated, it is likely that the abnormal painful states involve the increased sensitivity of small-diameter nociceptive sensory neurons; cells that are known to mediate the transmission of pain.

In NFI there is a mutation of one allele of the NFI gene (NFI+/−). This results in reduced expression of the protein product of the NFI gene, neurofibromin, in many cells, including neurons (Bollag and McCormick 1991; Cichowski and Jacks 2001; Largaespada et al. 1996; Zhang et al. 1998). Neurofibromin is a guanosine-triphosphatase-activating protein (GAP) that accelerates the conversion of the active form of the small G protein, Ras (Ras-GTP), to its inactive form (Ras-GDP) (Li et al. 1992; Martin et al. 1990; Wallace et al. 1990). In many cell types, mutation of the NFI gene or its mouse correlate (Nfi/−), frequently results in increased basal and cytokine-stimulated Ras-GTP and enhanced activity of the downstream effectors of the Ras transduction cascade. For example, investigators have shown that the level of Ras-GTP is elevated in human NF1 neurogenic tumors (Guha et al. 1996), in mast cells from mice with a heterozygous mutation of the NFI gene (Nfi+/−) (Ingram et al. 2001), and in Schwann cells from embryonic mice with a homozygous mutation of the NFI gene (Nfi/−) (Sherman et al. 2000). In addition, the sensory neurons from embryonic Nfi/− mice demonstrate increased Ras activity (Klesse and Parada 1998; Vogel et al. 2000).

Among the many growth factors that activate the Ras transduction cascade, nerve growth factor (NGF) has been explored extensively for its role in pain signaling. NGF plays a critical role in the development and maintenance of sensory neurons; however, a growing body of evidence has demonstrated that NGF is an important mediator of the enhanced pain sensation (hyperalgesia) that occurs with inflammation. The content of NGF is elevated in inflamed skin (Weskamp and Otten 1987) and peripheral tissue (Aloe et al. 1992a,b). Mendell and co-workers demonstrated that NGF produces both thermal and mechanical hyperalgesia (Lewin and Mendell 1993; Lewin et al. 1993). In addition, the hyperalgesia associated with inflammation is diminished by an anti-NGF antibody (Woolf et al. 1994). By using a skin-nerve preparation, Rueff and Mendell (1996) demonstrated that NGF can increase the firing frequency of isolated saphenous nerve in response to heat stimulation. NGF also enhanced the excitability of isolated sensory neurons in culture by increasing a TTX-resistant sodium current and by suppressing a delayed-rectifier potassium current (Zhang et al. 2002). Although it is clear that NGF can sensitize sensory neurons to noxious stimuli, the intracellular cascades by which NGF exerts its effects remain poorly understood. The stimulation of either the TrkA or p75 receptor by NGF can lead to the activation of Ras transduction cascade (Blochl et al. 2001).
2004; Corbett and Alber 2001; Huang and Reichardt 2003; Susen et al. 1999). In addition, recent studies have suggested that NGF can activate downstream effectors of the Ras transduction cascade to affect changes in adult sensory neurons (Bron et al. 2003; Ganju et al. 1998; Zhuang et al. 2004). For example, Bron and colleagues have shown that NGF-induced increases in phosphorylated extracellular signal-regulated kinase (pERK) and phosphorylated Akt (pAkt), two downstream effectors of Ras activation, are associated with increases in the expression of the heat- and capsaicin-activated receptor, TRPV1, in DRG neurons and that constitutively active Ras mimics the action of NGF to increase TRPV1 expression in isolated sensory neurons (Bron et al. 2003). Based on the hypothesis that NGF-induced alteration in peripheral pain signaling may, in part, be related to activation of the Ras transduction cascade, the enhanced painful sensations experienced by people with NF1 could result from altered control of the Ras cascade because of decreased neurofibromin levels.

To test the hypothesis that the Nf1 mutation results in increased sensory neuron excitability, we used a mouse model of NF1. These mice have a heterozygous mutation of the Nf1 gene (Nf1+/−), similar to that seen in the human disorder (Jacks et al. 1994). In this report, we demonstrate that capsaicin-sensitive sensory neurons from Nf1+/− mice exhibit enhanced excitability. Treatment of wild-type neurons with NGF mimics the increased excitability of Nf1+/− neurons. These results suggest that decreased GAP levels correlate with enhanced neuronal excitability and are consistent with the idea that GAP-regulated signaling pathways are important in the modulation of sensory neuron sensitivity.

METHODS

Animals

Mice heterozygous for the Nf1 mutation on a background of C57BL/6J were originally developed by Dr. Tyler Jacks (Jacks et al. 1994). All animals were housed and bred in the Indiana University Laboratory Animal Research Center and used in accordance with National Institute of Health Guide for Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80–23) revised 1996.

Reagents

Horse serum, F-12 medium, l-glutamine, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). NGF was purchased from Harlan Bioproducts for Science (Indianapolis, IN). Papain was purchased from Worthington Biochemical, and dispase was obtained from Roche (Indianapolis, IN). Collagenase, poly-D-lysine, laminin, 5′-fluoro-2′-deoxyuridine, uridine, and standard laboratory chemicals were from Sigma (St Louis, MO).

Isolation of sensory neurons from Nf1+/− or Nf1+/− adult mice

The isolation of sensory neurons from 1- to 2-week-old mice was accomplished using a modification of a method developed by Lindsay (1988). Briefly, the dorsal root ganglia were removed and transferred into a culture dish filled with sterilized Ca2+- and Mg2+-free Hank’s balanced salts solution (HBSS) consisting of (in mM) 171 NaCl, 6.7 KCl, 1.6 Na2PO4, 0.5 KH2PO4, 6 n-glucose, and 0.01% phenol red, pH 7.3. The ganglia were incubated for 10–15 min at 37°C in HBSS containing papain (10 ng/ml) and then transferred into F-12 media containing 1 mg/ml collagenase 1A and 2.5 mg/ml dispase. After a 10- to 15-min incubation at 37°C in the second set of enzymes, the tissue sample was centrifuged for 30 s before the enzyme-containing supernatant was removed. The pellet was resuspended in F-12 media and mechanically dissociated with a fire-polished pipette until all large pieces of tissue were gone. The isolated cell suspension was plated onto plastic cover slips that were coated with 0.5% poly-D-lysine and laminin (100 μg/ml). The sensory neurons were maintained in F-12 media supplemented with 10% horse serum, 2 mM glutamine, 100 μg/ml normocin, 50 μg/ml penicillin and streptomycin, 50 μM 5-fluoro-2′-deoxyuridine, 150 μM uridine at 37°C and 3% CO2. These cells were used for electrophysiological recordings within 5–12 h after isolation. NGF was added to the F-12 media at the time of plating where indicated.

Electrophysiology

Recordings were made using the whole cell patch-clamp technique as previously described (Evans et al. 1999; Zhang et al. 2002). Briefly, a cover slip with the sensory neurons was placed in a recording chamber where the neurons were bathed in normal Ringer solution of the following composition (mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, pH adjusted to 7.4 with NaOH. Recording pipettes were pulled from borosilicate glass tubing and typically had resistances of 2–5 MΩ when filled with the following solution (mM): 140 KCl, 5 MgCl2, 4 ATP, 0.3 GTP, 2.5 CaCl2, 5 EGTA (calculated free Ca2+ concentration of ~100 nM), and 10 HEPES, adjusted pH at 7.3 with KOH. Whole cell voltages were recorded with an Axopatch 200B amplifier (Axon Instruments). Only neurons that maintain resting membrane potentials more hyperpolarized than ~45 mV were used in this study. In the current-clamp experiments, two protocols were used to test the excitability. First, neurons were held at their resting potentials and injected with a 1-s ramp of depolarizing current that had a final amplitude of 1,000 pA. The sampling frequency was 2 kHz. Second, a series of current steps that were 200 ms in duration and of variable incremental amplitudes was used to determine the rheobase. The sampling frequency was 1.25 kHz. At the end of each recording, the neuron was superfused with a Ringer solution containing 100 nM capsacin, as sensitivity to this agent is believed to be an indicator of nociceptive sensory neurons (Holzer 1991). The results presented were obtained from capsacin-sensitive neurons only. All experiments were performed at room temperature (~23°C).

Data analysis

Summarized data are presented as the means +/- SE. Statistical significance between groups was determined using a t-test or a one-way ANOVA followed by Dunnett’s post hoc analysis, as appropriate and is specified in the text. Values of P < 0.05 were judged to be statistically significant.

RESULTS

Sensory neurons from Nf1+/− mice have higher excitability than neurons from wild-type mice

To determine whether nociceptive sensory neurons with reduced levels of neurofibromin have altered excitability, sensory neurons were isolated from Nf1+/− and wild-type mice. One measure of neuronal excitability is the number of APs elicited by a given amount of depolarizing current. Figure 1 shows representative recordings in response to the current ramp from single Nf1+/− (A) and Nf1+/− (B) neurons, respectively. As can be easily appreciated, identical ramps of...
respectively, of 8 neurons in the wild-type group and 11 neurons in the sensory neurons from Nf1- mice exhibited a more than twofold increase in the number of APs compared with wild-type neurons for identical stimulation (6.0 +/- 1.6 and 14.8 +/- 2.2 APs for Nf1+/+ and Nf1+-/ neurons, respectively, P < 0.05 using a t-test). All of the neurons of both genotypes were capsaicin-sensitive as determined at the end of the protocol.

Two additional parameters indicative of the level of neuronal excitability are the firing threshold and firing latency. These measurements were determined in the same neurons for which the number of APs evoked by the current ramp was assessed in the preceding text. The firing threshold is the membrane voltage at which the AP is generated and was determined as described in the legend. As summarized in Fig. 2B, neurons isolated from Nf1-/+ mice had a significantly lower firing threshold compared with that of Nf1+/+ neurons (-31.7 +/- 1.6 vs. -25.7 +/- 1.3 mV for Nf1+/+ and wild-type neurons, respectively, P < 0.05 using a t-test). A lower firing threshold suggests that the Nf1-/+ neurons are capable of generating APs at more hyperpolarized membrane potentials. Similarly, the firing latency, or the time from the onset of the current injection to the initiation of the first AP, was significantly shorter in the Nf1+/+ sensory neurons (Fig. 2C; 361 +/- 55 and 216 +/- 15 ms for Nf1+/+ and Nf1+-/ neurons, respectively, P < 0.05 using a t-test). However, there was no difference in the average resting membrane potentials between these genotypes (Fig. 2D; -62.7 +/- 1.8 and -60 +/- 2.0 mV for Nf1+/+ and Nf1+-/ neurons, respectively).

These results demonstrate that the firing threshold was reduced in neurons isolated from the Nf1-/+ mice. Consistent with this observation was our finding that the rheobase (the minimum amount of current required to evoke an AP) also was reduced in Nf1+/+ neurons. Representative tracings from a wild-type and Nf1+/+ neuron are shown in Fig. 3, A and B, respectively. As summarized in Fig. 3C, Nf1+/+ neurons had an almost threefold lower rheobase compared with wild-type neurons (56 +/- 9 and 154 +/- 36 pA for 11 Nf1+/+ and 8 Nf1+/+ neurons, respectively, using a t-test). As shown in Fig. 3D, the input resistance was not significantly different between the two genotypes (712 +/- 191 MΩ for Nf1+/+ neurons and 795 +/- 79 MΩ for Nf1+/+ neurons). Taken together, these data clearly demonstrate that capsaicin-sensitive sensory neurons isolated from mice that are heterozygous for the Nf1 mutation exhibit enhanced excitability compared with capsaicin-sensitive sensory neurons from wild-type mice and that this enhanced excitability is consistent across multiple electrophysiological parameters.

**Fig. 1.** Nf1+/+ sensory neurons generate more action potentials (APs) in response to a given stimulus than wild-type neurons. Tracings from representative sensory neurons isolated from either a wild-type (A) or a Nf1+/+ mouse (B) illustrate the APs evoked by the ramp of current. The neurons were held at their resting membrane potential and APs were evoked by injecting a 1-s ramp of depolarizing current that had a final amplitude of 1,000 pA. The dV/dt trace represents the differentiation of the membrane voltage. The baseline dV/dt is calculated as the average of all the values between the injection of the ramp current and just prior to the initiation of the AP. The firing threshold is the membrane voltage corresponding to the point at which dV/dt exceeds the baseline value by 20-fold. The firing latency is calculated as the time between the onset of the current ramp and the time at which the firing threshold is attained.

**Fig. 2.** Nf1+/+ sensory neurons exhibit increased excitability compared with wild-type neurons. ○, values from individual wild-type neurons (Nf1+/+); ●, values from individual Nf1+/+ neurons. To the right of each series of individual values is the mean +/- SE for the specific genotype (The error for the firing latency of Nf1+/+ neurons in F is small enough to be obscured by ●). The values in each panel are from the same 8 wild-type and 11 Nf1+/+ neurons. *, a statistical difference between genotypes using a t-test (P < 0.05).
Treatment with NGF enhances the excitability of wild-type sensory neurons and mimics the effects of the Nf1 mutation

Because NGF is a growth factor known to alter the excitability of nociceptive sensory neurons and is an activator of the Ras transduction cascade, we examined the actions of NGF on excitability in both wild-type and Nf1+/− sensory neurons. During the 5–12 h wherein the neurons were maintained in culture and prior to obtaining these recordings, the neurons were maintained in media containing either no added NGF or different concentrations of NGF. As described in the preceding text, the resting membrane potential, AP number, firing threshold, firing latency, and rheobase were measured under these different conditions. As shown in Fig. 4A, NGF caused a concentration-related increase in the number of APs elicited by a standard ramp of depolarizing current in capsaicin-sensitive sensory neurons isolated from wild-type mice. The number of evoked APs was significantly higher after treatment with NGF compared with that obtained in its absence. For example, after exposure to 100 ng/ml NGF, the number of evoked APs increased to 14.8 ± 2.8 (n = 6 neurons) compared with 5.9 ± 1.5 (n = 8 neurons) in the absence of NGF for wild-type neurons. Surprisingly, there was no difference in the number of APs elicited from Nf1+/− sensory neurons treated with NGF compared with those maintained in the absence of NGF. As a
consequence, the difference in AP number observed between the genotypes was abolished after treatments with the higher concentrations of NGF.

A similar concentration-related alteration of firing latency and rheobase were observed in the wild-type neurons treated with NGF (Fig. 4, C and D). These parameters were significantly lower in wild-type sensory neurons treated with 30 or 100 ng/ml NGF compared with neurons not exposed to NGF. Again, there was no effect of NGF on the firing latency or rheobase in Nf1+/− sensory neurons. Although the decrease in firing threshold in wild-type neurons treated with the higher concentrations of NGF was not statistically different from neurons that were not treated with NGF (Fig. 4B), the small decrease observed was sufficient to abolish the differences between genotypes seen in the absence of NGF or in the presence of 1 ng/ml NGF. These data demonstrate that NGF dramatically alters the excitability of capsaicin-sensitive sensory neurons from wild-type mice but has no effect on capsaicin-sensitive neurons heterozygous for the Nf1 mutation. Consequently, the increased excitability that is inherent in the Nf1+/− sensory neurons is mimicked in wild-type neurons by treatment with NGF.

**DISCUSSION**

In this report, we demonstrate that the small-diameter, capsaicin-sensitive sensory neurons with a heterozygous mutation of the Nf1 gene have augmented excitability compared with wild-type neurons. These data suggest that the activation of the Ras transduction cascade, as a consequence of mutation of the Nf1 gene, alters the state of modulation for ion channels that regulate the capacity of sensory neurons to fire APs as indicated by the increased number of evoked APs, a more hyperpolarized firing threshold, and a decreased rheobase. However, those channels that maintain the resting membrane potential and the resistance appear to be unaffected by the heterozygous mutation of the Nf1 gene and the consequences of its modifications of downstream transduction cascade(s).

There is evidence to support the modulation of ion channels by activation of the Ras transduction cascade. For example, Fitzgerald and Dolphin (1997) demonstrated that the microinjection of an activated K-Ras isoform enhances the voltage-gated calcium current in dorsal root ganglia (DRG) neurons from neonatal rats. Similarly, blocking Ras activation with a peptide that inhibits the interaction of Ras with the TrkA-Src complex or inhibiting Ras signaling with a neutralizing antibody, reduces these calcium currents. In addition, co-expression of constitutively active Ras and an inward-rectifier potassium channel in HEK cells causes a decrease in the inward-rectifying potassium current. This reduction in current is blocked by the mitogen-activated protein kinase/ERK kinase (Mek) inhibitor PD98059 (Giovannardi et al. 2002). These data are consistent with the ability of increased Ras activation to enhance neuronal excitability as observed in the isolated Nf1+/− sensory neurons. To elucidate the specific channels that are modulated by the Nf1 mutation, additional voltage-clamp studies are necessary.

Once Ras-GTP recruits the kinase, Raf, to the cell membrane, a cascade of downstream effectors is activated. The role of GAPs, such as neurofibromin, is to catalyze the hydrolysis of active Ras-GTP to inactive Ras-GDP and, thereby, halt the activation of downstream cascades (Li et al. 1992; Martin et al. 1990; Wallace et al. 1990). As a result of the Nf1 mutation, there is an increase in cellular levels of Ras-GTP, pERK, and pAkt, both at rest and when cells are exposed to growth factors that activate these cascades (Guha et al. 1996; Ingram et al. 2001; Klesse and Parada 1998; Sherman et al. 2000; Vogel et al. 2000). NGF is one such growth factor that can activate the Ras transduction cascade. Recently, several investigations demonstrated that the Ras transduction cascade and the downstream effectors of this pathway may play a key role in the actions of NGF to sensitize sensory neurons. For example, Zhuang et al. (2005) reported that in the spinal nerve ligation model of neuropathic pain, pERK levels were increased in spinal cord neurons and the DRG. NGF injected into the rat hindpaw also increased p-ERK labeling in TrkA-containing neurons in the DRG (Averill et al. 2001). In addition, peripheral inflammation increased the levels of phosphorylated p38 (p-p38), another member of the mitogen-activated kinase family, in nociceptive sensory neurons (Ji et al. 2002). This increase in p-p38 was correlated with an increase in expression of TRPV1 and thermal hyperalgesia, and all three of these responses were blocked by treatment with NGF antiserum prior to the initiation of the inflammation. Treatment with constitutively active Ras mimicked the action of NGF to increase TRPV1 expression in isolated sensory neurons and increased neuronal levels of pERK and pAkt (Bron et al. 2003). In addition, the Mek inhibitor, PD98059, reduced the capsaicin sensitivity of neurons that were treated with NGF for 1 wk (Ganju et al. 1998). Although most of these investigations have focused on the role of components of the Ras transduction cascade in NGF-mediated changes of TRPV1 expression and capsaicin responses, they do not exclude the possibility of other Ras-mediated changes in the excitability of nociceptive sensory neurons. In general, these observations are consistent with our findings that NGF increased the excitability of wild-type mouse sensory neurons and mimicked the enhanced excitability that was intrinsic to the Nf1+/− sensory neurons.

It is possible that a further enhancement of excitability in the Nf1+/− neurons treated with NGF was not observed because the mutated neurons had already attained their maximum ability to fire APs when stimulated by depolarizing currents. In this case, the Nf1+/− neurons would be unable to fire more APs when treated with NGF. Alternatively, there may be compensatory mechanisms modulating NGF-induced sensitization of the neurons with reduced neurofibromin. Potential compensatory mechanisms could include downregulation of the TrkA or p75 receptors or modulation of other downstream components of these cascades. Interestingly, Zhang et al. (2002) reported that NGF-induced sensitization of rat sensory neurons is mediated by ceramide, a product of the p75 receptor-activated transduction cascade (Dobrowsky et al. 1994). However, it is possible that there is overlap or cross-talk between the cascades activated by Trk A and those activated by p75. There is evidence that NGF can stimulate the Ras transduction cascade through activation of the p75 receptor in expression systems and neuronal cells that do not express TrkA (Blocilh et al. 2004; Susen et al. 1999). In addition, Hida and colleagues (1998) demonstrated that ceramide can activate Ras in cultured oligodendrocytes. Therefore, if NGF enhances neuronal excitability via Ras-dependent pathways mediated through TrkA or p75 or both, it is not surprising that this effect.
of NGF is lost in cells with constitutive activation of the Ras cascade, like those from NFI +/− mice. It would be very informative to examine the role of both of these receptors in the sensory neuronal excitability induced by activation of the Ras pathway.

These data clearly suggest that GAPs, such as neurofibromin, can play a key role in modulating the excitability of nociceptive sensory neurons. A clearer understanding of the mechanisms underlying the enhanced neuronal excitability in sensory neurons with the NFI mutation, similar to the human disorder NF1, and how this sensitization may be modified in injured cells would lead to better therapies for the painful conditions associated with NF1 or chronic painful conditions that arise from other ailments.

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