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Ras signaling pathways mediate NGF-induced enhancement of excitability of small-diameter capsaicin-sensitive sensory neurons from wildtype but not *Nf1*^{+/-} mice

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Abstract

Nerve growth factor (NGF) activates multiple downstream effectors, including Ras, phosphoinositide-3 kinase, and sphingomyelins. However, pathway mediating the NGF-induced augmentation of sensory neuronal excitability remains largely unknown. We previously reported that small-diameter sensory neurons with a heterozygous mutation of the *Nf1* gene (*Nf1*^{+/-}) exhibited increased excitability. The protein product of the *Nf1* gene is neurofibromin, a guanosine triphosphatase-activating protein (GAP) for p21ras (Ras) that accelerates the conversion of active Ras-GTP to inactive Ras-GDP. Thus, *Nf1*^{+/-} cells have augmented basal and stimulated Ras activity. To investigate whether NGF-induced increases in excitability of small-diameter sensory neurons are dependent on Ras signaling, an antibody that blocks the activation of Ras, Y13-259, was perfused into the cell. Under these conditions, the enhanced excitability produced by NGF was suppressed in wildtype neurons but the excitability of *Nf1*^{+/-} neurons was unaltered. In addition, expression of a dominant-negative form of Ras abolished the ability of NGF to increase the excitability of small-diameter sensory neurons. These results demonstrate that NGF enhances excitability of small-diameter sensory neurons in a Ras-dependent manner while the consequences of decreased expression of neurofibromin cannot be restored by blocking Ras signaling; suggesting that Ras-initiated signaling pathways can regulate both transcriptional and posttranslational control of ion channels important in neuronal excitability.

Keywords

dorsal root ganglia; nerve growth factor; neurofibromin; sensitization

Neurofibromatosis type 1 (NF1) results from a heterozygous mutation of the *NF1* gene (*NF1*^{+/-}), encoding the protein product, neurofibromin. Neurofibromin facilitates conversion from active Ras (Ras-GTP) to its inactive form (Ras-GDP) by serving as a GTPase-activating protein (GAP; 1). Decreased levels of neurofibromin result in increased Ras-GTP in human NF1-related tumors (2) and in *Nf1*^{+/-} mast cells (3), *Nf1*^{-/-} Schwann

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cells (4) and *Nf1*^{-/-} sensory neurons (5,6). Ras-GTP and its downstream signaling cascades modulate the activity of a number of ionic currents that regulate neuronal excitability (7,8,9). Indeed, sensory neurons isolated from mice having a heterozygous mutation of the *Nf1* gene (*Nf1*^{+/-}) exhibit significantly higher levels of action potential (AP) firing to a ramp of depolarizing current than do their wildtype littermates (10).

The Ras signaling cascade can be activated by many growth factors, most notable nerve growth factor (NGF). We previously showed that the number of APs elicited by a ramp of depolarizing current was increased after treatment with NGF (10,11). Because Ras modulates membrane currents that underlie the generation of APs (12,13,14), one could infer that elevated Ras-GTP levels confer increased excitability leading to enhanced painful sensation in people with NF1 and NGF-induced neuronal sensitization. However, in sensory neurons isolated from *Nf1*^{+/-} mice, NGF failed to further enhance the elevated AP firing (10). These results raise two important questions: 1) is NGF-induced increase in AP firing observed in wildtype mice mediated by the Ras signaling cascade and 2) can the elevated excitability exhibited by *Nf1*^{+/-} sensory neurons be reversed by inhibition of Ras activity?

Isolation of sensory neurons from 1–2 month old wildtype C57BL/6J and *Nf1*^{+/-} C57BL/6J littermates (15) was performed as previously (10). All animals were housed, bred, and had free access to food and water in the Indiana University Laboratory Animal Research Center and used in procedures approved by the Animal Use and Care Committee of the Indiana University School of Medicine. Isolated cells were plated onto plastic cover slips previously coated with poly-D-lysine and laminin. Cells were maintained in F-12 medium 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. Tissue culture supplies were purchased from Invitrogen (Carlsbad, CA). Cells were used within 12–48 hr.

Recordings were made using the whole-cell patch-clamp technique as previously described (10). Neurons were bathed in normal Ringers (mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 10 glucose, pH 7.4. Recording pipettes were filled with the following (mM): 140 KCl, 5 MgCl₂, 4 ATP, 0.3 GTP, 2.5 CaCl₂, 5 EGTA and 10 HEPES, pH 7.3. Whole-cell voltages were recorded with an Axopatch 200B amplifier and analyzed using pCLAMP 9.0 (Molecular Devices, Sunnyvale, CA). Only neurons that maintained resting membrane potentials more hyperpolarized than -45 mV were used. At the end of each recording, the neuron was superfused with 100 nM capsaicin, as an indicator of nociceptive phenotype (16). Results presented are only from neurons that depolarized with capsaicin exposure. All experiments were performed at ~23 °C.

The construction of the dominant-negative Ras lentivirus was recently published (17). Briefly, we used a lentivirus subcloned with a CMV promotor, internal ribosome entry site (IRES) and enhanced green fluorescent protein (eGFP) to infect mice (3–4 weeks old) by intrathecal (i.t.) injection. Under light halothane anesthesia, the dorsal fur of each mouse was shaved and a 31-gauge needle was inserted into the subarachnoid space between the L4 and L5 vertebrae. Correct positioning of the needle tip was confirmed by tail flick. Approximately 10⁹–10¹⁰ lentiviral particles (5 µl) of the dominant-negative Ras construct (CMV-Ras(17N)-IRES-EGFP) or eGFP alone (CMV-IRES-EGFP) was injected. Each animal received a total of 3 injections on alternate days. Five to six weeks later, L4-L6 DRG were collected and isolated for recording as above.

All values represent the mean ± standard error of the mean (SEM). Statistical differences were determined by using t-tests, analysis of variance (ANOVA), or a repeated-measure

ANOVA (RM ANOVA). Significant differences obtained with ANOVA were followed by Dunnett's post hoc analysis. Values of $P < 0.05$ were judged to be statistically significant.

As shown in Fig. 1A (left) and summarized in Fig. 1B, internal perfusion with 30 $\mu\text{g/ml}$ IgG did not alter the generation of APs evoked by a ramp of depolarizing current. IgG did not alter the resting membrane potential (control -53.5 ± 3.3 mV vs. IgG 20 min -50.6 ± 2.2 mV, $n=5$, $P=0.50$ paired t-test). After internal perfusion of IgG for 15 min, a 5 min exposure to 100 ng/ml NGF (Harlan Bioproducts for Science, Indianapolis, IN) significantly increased the number of evoked APs by about two-fold (Fig. 1A, right and Fig 1B); confirming our previous results demonstrating increased excitability after treatment with NGF (10).

To determine whether the enhanced excitability of wildtype neurons produced by NGF was dependent on the Ras signaling pathway, anti-v-H-Ras (Y13-259, EMD Chemicals Inc., San Diego, CA), a mouse monoclonal antibody that neutralizes the biological and biochemical activities of vertebrate Ras (18,19), was internally perfused via the recording pipette (30 $\mu\text{g/ml}$). The time necessary to achieve a "steady-state" concentration of intracellular Y13-259 was calculated as described by Pusch and Neher (20) and estimated to be 5–9 min. Thus, we chose to internally perfuse Y13-259 for 15 min before assessing the sensitizing effects of NGF. Figure 1B shows that Y13-259 had no effect on the number of evoked APs over 20 min. Y13-259 had no effect on the resting membrane potential (control -57.1 ± 3.8 mV vs. Y13-259 20 min -56.2 ± 2.4 mV, $n=7$, $P=0.84$ paired t-test). However, in the presence of Y13-259, exposure to NGF failed to augment the number of current-evoked APs. These results indicate that the Ras blocking antibody suppressed NGF's ability to enhance neuronal excitability.

Our previous work showed that *Nf1*^{+/-} sensory neurons exhibit augmented sodium current densities (21) and exposure to the same ramp current (1000 pA) elicited more APs from *Nf1*^{+/-} sensory neurons than wildtype neurons (10). To test the effects of the Ras blocking antibody at similar levels of AP production, variable ramp amplitudes were set to evoke between 5–7 APs. Using this protocol, the number of APs evoked by the current ramp and the corresponding firing thresholds were not different between neurons from the two genotypes (Fig. 1C). Although the average ramp amplitude necessary to generate 5–7 APs was not significantly different between the two genotypes (likely because of the large variance), there is a clear trend towards a lower current necessary to evoke AP firing in *Nf1*^{+/-} neurons. In addition, the rheobase was significantly lower whereas the membrane resistance (R_m) and the resistance at threshold (R_{Th}) were higher in *Nf1*^{+/-} neurons (Table 1). A measure of AP output per stimulus input (number of APs/ramp amplitude) demonstrated a significant two-fold increase in the *Nf1*^{+/-} neurons (Table 1) and supports our previous findings that the excitability of *Nf1*^{+/-} sensory neurons are indeed higher than wildtype sensory neurons. These observations raise the question as to whether suppression of Ras activity by Y13-259 could reverse the elevated excitability exhibited by *Nf1*^{+/-} neurons. As shown in Fig. 1C, neither IgG or Y13-259 altered the number of evoked APs in *Nf1*^{+/-} neurons. In addition, exposure to NGF failed to increase the number of APs in antiserum-treated *Nf1*^{+/-} neurons, consistent with our previous findings wherein NGF did not augment AP firing in *Nf1*^{+/-} neurons (10). Table 2 summarizes that Y13-259 had no significant effects on the parameters of excitability for 11 *Nf1*^{+/-} neurons after 20 min of internal perfusion. To exclude the possibility that the amplitude of the current ramp was insufficient to detect subtle changes in excitability, ramps that evoked 10–15 APs under control conditions were used. Y13-259 did not alter the number of APs elicited by the larger ramp in the *Nf1*^{+/-} neurons (control 12.6 ± 3.4 APs vs. Y13-259 12.0 ± 3.0 APs, $n=6$). These results suggest that direct and acute neutralization of Ras activity did not reduce neuronal excitability in *Nf1*^{+/-} neurons. The lack of an acute effect of Ras inhibition would

suggest that long-term enhancement of Ras activity leads to downstream transcriptional or protein expression changes not readily reversed by the neutralizing antibody.

To further investigate the role of NGF-mediated Ras activation in the increased excitability of small-diameter sensory neurons, lentiviral constructs containing a dominant-negative Ras-IRES-eGFP or eGFP, alone, were injected into the intrathecal space of wildtype mice. Five to six weeks after viral injection, sensory neurons from L4–L6 DRGs were isolated. Recordings were obtained from only green fluorescing neurons <25 μm in diameter. Figure 2 shows a representative micrograph of isolated sensory neurons after infection with the lentiviral eGFP construct (A: bright-field image, B: fluorescence image from the same field). Figure 2C is a current clamp recording obtained from the neuron indicated by the arrow in Fig. 2B under control conditions and after a 4 min exposure to 100 ng/ml NGF where the number of evoked APs was increased to 12. Recordings from six sensory neurons (all capsaicin-sensitive small-diameter cells) infected with the eGFP construct are summarized in Fig. 3A/B. Interestingly, three of six were not sensitized by NGF (A) whereas three others exhibited a ~4-fold increase in evoked APs after a 6 min exposure to NGF (B). Recordings obtained from neurons infected with the lentivirus dominant-negative Ras-eGFP construct are summarized in Fig. 3C. The dominant-negative Ras-eGFP construct did not affect the ability of these nine neurons to fire APs, but NGF failed to augment the number of evoked APs in these neurons. These results are consistent with the results obtained with Y13-259 wherein the sensitizing actions of NGF were also inhibited and together demonstrate that NGF causes an increase in excitability via a Ras-dependent signaling pathway in sensory neurons isolated from wildtype mice.

In contrast, Y13-259 did not reverse the elevated excitability exhibited by sensory neurons isolated from *Nf1*^{+/-} mice. Since functional levels of neurofibromin, a Ras GAP, are reduced in *Nf1*^{+/-} cells, it was expected that inhibition of Ras signaling with Y13-259 would restore the measures of excitability to wildtype levels. That acute inhibition of Ras in *Nf1*^{+/-} neurons did not reverse the increased excitability suggests that developmental loss of neurofibromin leads to long-lasting changes in transcription or protein expression, which are beyond acute correction of Ras activity. Consistent with this idea, Schwann cells isolated from DRG of embryonic *Nf1*-null mice exhibit enhanced I_A -type potassium current (22) but a five-day exposure to a farnesyltransferase inhibitor, which blocks Ras activity, or expression of dominant-negative Ras failed to reverse the increased I_A current. The cellular mechanisms responsible for this altered sensitivity or expression in cells with diminished neurofibromin are presently unknown.

NGF, via activation of the TrkA neurotrophin receptor, is known to activate the Ras signaling pathway (23,24). This notion is supported by the findings that NGF-induced increases in phosphorylated ERK levels were Ras-dependent in sensory neurons isolated from DRG (12,25). Not all actions of NGF on sensory neurons are mediated by Ras activation. For example, NGF-induced increases in calcitonin gene-related peptide (CGRP) content are mediated by Ras activation but the acute actions of NGF to increase stimulus-evoked release of CGRP are not Ras-dependent (17). Interestingly, modulation of Ras activity can impact membrane excitability both acutely and chronically. Injection of Ras into Hermissenda photoreceptors decreased an I_A -type potassium current within 30 min (26). In differentiated NG108-15 cells, overexpression of dominant-negative Ras blocked AP firing compared to the controls or cells overexpressing a constitutively-active form of Ras (27). K_{ATP} channels expressed in HEK293 cells can be activated by nitric oxide; this increase in channel activity was blocked by dominant-negative Ras whereas constitutively-active Ras increased basal channel activity (28). The frequency of miniature excitatory post-synaptic currents conducted by AMPA receptors was significantly increased in recordings from cortical layers II/III in mice overexpressing a constitutively-active Ras (29). Interestingly,

expression of a GAP-resistant Ras in AtT20 cells induced activation of a potassium channel that greatly shortened the duration of the AP and lead to repetitive firing (30). Together, these studies demonstrate that Ras signaling can play a critical role in regulating the expression of ion channels that set the level of neuronal excitability. In sensory neurons from *Nf1*^{+/-} mice, it seems likely that developmentally decreased neurofibromin expression results in a nonreversible increase in the excitability by changing the expression of ion channels. Indeed, there is a significant increase in tetrodotoxin-sensitive and tetrodotoxin-insensitive sodium currents in *Nf1*^{+/-} sensory neurons but no difference in a variety of potassium currents (30). Further studies are warranted to determine which ion channels have altered expression in *Nf1*^{+/-} neurons and what impact that has on the neurological phenotypes of those with NF1.

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Abbreviations

AP	action potential
CGRP	calcitonin gene-related peptide (CGRP)
DRG	dorsal root ganglion
FT	firing threshold
GAP	GTPase activating protein
eGFP	enhanced green fluorescent protein
IRES	internal ribosome entry site
i.t.	intrathecal
NF1	neurofibromatosis type 1
<i>NF1</i>	neurofibromin gene (human)
<i>Nf1</i>	neurofibromin gene (mouse)
NGF	nerve growth factor
R_{amp}	amplitude of ramp
R_m	membrane resistance
R_{Th}	resistance at threshold
RM ANOVA	repeated-measures analysis of variance
SEM	standard error of the mean

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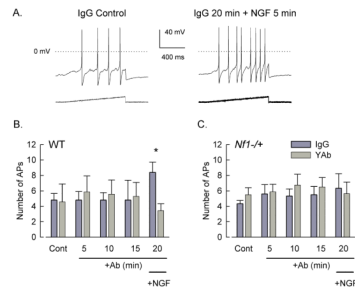


Figure 1.

Y13-259 blocks NGF-mediated increases in excitability in wildtype but not *Nf1*^{+/-} neurons.

A: Representative recording after internal perfusion with 30 μ g/ml IgG (left) and an additional 5 min exposure to 100 ng/ml NGF via the bath (right). B and C: Results obtained from sensory neurons isolated from wildtype (n = 5–7 neurons) and *Nf1*^{+/-} (n = 6–8 neurons) mice. APs were measured over 20 min during internal perfusion with either IgG (dark gray) or Y13-259 (light gray). During the final 5 min of recording, 100 ng/ml NGF was added to the bath. The asterisk indicates a statistically significant difference from control numbers of APs ($P < 0.05$, RM ANOVA).

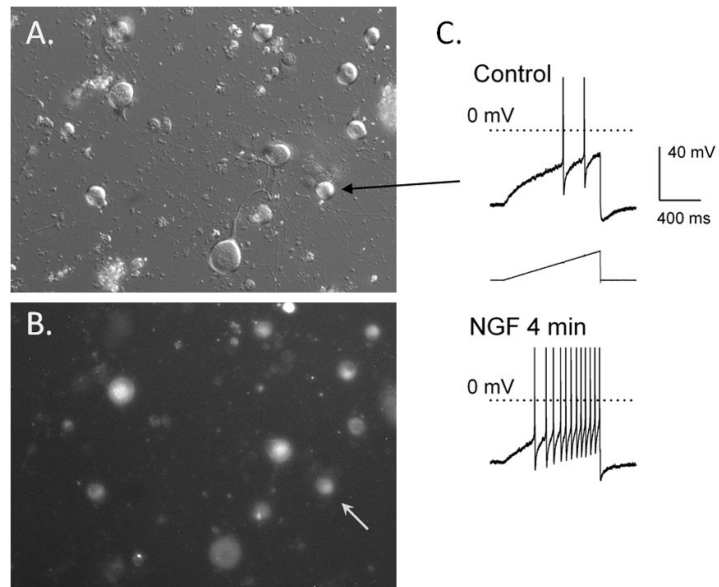


Figure 2. Lentiviral-driven expression of eGFP does not alter NGF-induced sensitization. A: Representative bright-field image of sensory neurons from a wildtype mouse receiving intrathecal injection of CMV-IRES-EGFP. B: Fluorescence image obtained from the same cover slip. Images were taken through a 10X objective. C: Recording obtained from an eGFP expressing neuron (indicated by the arrow in B).

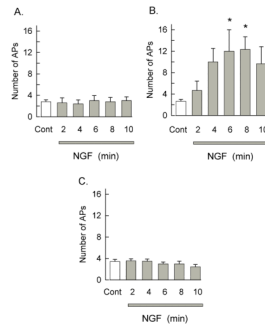


Figure 3.

Expression of DN-Ras-IRES-eGFP blocks the enhancement of AP firing produced by NGF in wildtype sensory neurons. A and B: The number of APs generated by a ramp of depolarizing current in 3 of 6 fluorescing small-diameter neurons from wildtype mice receiving i.t. CMV-IRES-EGFP that were insensitive to 100 ng/ml NGF (A) or that exhibited sensitization in response to 100 ng/ml NGF (B). C: Summarizes the lack of sensitization produced by NGF in nine small-diameter sensory neurons expressing DN-Ras-IRES-eGFP. Asterisks indicate a statistically significant difference from control number of APs ($P < 0.05$, RM ANOVA).

Table 1Parameters of excitability for wildtype vs. *Nf1*^{+/-} mice

	Wildtype	<i>Nf1</i> ^{+/-}	P
APs ramp	4.3 ± 0.5	5.8 ± 0.8	0.08
FT (mV)	-18.5 ± 1.7	-22.9 ± 2.0	0.13
Rheobase (pA)	329 ± 77	116 ± 34	0.03
R _{Th} (MΩ)	376 ± 72	624 ± 141	0.02
R _m (MΩ)	517 ± 59	778 ± 119	0.04
Ramp (pA)	900 ± 162	469 ± 114	0.06
APs/Ramp pA	0.019 ± 0.006	0.038 ± 0.012	0.02
n	38	17	

APs: action potentials

FT: firing threshold

R_{Th}: resistance at thresholdR_m: membrane resistance

Ramp: amplitude of ramp

n: number of neurons

P: statistical probability, wildtype vs. *Nf1*^{+/-}, Student's t-test

Table 2Effects of Y13-259 on parameters of excitability for *Nfl*^{+/-} neurons

	Control	20 min
APs ramp	6.6 ± 1.2	5.5 ± 1.1
FT (mV)	-22.9 ± 2.9	-20.2 ± 3.4
Rheobase (pA)	132 ± 51	77 ± 21
R _{Th} (MΩ)	689 ± 211	796 ± 234
R _m (MΩ)	780 ± 167	617 ± 91

APs: action potentials

FT: firing threshold

R_{Th}: resistance at thresholdR_m: membrane resistance

n=11