Augmented Sodium Currents Contribute to the Enhanced Excitability of Small Diameter Capsaicin-Sensitive Sensory Neurons Isolated From Nfi1+/- Mice

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Wang Y, Duan JH, Hingtgen CM, Nicol GD. Augmented sodium currents contribute to the enhanced excitability of small diameter capsaicin-sensitive sensory neurons isolated from Nfi1+/- mice. J Neurophysiol 103: 2085–2094, 2010. First published February 17, 2010; doi:10.1152/jn.01010.2009. Neurofibromin, the product of the Nfi1 gene, is a guanosine triphosphatase activating protein (GAP) for p21ras (Ras) that accelerates conversion of active Ras-GTP to inactive Ras-GDP. Sensory neurons with reduced levels of neurofibromin likely have augmented Ras-GTP activity. We reported previously that sensory neurons isolated from a mouse model with a heterozygous mutation of the Nfi1 gene (Nfi1+/−) exhibited greater excitability compared with wild-type mice. To determine the mechanism giving rise to the enhanced excitability, differences in specific membrane currents were examined. Consistent with the enhanced excitability of Nfi1+/− neurons, peak current densities of both tetrodotoxin-resistant sodium current (TTX-R I\textsubscript{Na}) and TTX-sensitive (TTX-S) I\textsubscript{Na} were significantly larger in Nfi1+/− than in wild-type neurons. Although the voltages for half-maximal activation (V\textsubscript{0.5}) were not different, there was a significant depolarizing shift in the V\textsubscript{0.5} for steady-state inactivation of both TTX-R and TTX-S I\textsubscript{Na} in Nfi1+/− neurons. In addition, levels of persistent I\textsubscript{Na} were significantly larger in Nfi1+/− neurons. Neither delayed rectifier nor A-type potassium currents were examined. Consistent with the enhanced excitability of Nfi1+/− neurons, peak current densities of both tetrodotoxin-resistant sodium current (TTX-R I\textsubscript{Na}) and TTX-sensitive (TTX-S) I\textsubscript{Na} were significantly larger in Nfi1+/− than in wild-type neurons. Although the voltages for half-maximal activation (V\textsubscript{0.5}) were not different, there was a significant depolarizing shift in the V\textsubscript{0.5} for steady-state inactivation of both TTX-R and TTX-S I\textsubscript{Na} in Nfi1+/− neurons. In addition, levels of persistent I\textsubscript{Na} were significantly larger in Nfi1+/− neurons. Neither delayed rectifier nor A-type potassium currents were altered in Nfi1+/− neurons. These results demonstrate that enhanced production of action potentials in Nfi1+/− neurons results, in part, from larger current densities and a depolarized voltage dependence of steady-state inactivation for I\textsubscript{Na} that potentially leads to a greater availability of sodium channels at voltages near the firing threshold for the action potential.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is an autosomal dominant disease with an incidence of 1 in 3,000 people and is characterized by numerous abnormalities including neurofibromas (benign complex tumors composed of axonal processes, Schwann cells, and mast cells) as well as malignant peripheral nerve sheath tumors (MPNSTs), astrocytomas, and myeloid leukemias (Friedman 1999). Some people with NF1 also experience much more intense and exaggerated painful responses (Creange et al. 1999; North et al. 1997; Ozonoff 1999; Trovo-Marqui et al. 2005) indicate that the single active NF1 allele does not generate sufficient functional neurofibromin to fulfill its normal biological role in the nervous system. Neurofibromin facilitates switching the active form of Ras (Ras-GTP) to its inactive form (Ras-GDP) by serving as a GTPase activating protein (GAP). Mutation of NF1 frequently results in enhanced basal and cytokine-stimulated Ras activity in many cell types, including sensory neurons. For example, investigators have shown that the level of Ras-GTP was elevated in MPNSTs and neurofibromas from humans with NF1 (Guha et al. 1996), in mast cells from mice with a heterozygous mutation of NF1 (Nfi1+/−) (Ingram et al. 2001), and in Schwann cells from embryonic mice with a homozygous mutation of NF1 (Sherman et al. 2000). In addition, sensory neurons from embryonic Nfi1+/− mice exhibit increased Ras activity (Klesse and Parada 1998; Vogel et al. 2000). Therefore it is reasonable to speculate that a higher level of Ras-GTP in adult sensory neurons with a heterozygous mutation of the NF1 gene could promote changes in neuronal function.

Previous evidence suggests that the Ras transduction cascade can modulate the activity of ion channels that could contribute to the generation of an action potential (AP). Fitzgerald and Dolphin (1997) demonstrated that microinjection of an activated K-Ras isoform enhanced the voltage-gated calcium current in neurons of the dorsal root ganglia (DRG) from neonatal rats. In addition, we reported previously that small diameter, capsaicin-sensitive sensory neurons isolated from Nfi1+/− mice exhibited augmented excitability (Wang et al. 2005). To determine the mechanisms giving rise to the augmented neuronal firing in Nfi1+/− neurons, the differences in specific membrane currents were examined in this study. It is possible that the heterozygous deletion of the NF1 gene somehow confers a change in channel activity that leads to increased excitability and may underlie the onset of enhanced painful sensation in people with NF1.

METHODS

Animals

Mice heterozygous for the Nfi1 mutation on a background of C57BL/6J were originally developed by Dr. Tyler Jacks (Jacks et al. 1994). All animals were housed, bred, and had free access to food and water in the Indiana University Laboratory Animal Research Center and used in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals. Nfi1+/− mice were generated by crossing wild-type mice with mice heterozygous for the Nfi1 mutation (Nfi1+/-). The typical survival time for Nfi1−/− mice is around 4 months. The average age of the Nfi1−/− mice used in this study was 12 months. The experiments were performed in accordance with the guidelines of the Indiana University Laboratory Animal Research Center and the Indiana University Institutional Animal Care and Use Committee. The protocol for the experiments was approved by the Institutional Animal Care and Use Committee of Indiana University.

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Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80–23, revised 1996).

Isolation and maintenance of sensory neurons

Isolation of sensory neurons from young adult mice (1–2 mo of age) used the procedure developed by Lindsay (1988) with slight modification. The wild-type and Nf1+/− mice used in these studies were littersmates. Briefly, male mice were killed by placing them in a CO2 chamber. The isolated spinal column was dissected, the spinal cord was removed, and the dorsal root ganglia (DRGs) were collected and 3% CO2. The cells were used within 4–12 h for electrophysiology. Only neurons that maintained resting membrane potential. Results were not corrected for the liquid junction. The data were acquired and analyzed using pCLAMP 9.0 after performing the compensation for capacitance and series resistance.

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Electrophysiology

Recordings were made using the whole cell patch-clamp technique as previously described (Hamill et al. 1981; Wang et al. 2005). Briefly, a cover slip with the sensory neurons was placed in a recording chamber where the neurons were bathed in normal Ringers of the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, pH adjusted to 7.4 with NaOH. Whole cell currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The whole cell recording configuration was established in normal Ringer. Both capacitance and series resistance compensation (typically 80%) were used. The membrane capacitance was read directly from the patch-clamp amplifier after performing the compensation for capacitance and series resistance. The data were acquired and analyzed using pCLAMP 9.0 (Molecular Devices). Results were not corrected for the liquid junction potential. Only neurons that maintained resting membrane potentials more hyperpolarized than −45 mV were used in this study. At the end of each recording, the neuron was superfused with normal Ringer containing 100 nM capsaicin as sensitivity to this agent is believed to be an indicator of nociceptive sensory neurons (Holzer 1991). Neurons were judged to be capsaicin sensitive if they depolarized in response to 100 nM capsaicin. However, the correlation between capsaicin sensitivity and whether a neuron is a nociceptor is not absolute. Some nociceptive neurons are insensitive to capsaicin, whereas some capsaicin-sensitive neurons are not nociceptors (see Petruska et al. 2000). Therefore this agent was used to define a population of small diameter sensory neurons that could serve a nociceptive function. The results reported here were obtained from capsaicin-sensitive neurons only. All experiments were performed at room temperature (−23°C).

To isolate the potassium current (I\textsubscript{K}), neurons were superfused with a Ringer solution wherein NaCl was substituted with equimolar N-methyl-glucamine (NMG) chloride and was composed of (in mM) 140 NMG chloride, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, pH adjusted to 7.4 with KOH. Recording pipettes were pulled from borosilicate glass tubing and typically had resistances of 2–5 MΩ when filled with the following solution (in mM): 140 KCl, 5 MgCl\textsubscript{2}, 4 ATP, 0.3 GTP, 2.5 CaCl\textsubscript{2}, 5 EGTA (calculated free Ca\textsuperscript{2+} concentration of ~100 nM, MaxChelator), and 10 HEPES, adjusted pH at 7.3 with KOH. The membrane was held at −60 mV; this value was chosen so that current measurements could be ascertained at a voltage that reflected the normal resting potential in these sensory neurons. The data were acquired at 500 Hz. Leak subtraction was not used for the measurement of I\textsubscript{K} so that any effects on the holding current could be determined. Activation of I\textsubscript{K} was determined by voltage steps 300 ms in duration, which were applied at 5-s intervals in +10 mV increments from −80 to +60 mV. Steady-state inactivation of I\textsubscript{K} was measured by applying a 15-s conditioning prepulse (−100 to +20 mV in +20 mV increments) after which the voltage was stepped to +60 mV for 200 ms; a 20-s interval separated each acquisition. The fast inactivating I\textsubscript{K} known as I\textsubscript{Ks} (Connor and Stevens 1971; Neher 1971) was isolated by subtraction of the currents obtained for a conditioning prepulse to −40 mV from those obtained for a prepulse to −100 mV; additionally, a 4-s prepulse to −100 mV was applied followed by voltage steps of 500 ms that ranged from −80 to +40 mV in +20 mV increments at 15-s intervals. This was followed by an identical voltage protocol using a prepulse to −40 mV. I\textsubscript{As} was obtained by digital subtraction of these current traces as shown in Fig. 3. Inactivation of I\textsubscript{K} was determined by using a series of 4-s prepulses that ranged from −100 to −40 mV (+10 mV increments) that were immediately followed by a 200-ms step to −40 mV. The peak values of I\textsubscript{K} were determined isochronally. At the end of each recording, the neuron was exposed to 100 nM capsaicin.

To isolate the sodium current (I\textsubscript{Na}), a Ringer solution composed of (in mM) 110 NaCl, 30 TEACl, 0.1 CaCl\textsubscript{2}, 5 MgCl\textsubscript{2}, and 10 HEPES, pH adjusted to 7.4 with HCl and TEAOH was used. The osmolality was adjusted to 300–310 mosM/l using glucose. The pipettes were filled with a Cs fluoride-based solution composed of (in mM) 110 CsF, 25 CsCl, 10 NaCl, 5 MgCl\textsubscript{2}, 4 ATP, 0.3 GTP, 1 CaCl\textsubscript{2}, 10 EGTA, 10 glucose, and 10 HEPES at pH 7.3 adjusted with CsOH. The data were acquired at 10 kHz and filtered at 5 kHz. Leakage currents were subtracted by using the P4 protocol. Pipettes used for the recording of I\textsubscript{Na} had an average resistance of 2.02 M\textOmega. The peak values of I\textsubscript{Na} were obtained without the prepulse to yield the uncompensated series resistance. The mean series resistance before compensation was 5.4 ± 0.4 M\textOmega (n = 20). For the peak values of TTX-R I\textsubscript{Na}, the uncompensated series resistance gave a voltage error of approximately −2.2 ± 0.3 mV (n = 20). Activation of I\textsubscript{Na} was determined by using a holding voltage at −100 mV with voltage steps of 30 ms applied at 10-s intervals in +5-mV increments from −80 to +40 mV; a 5-s interval separated each prepulse sweep. After obtaining the control recording of total I\textsubscript{Na}, the superfusate was changed to Ringer containing 500 nM TTX and superfused for the appropriate times. TTX-S I\textsubscript{Na} was obtained by digital subtraction of the current traces recorded before and after TTX treatment; these values were used to determine the current-voltage relation for activation. Steady-state inactivation was determined by applying a 200-ms conditioning prepulse that ranged from −100 to +10 mV in +10-mV increments after which the voltage was stepped to 0 mV for 30 ms; a 5-s interval separated each prepulse sweep. However, to determine the steady-state inactivation of TTX-S I\textsubscript{Na}, the prepulse inactivation protocol described by Cummins and Waxman (1997) was used to rapidly separate TTX-S and TTX-R components. Briefly, the TTX-R I\textsubscript{Na} obtained by using a prepulse (typically −35 to −40 mV) that selectively induced inactivation of TTX-S currents was digitally subtracted from the total I\textsubscript{Na} obtained without the prepulse to yield the TTX-S I\textsubscript{Na}.

Data analysis

All values represent the means ± SE. The voltage dependence for activation of I\textsubscript{K} or I\textsubscript{Na} was determined by fitting the conductance-voltage curve with the Boltzmann relation wherein G/G\textsubscript{max} =
\[ I = \frac{G}{[1 + \exp(V_{0.5} - V_m)/k]} \], where \( G \) is the conductance \[ G = I/(V_m - E_{Rev}) \], \( G_{\text{max}} \) is the maximal conductance obtained from the Boltzmann fit under control conditions, \( V_{0.5} \) is the voltage for half-maximal activation, \( V_m \) is the membrane potential, and \( k \) is a slope factor. \( E_{\text{Rev}} \) is the reversal potential. For \( I_{K}\), the calculated \( E_{\text{K}} \) value of \(-84 \text{ mV}\) was used. For \( I_{\text{Na}} \), \( E_{\text{Na}} \) was determined for each neuron wherein the current values around the reversal potential were fit with a linear regression line to determine the voltage at which the current was zero. This reversal potential was used in the Boltzmann calculation. The average value for \( E_{\text{Na}} \) for wild-type TTX-S was \( 45.5 \pm 1.2 \text{ mV} \) \( (n = 5) \) versus \( Nf1^{-/-} \) \( 50.2 \pm 3.4 \text{ mV} \) \( (P = 0.25, \text{ Student’s } t\)-test) and for TTX-R, \( E_{\text{Na}} \) for wild-type neurons was \( 42.9 \pm 2.2 \text{ mV} \) \( (n = 8) \) versus \( Nf1^{-/-} \) \( 47.6 \pm 1.9 \text{ mV} \) \( (n = 9; P = 0.12 \text{ Student’s } t\)-test). The Boltzmann parameters were determined for each individual neuron and were used to calculate the means ± SE. Fits were performed using SigmaPlot 9.0 (Systat Software, San Jose, CA). To fit the inactivation curves, the Boltzmann relation \[ G/G_{\text{max}} = c + [(1 - c)/(1 + \exp(V_{0.5} - V_m)/k)] \] was used where \( c \) is the fraction of noninactivating current. For \( I_{K}\), \( c \) is defined as the peak current obtained at \(+60 \text{ mV}\) for the prepulse to \(+20 \text{ mV}\), whereas for \( I_{\text{Na}} \), \( c \) is defined by the peak current obtained at \( 0 \text{ mV}\) for the prepulse to \(+10 \text{ mV}\). The other parameters are as defined in the preceding text. Statistical differences between the control recordings and those obtained under various treatment conditions were determined by using either a Student’s \( t\)-test or an ANOVA. When a significant difference was obtained with an ANOVA, post hoc analyses were performed using a Tukey test. Values of \( P < 0.05 \) were judged to be statistically significant.
Chemicals

Tissue culture supplies were purchased from Invitrogen (Carlsbad, CA). Papain was purchased from Worthington Biochemical (Lake-wood, NJ), and dispase was obtained from Roche Diagnostics (Indi-anaolis, IN). All other chemicals were obtained from Sigma Chem-ical (St Louis, MO). Capsaicin was dissolved in 1-methyl-2-pyro-lidinone (MPL) to obtain a 1 mM stock solution that was then diluted with normal Ringer to yield final concentration of 100 nM. Previous studies from this laboratory have shown that MPL does not affect either $I_K$ or $I_{Na}$ (Zhang et al. 2002, 2006a,b).

RESULTS

Delayed-rectifier like potassium currents in wild-type and Nf1+/− sensory neurons are not different

Exposure of small diameter rat sensory neurons to pro-inflammatory agents suppresses $I_K$ and leads to enhanced excitability (Evans et al. 1999; Nicol et al. 1997; Zhang et al. 2002). Because neurons isolated from Nf1+/− mice exhibited augmented excitability compared with wild-type mice (Wang et al. 2005), experiments were performed to determine whether $I_K$ recorded from Nf1+/− neurons was different from that of wild-type neurons. In these recordings from mouse neurons, two distinct phenotypes of $I_K$ were observed. One exhibited rapid activation with little time-dependent inactivation, whereas the other type showed rapid activation with more rapid inactivation kinetics. Both phenotypes of $I_K$ were observed in each genotype. Figure 1A, top, shows representative current traces that exhibited rapid activation with little time-dependent inactivation. These currents were obtained from a Nf1+/− neuron wherein the peak $I_K$ was 7.21 nA, which occurred at the end of the voltage step. Figure 1A, bottom, demonstrates the results obtained for steady-state inactivation of $I_K$ in this neuron. Note that there is little reduction in the current during the prepulses even after 15 s. Figure 1B, top, illustrates representative currents that showed rapid activation with faster inactivation kinetics and were obtained from a different Nf1+/− neuron. The peak $I_K$ measured at +60 mV was 10.49 nA and occurred at 70 ms. The steady-state inactivation for this neuron is shown in Fig. 1B, bottom. Note the large amount...
of current decay during the prepulse. Figure 1C, left, compares
the inactivation kinetics for the traces obtained for the step to
+60 mV after prepulses to −100 mV from the neurons shown to
A and B. The right panel represents the subtraction of the
slowly inactivating trace (A) from the more rapidly inactivating
trace (B); this yields a rapidly inactivating current that has
many of the hallmarks of I_A. The decay of the current shown in
Figure 1C, right, was fit with a double exponential where the
slow τ1 was 97 ms and the fast τ2 was 33 ms (A1 was 1,696,
A2 was 1,659, correlation coefficient was 0.989). These time
constants are consistent with the values described for those
obtained for isolated I_K currents (see following text). Thus it is
possible that the more rapid inactivation of I_K as typified by the
neuron shown in B results from the contribution of I_A-type
currents to the total outward I_K.

To minimize the variance of currents obtained from these
different neurons, currents were normalized for cell surface
area and expressed as current density (pA/pF). The current
density-voltage relations for 11 wild-type and 13 Nf1+/−
neurons are summarized in Fig. 2A. The current density-
voltage relations for the peak (left) and steady-state (right) I_Ks
in wild-type and Nf1+/− neurons were nearly identical (the
average peak current density in wild-type and Nf1+/− neurons
was 569.2 ± 96.3 and 570.3 ± 71.1 pA/pF, respectively, for
the step to +60 mV). The current values were transformed to
conductance (G), the conductance-voltage relation was fit with
the Boltzmann relation, and the conductance for each neuron
was then normalized to the maximal value of G (G_max
obtained from the fit. The G/G_max-voltage relation is summarized
in Fig. 2B and indicates there is little difference in either the
peak or steady-state values between the two genotypes. The
Boltzmann fitting parameters, V_0.5 and k, for the peak and
steady-state measurements are shown in Table 1. Similar to I_K
activation, there were no significant differences in the proper-
ties of steady-state inactivation for I_K between neurons of the
two genotypes (see Fig. 2C). For example, in wild-type cells,
I_K was inactivated by 70.2 ± 5.4% (n = 7) after the condi-
tioning prepulse to +20 mV, which was not different from the
74.5 ± 7% (n = 6) in Nf1+/− neurons. The Boltzmann
parameters for inactivation are summarized in Table 1. Based
on the close overlap between the relations in the wild-type and
Nf1+/− neurons for the current-voltage, the G/GMax-voltage,
and steady-state inactivation it seems unlikely that either the
total I_K or the voltage dependence of activation/inactivation of
I_K is altered in Nf1+/− neurons and therefore differences in I_K
do not account for the enhanced excitability previously shown
(Wang et al. 2005).

A-type I_K does not contribute to the augmented excitability of
Nf1+/− neurons

The rapidly inactivating type of I_K known as I_A controls
neuronal excitability by its modulation of the frequency of
firing (Connor and Stevens 1971; Neher 1971). Because
Nf1+/− neurons fire APs at a higher frequency when stimu-
lated by a depolarizing ramp of current compared with neurons
from wild-type mice (Wang et al. 2005), experiments were
performed to determine whether I_K currents were different in
neurons from the two genotypes. Interestingly, not every small
diameter, capsaicin-sensitive sensory neurons of either geno-
type exhibited an obvious I_A-type current. As shown in Fig. 3,
I_A was isolated (described in METHODS) by subtracting the
currents obtained at a holding potential of −100 mV (A) from
those obtained at −40 mV (B); the results for a representative
wild-type neuron are shown in C. All isolated I_K traces,
regardless of genotype had a peak current that rapidly decayed
time and then reached a stable plateau (Fig. 3C). When the
currents were normalized to cell capacitance, the pA/pF-volt-
age relations were not different (data not shown). In wild-type
neurons, the peak value I_A was 238.7 ± 94.7 pA/pF (for
the step to +40 mV, n = 14) and was not significantly differ-
ent from that in Nf1+/− neurons (340.9 ± 114.7 pA/pF, n = 11,
P = 0.49 Student’s t-test). The G/G_max-voltage relations are
summarized in Fig. 3D and demonstrate the similarities in
voltage dependence for both activation and inactivation of I_A in
these two genotypes. The relations for G/G_max-voltage were
fitted by the Boltzmann relation and are summarized in Table
1 wherein the values for V_0.5 and k for the activation of I_A in
the two genotypes were not different.

To determine whether altered kinetics of I_A in Nf1+/−
neurons could account for the enhanced AP firing, the decay of
the current for the voltage step to +40 mV was fitted with an
exponential function by using Clampfit 9. In six wild-type
neurons, the decay kinetics were well fitted with a double
exponential having a fast tau (τ) of 34 ± 13 ms and a slow τ
of 246 ± 75 ms. Similarly, in four of five Nf1+/− neurons, the
deay of I_A was well fitted wherein the fast τ was 52 ± 18 ms
and the slow τ was 470 ± 151 ms. These values were not
significantly different (P = 0.44 and 0.29 for the fast and slow
τs, respectively, Student’s t-test). In one of the five Nf1+/−
cells, the decay phase was best fitted with a single exponential
wherein τ was 160 ms and was within the range observed for
the slow τs for the two exponential fits. Taken together, it is
unlikely that I_A contributes to the augmented neuronal excit-
ability observed previously for Nf1+/− neurons.

TTX-R and TTX-S I_Na are augmented in Nf1+/− neurons

The properties of sodium channels, such as distribution,
density, trafficking as well as the threshold of activation and
repriming characteristics can all influence the firing patterns of

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<th>TABLE 1. Boltzmann fitting parameters for I_K</th>
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All values are means ± SE.
The average peak current density in Nf1+/− neurons was 

\(-1.047 ± 85\) pA/pF \((n = 6)\) for the step to 

\(-20\) mV and was significantly larger than the 

\(-721 ± 118\) pA/pF for the wild-type neurons \((n = 5,\ Student’s t-test)\). Figure 5A, right, 

represents the \(G/G_{\text{max}}\)-voltage relation for both activation and 

inactivation. These results show that the voltage dependence 

for activation of TTX-S \(I_{\text{Na}}\) in Nf1+/− neurons was not 

different from that in wild-type neurons (see Table 2). 

However, the \(V_{1/2}\) for steady-state inactivation of TTX-S \(I_{\text{Na}}\) 

determined in Nf1+/− neurons was shifted to more depolarized 

values by 12 mV (see Table 2) although the value of \(k\) was 

unchanged. These results suggest that the rightward shift in 

the inactivation for TTX-S \(I_{\text{Na}}\) may, in part, contribute to 

the greater current densities in Nf1+/− neurons.

In addition, augmentation of TTX-R \(I_{\text{Na}}\) has been proposed 

to be an important underlying mechanism in neuronal sensiti-

zation (England et al. 1996; Gold et al. 1996, 2002; Jeftinija 

1994; Tanaka et al. 1998; Waxman et al. 1999; Zhang et al. 

2002). Therefore the possible contribution of TTX-R \(I_{\text{Na}}\) to the 

augmented \(I_{\text{Na}}\) measured from Nf1+/− neurons was examined. 

TTX-R \(I_{\text{Na}}\) was isolated by using the voltage protocols 

described in the preceding text in the presence of 500 nM TTX. 

The current density of TTX-R \(I_{\text{Na}}\) in Nf1+/− neurons was 

significantly larger than that measured in wild-type neurons.

**FIG. 3.** The rapidly inactivating \(I_{\text{Na}}\) current in wild-type and 

Nf1+/− neurons is not different. Representative current traces 

for a wild-type neuron \((\text{left})\) was 

isolated by using the voltage protocols de-

scribed in the preceding text in the presence of 500 nM TTX. 

The current density of TTX-R \(I_{\text{Na}}\) in Nf1+/− neurons was 

significantly larger than that measured in wild-type neurons.

sensory neurons (Blair and Bean 2002, 2003; Herzog et al. 

2001; Schild and Kunze 1997; Waxman et al. 1999). To 
determine whether the enhanced firing of Nf1+/− neurons 

results from alterations of \(I_{\text{Na}}\), studies were performed to 

measure the properties of this current in both wild-type and 

Nf1+/− sensory neurons. In small diameter sensory neurons, 
tetrodotoxin (TTX) can be used to separate total \(I_{\text{Na}}\) into those 
currents that are sensitive (TTX-S) and resistant (TTX-R) to 
blockage by this toxin (Caffrey et al. 1992; Campbell 1993; 
Elliott and Elliott 1993; Ogata and Tatebayashi 1992; Roy and 
Narahashi 1992). Representative traces for total, TTX-S, and 
TTX-R \(I_{\text{Na}}\) in wild-type neurons and Nf1+/− neurons are 
shown in Fig. 4. For the wild-type neuron (left), the peak 
value for the total \(I_{\text{Na}}\) was 

\(-10.41\) nA and occurred at 

\(-15\) mV, whereas the peak current for the Nf1+/− neuron (right) 

was 

\(-12.38\) nA and occurred at 

\(-10\) mV. The current density for 

TTX-S \(I_{\text{Na}}\) in Nf1+/− neurons was significantly larger compared 

with the values determined from wild-type neurons (Fig. 5A, left).
As illustrated in Fig. 5B, left, the average peak value of TTX-R \( I_{\text{Na}} \) measured for the step to \(-5\) mV in \( \text{Nf1}^{+/+} \) neurons was \(-442 \pm 55\) pA/pF \((n = 9),\) which was significantly larger than that in wild-type neurons \((-287 \pm 52\) pA/pF, \(n = 8,\) Student’s \(t\)-test). The \( G/G_{\text{max}} \)-voltage relations for activation and inactivation in the wild-type and \( \text{Nf1}^{+/+} \) sensory neurons are shown in Fig. 5B, right. The voltage dependence for activation of TTX-R \( I_{\text{Na}} \) in \( \text{Nf1}^{+/+} \) neurons is nearly the same as wild-type neurons (see Table 2). However, the \( V_{0.5} \) for TTX-R \( I_{\text{Na}} \) determined for steady-state inactivation in \( \text{Nf1}^{+/+} \) neurons was shifted to more depolarized values by 6 mV (see Table 2) although the value of \( k \) was unchanged.

Because the inactivation of TTX-S and TTX-R \( I_{\text{Na}} \) in \( \text{Nf1}^{+/+} \) neurons was altered whereas the activation was not, this depolarization shift in the inactivation of \( I_{\text{Na}} \), resulted in a larger window current suggesting that over this voltage range there are more conducting sodium channels. To examine a potential consequence of this idea, the levels of the persistent \( I_{\text{Na}} \) were measured. In recordings to assess the inactivation of \( I_{\text{Na}} \), measurements of the current 100 ms after the onset of the prepulse indicated that there was greater current in the \( \text{Nf1}^{+/+} \) neurons compared with the wild-type (see Fig. 6). Representative traces of the persistent \( I_{\text{Na}} \) are shown in Fig. 6A where the vertical bar indicates the point at which the current was determined; the peaks have been truncated to better illustrate the persistent component. The results obtained under control recording conditions (total \( I_{\text{Na}} \)) and after treatment with TTX (TTX-R \( I_{\text{Na}} \)) for the two genotypes are summarized in Fig. 6B.

In wild-type neurons, the persistent \( I_{\text{Na}} \) had an average value of \(-0.42 \pm 0.08\) nA \((n = 10,\) data not shown) and peaked at \(-40\) mV, whereas in \( \text{Nf1}^{+/+} \) neurons the persistent current had an average value of \(-0.68 \pm 0.32\) nA \((n = 12)\) that peaked at \(-20\) mV. Values for the persistent \( I_{\text{Na}} \) in wild-type neurons are consistent with previous observations (Baker and Bostock 1997; Cepeda et al. 1995; Crill 1996; French et al. 1990; Kiernan et al. 2003; Wu et al. 2005). For the TTX-R persistent \( I_{\text{Na}} \), there was no difference between the two genotypes. In wild-type neurons, the current had a peak value of \(-0.48 \pm 0.15\) nA \((n = 12,\) data not shown) at \(-40\) mV and in \( \text{Nf1}^{+/+} \) neurons the average value was of \(-0.51 \pm 0.11\) nA \((n = 12)\) that peaked at \(-40\) mV. To account for cell-to-cell variance in the levels of total \( I_{\text{Na}} \), the persistent \( I_{\text{Na}} \) was normalized as the percentage of the maximal transient current for each neuron. When measured as the percent of the peak transient current, the persistent \( I_{\text{Na}} \) was significantly larger (Student’s \(t\)-test) for the \(-30-\) and \(-20-mV\) steps compared with wild-type neurons (left); however, there was no difference for TTX-R \( I_{\text{Na}} \) (right). For wild-type neurons at \(-30\) mV, the persistent \( I_{\text{Na}} \) was 3.2 \pm 0.6% of the peak \((n = 10,\) range: \(-0.5-6.0\%\)), whereas for \( \text{Nf1}^{+/+} \) neurons, the persistent \( I_{\text{Na}} \) was 7.8 \pm 1.7% \((n = 12,\) range: 2.6-17.6%). Not all \( \text{Nf1}^{+/+} \) neurons exhibited an augmented persistent current; 3 of the 12 \( \text{Nf1}^{+/+} \) neurons had

![Graphs showing voltage dependence for activation and inactivation of sodium current in wild-type and 

**Fig. 5.** The current density of both TTX-R and TTX-S \( I_{\text{Na}} \) in \( \text{Nf1}^{+/+} \) neurons is significantly larger than those in wild-type neurons. A: the current density for TTX-S \( I_{\text{Na}} \) was significantly larger in the 6 \( \text{Nf1}^{+/+} \) neurons compared with 5 wild-type neurons. The values were significantly different between \(-20\) and \(+20\) mV \((P < 0.05,\) Student’s \(t\)-test). Right: the \( G/G_{\text{max}} \)-voltage relation for TTX-S \( I_{\text{Na}} \) in these neurons and shows that there is no difference in the voltage-dependence of activation; however, the steady-state inactivation of the 12 \( \text{Nf1}^{+/+} \) neurons was shifted to more depolarizing potentials compared with the 10 wild-type neurons. The values for inactivation were significantly different for the prepulse voltages of \(-50\) and \(-40\) mV \((P < 0.05,\) Student’s \(t\)-test). The continuous lines through the points are the Boltzmann fits for the wild-type (black line) and \( \text{Nf1}^{+/+} \) (gray line) neurons. B: left; the current density for TTX-R \( I_{\text{Na}} \) obtained from 9 \( \text{Nf1}^{+/+} \) neurons was significantly larger compared with 8 wild-type neurons. The current values were significantly different between 0 and \(+25\) mV \((P < 0.05,\) Student’s \(t\)-test). Right: the \( G/G_{\text{max}} \)-voltage relation for TTX-R \( I_{\text{Na}} \) in these neurons and shows that there is no difference in the voltage dependence of activation; however, the steady-state inactivation of the \( \text{Nf1}^{+/+} \) neurons was shifted to more depolarizing potentials. The continuous lines through the points are the Boltzmann fits for the wild-type (black line) and \( \text{Nf1}^{+/+} \) (gray line) neurons. The values of \( G/G_{\text{max}} \) for the \( \text{Nf1}^{+/+} \) neurons were significantly different from the wild-type neurons for prepulse voltages from \(-60\) to \(-30\) mV \((P < 0.05,\) Student’s \(t\)-test). The fitting parameters are described in Table 2.
ties for either the rapidly inactivating $I_{Na}$ in neurofibromin-deficient Schwann cells (SCs). Previous work showed that $I_{Ks}$ were upregulated in SCs, which may play an important role in the tumorogenesis of neurofibromas. However, the currents modulated as a consequence of these mutations appeared to be different. Both normal isolated human SCs and neurofibromin-deficient human MNP NST cell lines exhibited a prominent rapidly inactivating $I_{K}$, the densities of which were the same; however, in tumor cells, $I_{K}$ exhibited slower inactivation kinetics (Fieber 1998). These mutant cells exhibited a TEA- and 4-AP-sensitive noninactivating type $I_{K}$ that was not observed in the normal SCs. In contrast, SCs isolated from $Nf1^{-/-}$ embryonic mice had a greater current density for $I_{K}$-like currents compared with wild-type mice (Xu et al. 2002). The origins for the differences in the types of $I_{K}$ in the neurofibromin-deficient SCs are unclear; they may arise from differences in species (human vs. mouse), developmental stage of the SCs (adult vs. embryonic) or be related to other alterations intrinsic to the malignant tumor cells. We found no differences in $I_{K}$ for sensory neurons isolated from wild-type and $Nf1^{-/-}$ mice, suggesting that there are significant differences in the regulation of membrane currents by neurofibromin in SCs and sensory neurons.

Our observations that the current density for $I_{Na}$ was significantly larger in $Nf1^{+/+}$ sensory neurons raise an important question as to whether the increased current densities result from differences in the levels of expression of sodium channels or in altered modulatory activities of signaling pathways. It is well documented that inflammatory mediators such as prostaglandin E2, 5-HT, and endothelin-1 augment the peak amplitude of TTX-R $I_{Na}$ by shifting the voltage dependence for activation to more hyperpolarized voltages (England et al. 1996; Gold et al. 1996; Zhou et al. 2002). Because the voltage dependences for activation of TTX-S or TTX-R $I_{Na}$ were not different between the genotypes, our results imply that the larger current densities in $Nf1^{+/+}$ neurons did not result from posttranslational modifications mediated by intracellular signaling pathways. However, tumor necrosis factor α, via activation of p38 MAP kinase, enhanced TTX-R $I_{Na}$ without altering the voltage dependences for either activation or inactivation in sensory neurons isolated from the mouse (Jin and Greane 2006). In addition to the transient TTX-S and TTX-R $I_{Na}$, sensory neurons from $Nf1^{+/+}$ mice exhibited significantly increased persistent $I_{Na}$ as measured 100 ms after the onset of prepulse voltage steps. For the persistent $I_{Na}$ (total $I_{Na}$ conditions), the currents were significantly enhanced by about twofold at −30 and −20 mV. Our previous study (Wang et al. 2005) determined that the firing threshold for $Nf1^{+/+}$ neurons was approximately −32 mV compared with −26 mV for the wild-type neurons (based on APs evoked with a depolarizing current ramp). Thus over this voltage range, an augmented persistent $I_{Na}$ that overlapped the firing threshold could have a significant impact on neuronal excitability. Consistent with this notion, previous reports demonstrated that activation of a persistent $I_{Na}$ led to increased AP firing (Cepeda et al. 1995; Crill 1996; Do and Bean 2003; French et al. 1990; Stafstrom et al. 1982, 1984; Taddeis and Bean 2002; Theiss et al. 2007; Wu et al. 2005). Conditions associated with the $Nf1^{+/+}$ genotype could produce transcriptional modifications that alter levels of expression for critical modulators of channel activity. Consistent with this idea are studies examining the interactions

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\[\text{A} \quad Nf1^{+/+} \quad Nf1^{-/-} \]

\[\text{B} \quad \text{Total } I_{Na} \quad \text{TTX-R } I_{Na} \]

**FIG. 6.** $Nf1^{+/+}$ sensory neurons exhibit larger persistent $I_{Na}$ compared with wild-type neurons. A: representative current traces for the total $I_{Na}$ obtained from a wild-type neuron (left) and from an $Nf1^{+/+}$ neuron (right). The currents were evoked by a series of 200 ms voltage pulses that ranged from −120 to +10 mV. The values for the persistent $I_{Na}$ were obtained 100 ms after the onset of the prepulse (noted by the vertical bar labeled 100 ms). The peak currents have been truncated for clarity of the persistent $I_{Na}$. B: summary for the voltage dependence of the persistent $I_{Na}$ measured as the percent of the maximum transient current for wild-type ($n=10$) and $Nf1^{-/-}$ ($n=12$) neurons. Left: the persistent current for the total $I_{Na}$; right: current remaining after exposure to TTX. *, significant difference between wild-type and $Nf1^{-/-}$ neurons ($P<0.05$).
between the conducting $\alpha$ subunit of TTX-S sodium channels and the auxiliary $\beta$ subunits. Overexpression of $\beta_2$, $\beta_3$, or $\beta_4$ with Nav1.1 or Nav1.2 in heterologous expression systems had little effect on peak current amplitudes; however, expression of these $\beta$ subunits gave rise to a threefold increase in the persistent $I_{Na}$ (Aman et al. 2009; Qu et al. 2001). The $\beta_3$ subunit was expressed in high levels in small and medium diameter sensory neurons of the DRG (Qu et al. 2001). Thus the elevated levels of persistent $I_{Na}$ observed in the $\text{Nf1}^+/−$ sensory neurons might result from increased expression of key $\beta$ subunits. Currently very little is understood about potential differences in the efficacies of different signaling cascades in sensory neurons of $\text{Nf1}^+/−$ mice with reduced activities of neurofibromin. Thus the regulation by intracellular signaling pathways of $I_{Na}$ and other membrane currents important in controlling excitability in $\text{Nf1}^+/−$ mice could be an important area for future investigation.

Previous studies have established important connections between neurofibromin and cyclic AMP signaling. In PC12 cells deficient in PKA, treatment with NGF increased the levels of both mRNA and saxitoxin binding for type II sodium channels; however, recordings showed no detectable $I_{Na}$ in these cells, indicating that PKA was critical in the expression of functional channels (Ginty et al. 1992). Although early studies demonstrated that NGF rapidly increased intracellular levels of cyclic AMP (Higuchi et al. 2003; Knipper et al. 1993), establishing a causal link has been elusive. Recent studies in PC12 cells showed that NGF elevated cyclic AMP levels through activation of soluble rather than transmembrane adenylcycl cyclase and that this cyclic AMP increased the activity of the small G protein, Rap1 (Stressin et al. 2006). Furthermore, enhancement of $I_{K}$ in neurofibromin-deficient SCs was dependent on activation of the cyclic AMP signaling pathway (Fieber 1998; Xu et al. 2002), suggesting an important interaction between neurofibromin and cyclic AMP. However, the role of neurofibromin in regulating the activity of the cyclic AMP pathway appears to depend on cell type. In SCs, cyclic AMP was mitogenic and cyclic AMP levels were negatively regulated by neurofibromin (Kim et al. 2001), whereas in astrocytes, cyclic AMP was anti-mitogenic and neurofibromin positively regulated cyclic AMP production (Dasgupta et al. 2003). Likewise, in isolated cortical neurons, increased cyclic AMP elevated the levels of phosphorylated Erk and activation of Rap1, whereas in cortical astrocytes, this elevation in cyclic AMP decreased phosphorylated Erk (Dugan et al. 1999). The exact nature of the interaction between neurofibromin and adenyl cycl cyclase remains unclear.

Taken together, the role of neurofibromin in regulating/modulating the activities of a variety of signaling pathways is poorly understood. It is difficult to mechanistically infer the regulation of intracellular transduction cascades based on results obtained in other cell systems. Thus understanding the role of neurofibromin in regulating the expression levels of ion channels as well as the modulation of their activity will be the focus of future investigations. A better appreciation of the mechanisms underlying the enhanced neuronal excitability in sensory neurons with the $\text{Nf1}$ mutation, similar to the human disorder NF1, and how this sensitization may be modified in inflammation or injury could lead to better therapies for the painful conditions associated with NF1.

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Disclosures

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References


Guha A, Lau N, Huvar I, Gutmann D, Provias J, Pawson T, Boss G.
Klesse LJ, Parada LF.
Jin X, Gereau RW 4th.
Bradykinin excites tetrodotoxin-resistant primary afferent fibers.
Jeftinija S.
Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA.

Gold MS, Reichling DB, Shuster MJ, Levine JD.
Ginty DD, Fanger GR, Wagner JA, Maue RA.
Friedman JM.

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Cognitive impairment in neurofibromatosis type 1.
Herzog RI, Cummins TR, Waxman SG.


Y. WANG, J.-H. DUAN, C. M. HINGTGEN, AND G. D. NICOL

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