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STIMULUS-EVOKED RELEASE OF NEUROPEPTIDES IS ENHANCED IN SENSORY NEURONS FROM MICE WITH A HETEROZYGOUS MUTATION OF THE *Nf1* GENE

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Abstract—Neurofibromatosis type I is a common autosomal dominant disease characterized by formation of multiple benign and malignant tumors. People with this disorder also experience chronic pain, which can be disabling. Neurofibrinomin, the protein product of the *NF1* gene (neurofibromin gene (human)), is a guanosine triphosphate activating protein for p21^{ras}. Loss of *NF1* results in an increase in activity of the p21^{ras} transduction cascade. Because of the growing evidence suggesting involvement of downstream components of the p21^{ras} transduction cascade in the sensitization of nociceptive sensory neurons, we examined the stimulus-evoked release of the neuropeptides, substance P and calcitonin gene-related peptide, from primary sensory neurons of mice with a mutation of the *Nf1* gene (neurofibromin gene (mouse)) (*Nf1*+/−). Measuring immunoreactive substance P and immunoreactive calcitonin gene-related peptide by radioimmunoassay, we demonstrated that capsaicin-stimulated release of neuropeptides is three to five-fold higher in spinal cord slices from *Nf1*+/− mice than from wildtype mouse tissue. In addition, the potassium and capsaicin-stimulated release of immunoreactive calcitonin gene-related peptide from cultures of sensory neurons isolated from *Nf1*+/− mice was more than double that from cultures of wildtype neurons. Treatment of wildtype sensory neurons with nerve growth factor for 5–7 days mimicked the enhanced stimulus-evoked release observed from the *Nf1*+/− neurons. When nerve growth factor was removed 48 h before conducting release experiments, nerve growth factor-induced augmentation of immunoreactive calcitonin gene-related peptide release from *Nf1*+/− neurons was more pronounced than in *Nf1*+/− sensory neurons that were treated with nerve growth factor continuously for 5–7 days. Thus, sensory neurons from mice with a heterozygous mutation of the *Nf1* gene that is analogous to the human disease neurofibromatosis type I, exhibit increased sensitivity to chemical stimulation. This augmented responsiveness may explain the abnormal pain sensations experienced by people with neurofibromatosis type I and suggests an important role for guanosine triphosphate activating proteins, in the regulation of nociceptive sensory neuron sensitization. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: calcitonin gene-related peptide, dorsal root ganglia, nerve growth factor, neurofibromatosis, Ras, substance P.

Neurofibromatosis type 1 (NF1) is one of the most common genetic diseases in humans, with an incidence of one in 3500 people (Lakkis and Tennekoon, 2000). It is a pandemic, autosomal dominant disease and the hallmark of NF1 is the formation of various benign and malignant tumors. However, other common problems experienced by people with NF1 include abnormal painful sensations and painful itching or pruritus (Riccardi and Eichner, 1992; Creange et al., 1999; Wolkenstein et al., 2001). Although the mechanisms by which the human neurofibromin gene (*NF1*) mutation causes these debilitating symptoms have not been elucidated, it is likely that these abnormal pain states involve altered function of small diameter nociceptive sensory neurons that mediate the transmission of pain and itch (Besson and Chaouch, 1987; Schmelz et al., 1997). Numerous studies have examined the ability of nociceptive neurons to undergo sensitization to thermal, mechanical or chemical stimuli, resulting in states of hyperalgesia and allodynia (Treede et al., 1992). These behavioral changes correlate with increased excitability as measured by electrophysiological methods and by the enhanced release of the peptide transmitters, substance P (SP) and calcitonin gene-related peptide (CGRP). These neuropeptides are localized in small diameter A-delta and C sensory neurons (Willis and Coggeshall, 1991) and are released from both peripheral and central terminals by noxious stimulation (Kuraishi et al., 1985; Linderoth and Brodin, 1988; Hua and Yaksh, 1992). Therefore, one mechanism for an increase in pain signaling observed in people with NF1 could be an enhanced release of SP and CGRP from primary sensory neurons.

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Abbreviations: ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; ERK, extracellular signal-regulated kinase; GAP, guanosine triphosphatase activating protein; GTP, guanosine triphosphate; iCGRP, immunoreactive calcitonin gene-related peptide; iSP, immunoreactive substance P; MAPK, mitogen activating protein kinase; Mek, mitogen-activated protein kinase kinase; NF1, neurofibromatosis type 1; *NF1*, neurofibromin gene (human); *Nf1*, neurofibromin gene (mouse); NGF, nerve growth factor; pAkt, phosphorylated protein kinase B; PCMS, *p*-chloromercuriphenyl sulfonic acid; pERK, phosphorylated extracellular signal-related kinase; PI-3K, phosphoinositide-3-kinase; Ras, p21^{ras}; RIA, radioimmunoassay; S.E.M., standard error of the mean; SP, substance P.

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The mutation in NF1 causes decreased production of functional neurofibromin, the protein product of the *NF1* gene. Neurofibromin is a guanosine triphosphatase activating protein (GAP) for $p21^{ras}$ (Ras; Martin et al., 1990; Wallace et al., 1990; Li et al., 1992). The Ras transduction pathway is activated by many cytokines and growth factors, as indicated by an increase in Ras-guanosine triphosphate (GTP) levels, and this transduction cascade is important for cell survival, growth and proliferation in many cells types (Bar-Sagi and Hall, 2000; Kolch, 2000). Active Ras-GTP recruits the kinase, Raf, to the membrane and thereby activates a cascade of downstream effectors such as extracellular signal-regulated kinase (ERK), a member of the mitogen activating protein kinase (MAPK) pathway, and phosphoinositide-3-kinase (PI-3K; Wennstrom and Downward, 1999). Ras-GTP is converted to the inactive form, Ras-guanosine diphosphate, through hydrolysis and this reaction is accelerated thousands of fold by neurofibromin, thereby limiting the strength and duration of downstream signals. Loss of *NF1* frequently results in an increase in both basal and cytokine-stimulated Ras activity (Bollag and McCormick, 1991; Largaespada et al., 1996; Zhang et al., 1998; Cichowski and Jacks, 2001).

To elucidate the pathologic mechanisms in NF1, mouse models were developed in which the mouse neurofibromin gene (*Nf1*) was disrupted (Jacks et al., 1994; Largaespada et al., 1996). Although mice with a homozygous mutation of the *Nf1* gene (*Nf1*−/− mice) die in utero, mice with a heterozygous mutation (*Nf1*+/− mice), that is analogous to the human autosomal dominant disorder, have a normal lifespan. Neurons, mast cells, fibroblasts and Schwann cells from *Nf1*+/− mice exhibit some of the abnormal characteristics observed in similar cells isolated from human neurofibromas. Sensory neurons from embryonic *Nf1*+/− and *Nf1*−/− mice do not require nerve growth factor (NGF) for survival, unlike wild-type sensory neurons (Vogel et al., 1995). Sensory neurons with the *Nf1* mutation also exhibit increased amounts of Ras-GTP and activation of the PI-3K pathway (Vogel et al., 1995; Klesse and Parada, 1998).

There is growing evidence that NGF and other growth factors can activate downstream effectors of the Ras transduction cascade to affect changes in adult sensory neurons. Electrical stimulation of the sciatic nerve or injection of capsaicin into the paw results in an increase in phosphorylated extracellular signal-related kinase (pERK) in dorsal root ganglia (DRG) cells (Dai et al., 2002), as does peripheral inflammation (Obata et al., 2003). NGF-induced increases in pERK and phosphorylated protein kinase B (pAkt, a downstream effector of the PI-3K pathway) are associated with increases in the expression of the heat- and capsaicin-activated receptor, TRPV1, in DRG neurons (Bron et al., 2003). The inhibitor of mitogen-activated protein kinase kinase (Mek), PD98059, reduces the capsaicin sensitivity of neurons treated with NGF (Ganju et al., 1998) and Zhuang and coworkers (2004) demonstrate that both Mek and PI-3K activity are important in NGF-mediated changes in capsaicin currents and thermal hyperalgesia. This suggests that many of the NGF-mediated changes in capsaicin sensitivity in primary sensory neurons involve Ras-activated pathways.

Because of the evidence that downstream effectors of the Ras transduction cascade are involved in enhanced sensitivity of sensory neurons, or peripheral sensitization, it is important to examine regulation of this cascade. The GAP, neurofibromin, is a key regulator of Ras-GTP levels and, therefore, we investigated whether the reduction in neurofibromin in *Nf1*+/− mice, could augment the sensitivity of primary sensory neurons. We measured stimulus-evoked neuropeptide release from both spinal cord slices and isolated sensory neurons from wildtype (*Nf1*+/+) and *Nf1*+/− mice as an indicator of sensory neuron sensitivity. Our data demonstrate an enhanced stimulus-evoked release of neuropeptide transmitters from *Nf1*+/− sensory neurons. This is the first demonstration of an alteration in the sensitivity of adult sensory neurons in response to changes in GAP expression. It is clear, therefore, that neurofibromin can modulate sensory neuron responsiveness and play an important role in peripheral sensitization. These mechanisms may account for the dysesthesias, pain and pruritus experienced by patients with NF1.

EXPERIMENTAL PROCEDURES

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 Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. All procedures were reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. These procedures involve measures to minimize the number of animals used and the suffering of animals.

Horse serum, F-12 medium, L-glutamine, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). NGF was purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN, USA). Collagenase, poly-D-lysine, laminin, 5′-fluoro-2′-deoxyuridine, uridine and standard laboratory chemicals were from Sigma (St. Louis, MO, USA). Antibodies to substance P and calcitonin gene-related peptide were generously provided by Michael R. Vasko (Indiana University School of Medicine, Indianapolis, IN, USA).

To establish sensory neuron cultures, the DRG were removed from adult mice in a manner similar to that previously published (Southall and Vasko, 2000; Burkey et al., 2004). DRG were dissociated by mechanical agitation after digestion in 0.1% collagenase for 45 min at 37 °C. Cells were plated in wells of 24-well Falcon culture dishes coated with poly-D-lysine and laminin at a density of 40,000–50,000 cells/well. Cultures were maintained at 37 °C in a 5% CO_2 atmosphere in F12 media supplemented with 2 mM glutamine, 50 μg/ml penicillin and streptomycin, 10% heat-inactivated horse serum and mitotic inhibitors (50 μM 5-fluoro-2-deoxyuridine and 150 μM uridine). Growth medium was changed every 2–3 days.

The stimulus-evoked release and content of SP and CGRP was measured from spinal cord slices as previously published (Southall et al., 1998) using radioimmunoassay (RIA) and is reported as immunoreactive peptide (immunoreactive substance P, iSP; or immunoreactive calcitonin gene-related peptide, iCGRP). The minimal amount of iSP and iCGRP detected by the respective RIAs is 5 fmol with a 95% confidence interval (Chen et al., 1996). The entire spinal cord from each animal was removed, weighed and chopped parasagitally and transversely into 300 μm cross-

sections using a McIllwain Tissue Chopper. The average amount of protein per spinal cord was 92 μg (standard deviation of 28 μg for 20 animals). There was no significant difference in spinal cord weight between the genotypes. The sections from each spinal cord were placed into individual chambers and perfused at a rate of 0.5 ml/min for 15 min with Krebs buffer consisting of (in mM): 135 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2$, 20 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 3.3 mM dextrose, 0.1% (w/v) bovine serum albumin, 100 μM Phe-Ala, and 50 μM *p*-chloromercuriphenyl sulfonic acid (PCMS), pH 7.4, aerated with 95% O_2/5% CO_2. The compounds, Phe-Ala and PCMS are used as peptidase inhibitors (Chen et al., 1996). After the equilibration period, serial 3 min collections (1.5 ml of perfusate) were obtained from each spinal cord. After three basal collection periods (total of 9 min), the perfusate was changed to Krebs buffer containing 250 nM capsaicin for 9 min to measure stimulus-evoked release. Upon completion of the stimulus, a 15 min perfusion with Krebs buffer alone was performed to allow a return to resting conditions. An aliquot from each 3 min collection period was assayed for iSP and iCGRP using RIA. After the release protocol was complete, the peptide content of the tissue was determined by homogenizing the residual spinal cord tissue in 0.1 N HCl. The homogenized tissue was centrifuged and the supernatant was serially diluted with Krebs buffer. Peptide content was determined from these diluted samples by RIA. This value was added to the amount of iSP or iCGRP released during the perfusion to obtain the total peptide content. The release of iSP or iCGRP is expressed as percent of the total neuropeptide content per min of perfusion.

The stimulus-evoked release and content of iCGRP were measured from cells in culture as previously published (Hingtgen and Vasko, 1994) using RIA. Because basal levels of iSP release were below the level of detection in cultures of sensory neurons grown in the absence of NGF, iSP release was not measured in these experiments. Sensory neurons were maintained in culture for 5–7 days prior to the release experiments. Medium was removed and the cells were washed with HEPES buffer consisting of (in mM): 25 HEPES, 135 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1 Mg_2Cl_2, 3.3 dextrose, and 0.1% (w/v) bovine serum albumin, pH 7.4, and maintained at 37 °C. Cells initially were incubated in HEPES buffer for 10 min to determine resting or basal release levels iCGRP. The cells underwent subsequent 10 min incubations in HEPES buffer containing stimulants (50 mM KCl or 50 nM capsaicin) or HEPES buffer alone. Aliquots of the incubation samples were assayed for iCGRP using RIA. Peptide content of the sensory neurons in culture was determined by exposing the wells of cells to 2 N acetic acid for 10 min. Aliquots of the acid solution were diluted in HEPES buffer and assayed for iCGRP using RIA. This value was added to the amount of iCGRP released in the previous 10 min periods to obtain the total iCGRP content per well of cells. Each manipulation was repeated in cells from at least three different preparations.

Results are expressed as the mean±standard error of the mean (S.E.M.). Differences in neuropeptide release and content were determined by *t*-tests or analysis of variance (ANOVA) with post hoc analysis, as appropriate. The significance level for all statistical tests was $P<0.05$.

RESULTS

Neuropeptide release from spinal cord slice preparations

Mice with disruption of the *Nf1* gene (*Nf1*+/−) that encodes for the GAP, neurofibromin, were developed to study the human disorder, NF1. Sensory neurons with the *Nf1* mutation also exhibit increased amounts of Ras and activation of the PI-3-K pathway (Vogel et al., 1995; Klesse and Parada, 1998). Since these cascades have been implicated in sensory neuron activation, one might hypothesize that sensory neurons from *Nf1*+/− mice have enhanced sensitivity to a given stimulus. Therefore, to determine if a reduction in neurofibromin results in sensitization of nociceptive sensory neurons, the capsaicin-stimulated release of the neuropeptide transmitters, iSP and iCGRP, was measured from spinal cord slice preparations obtained from wildtype and *Nf1*+/− mice. As seen in Fig. 1,

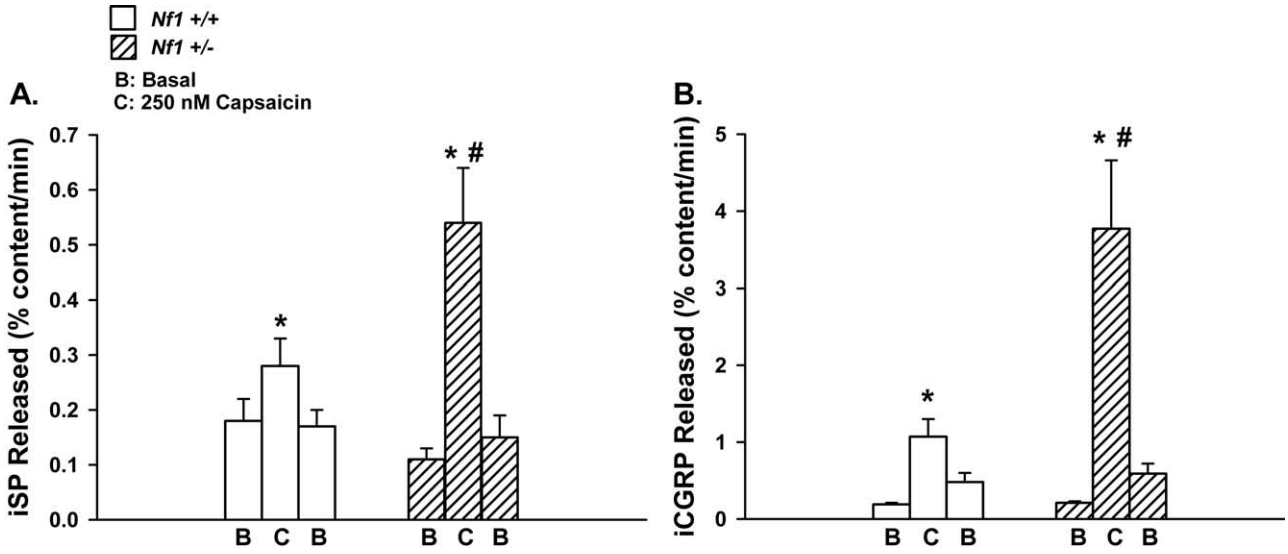

Fig. 1. Capsaicin-stimulated release of iSP and iCGRP is greater from *Nf1*+/− spinal cord preparations than from wildtype tissue. Adult mouse spinal cord slices were perfused with Krebs buffer for 9 min (Basal, B), Krebs buffer containing 250 nM capsaicin (C) for 9 min and then Krebs buffer alone again for 15 min (B). The prefusate was assayed for iSP (represented in panel A) and iCGRP (represented in panel B) using RIA. The columns represent the mean release of peptide expressed as % peptide content/min of perfusion±S.E.M. for nine animals per genotype. An asterisk indicates a statistically significant difference between resting and capsaicin-stimulated release of peptide whereas # indicates a statistically significant difference between genotypes using a *t*-test ($P<0.05$).

there was no significant difference in basal release of iSP or iCGRP between spinal cord slices obtained from *Nf1*+/+ and *Nf1*+/− mice. The basal release of iSP and iCGRP from wildtype mouse spinal cord slices was 0.18±0.04 and 0.19±0.07% total peptide content/min, respectively, whereas the basal release of iSP and iCGRP from *Nf1*+/− mouse spinal cord slices was 0.11±0.02 and 0.21±0.02% total peptide content/min, respectively. In contrast, there is a significant increase in the capsaicin-stimulated release of iSP and iCGRP from *Nf1*+/− mouse spinal cord slices compared with those from wildtype animals. The release of iSP evoked by 250 nM capsaicin was two-fold higher from *Nf1*+/− mouse spinal cord slices than from those from wildtype mice (0.28±0.05 versus 0.54±0.1% total peptide content/min for wildtype and *Nf1*+/− mice, respectively). Similarly, the capsaicin-evoked release of iCGRP from the same preparations was almost four-fold higher from *Nf1*+/− mouse spinal cord slices (1.1±0.2 versus 3.8±0.9% total peptide content/min for wildtype and *Nf1*+/− mice, respectively). After capsaicin was removed from the perfusion buffer, neuropeptide release returned to basal levels. In the spinal cord preparation, the total content of iSP and iCGRP was not significantly different between the genotypes; the content of iSP was 2700±120 and 2900±150 fmol/spinal cord for *Nf1*+/+ and *Nf1*+/− mice, respectively (mean±S.E.M., $n=9$ animals). These data clearly demonstrate that capsaicin-stimulated release of iSP and iCGRP is enhanced in spinal cord tissue from *Nf1*+/− mice.

Neuropeptide release from isolated sensory neurons

To demonstrate that the enhanced release of neuropeptides from *Nf1*+/− spinal cord slice preparations was a

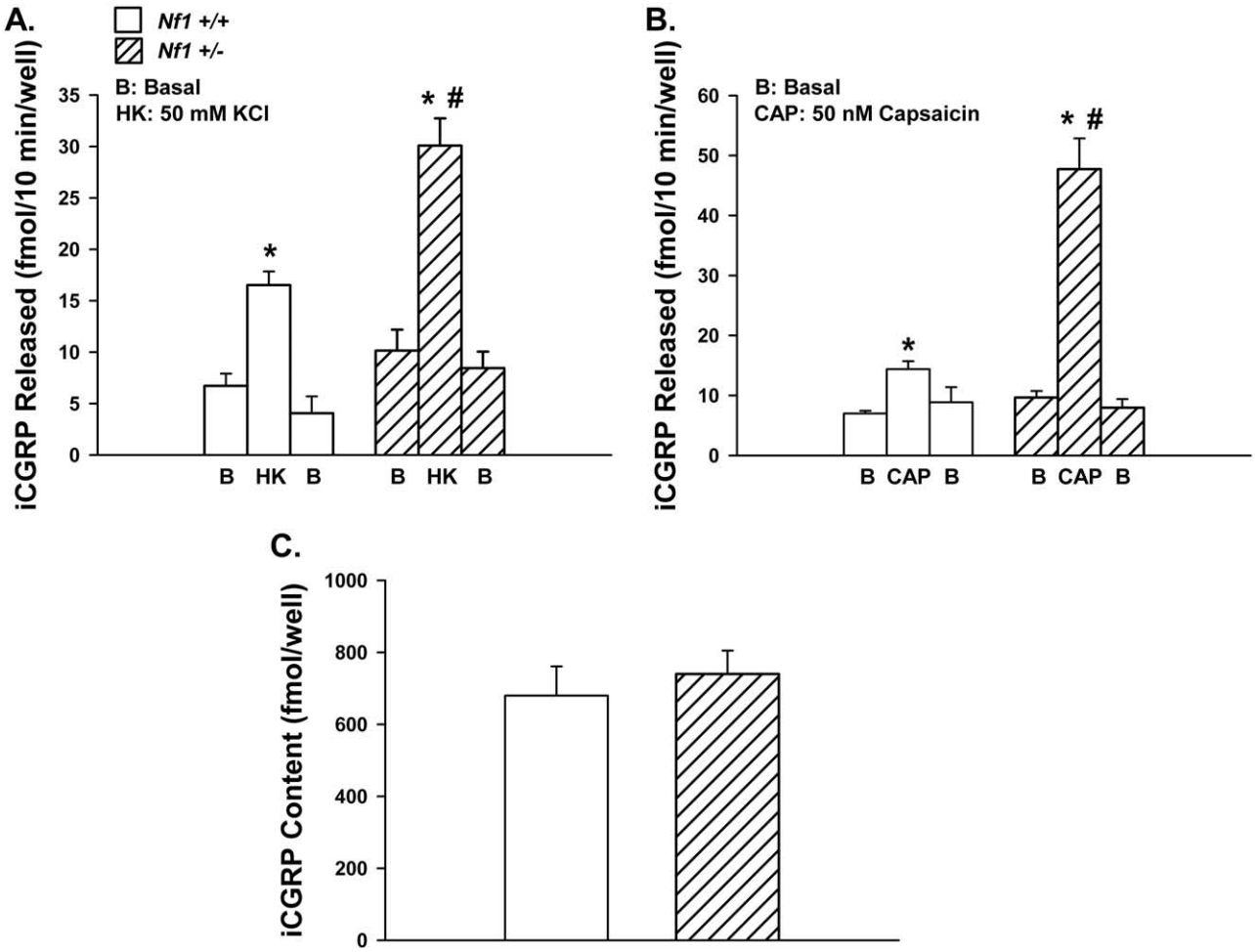

Fig. 2. Sensory neurons isolated from *Nf1*+/− mice exhibit enhanced stimulus-evoked iCGRP release compared with wildtype neurons. Adult mouse sensory neurons were maintained in culture for 5–7 days. Panel A depicts iCGRP release from cells treated with successive 10 min incubations of normal HEPES buffer (basal, B), HEPES buffer containing 50 mM KCl (HK), and HEPES buffer alone, again. Panel B depicts iCGRP release from cells treated with successive 10 min incubations of normal HEPES buffer (basal, B), HEPES buffer containing 50 nM capsaicin (CAP) and HEPES buffer alone, again. In both panels, the columns represent the mean±S.E.M. peptide release from neurons from at least three separate preparations ($n=6$–20 wells per condition). Asterisks (*) indicate statistically significant differences between the basal release and release caused by HK or CAP using a *t*-test ($P<0.05$), whereas # indicates a statistically significant difference between genotypes using a *t*-test ($P<0.05$). Panel C depicts the total content of iCGRP measured at the end of the release experiments ($n=28$–30 wells per genotype). There was no significant difference in iCGRP content between genotypes using a *t*-test.

direct result of increased responsiveness of the primary sensory neuron terminals and not related to other cells in the spinal cord, the stimulus-evoked release of iCGRP was measured in isolated mouse sensory neurons from *Nf1*+/+ and *Nf1*+/− mice. For these experiments, the sensory neurons were maintained in culture for 5–7 days prior to conducting the release experiments. As shown in Fig. 2a, the initial basal or resting release of iCGRP was not significantly different between genotypes (6.7±1.2 and 10±2.0 fmol/well for wildtype and *Nf1*+/− sensory neurons, respectively, n=6–15 wells). In contrast, potassium-stimulated iCGRP release was almost two-fold higher from the *Nf1*+/− cells compared with *Nf1*+/+ cells; 30±2.6 versus 16±1.3 fmol/well, respectively (n=8–20 wells for each genotype, P value for t-test <0.05). Similarly, in a separate series of studies, the release of iCGRP in response to 50 nM capsaicin was almost three-fold higher from *Nf1*+/− cells compared with wildtype cells (Fig. 2b). The initial basal release of iCGRP was 7.0±0.44 fmol/well from *Nf1*+/+ cells and increased to 14±1 fmol/well with a 10 min exposure to capsaicin. Although basal release of iCGRP was not different in *Nf1*+/− sensory neuron preparations (9.6±1.1 fmol/well), capsaicin-stimulated release was significantly higher from *Nf1*+/− sensory neurons (48±5 fmol/well, n=6–15 wells for each genotype, P value for t-test <0.05). In all instances, iCGRP release returned to resting levels when the cells were incubated in HEPES buffer alone following the stimulation period. The increase in stimulus-evoked release of iCGRP from *Nf1*+/− neurons was not a result of an increased content of iCGRP as there was no significant difference in neuropeptide content between genotypes in these preparations (Fig. 2c, 680±81 fmol/well for *Nf1*+/+ preparations versus 740±65 fmol/well for *Nf1*+/− preparations, n=28–30 wells). As observed in the spinal cord slice preparations, there was an enhanced stimulus-evoked release of iCGRP from isolated sensory neurons with a heterozygous mutation of the *Nf1* gene compared with wildtype neurons, confirming that sensitization of sensory neurons occurs secondary to the genetic trait.

Neuropeptide release from isolated sensory neurons treated with NGF

The neurotrophin, NGF can activate TrkA receptors on sensory neurons and thereby activate the Ras transduction cascade (Kaplan et al., 1991; Sofroniew et al., 2001). Recently, studies have focused on the ability of downstream components of this cascade to alter sensory neuron signaling. To determine if sensory neurons from *Nf1*+/− mice have an altered response to NGF because of a decrease in neurofibromin production, isolated sensory neurons from *Nf1*+/− and wildtype mice were maintained in culture in the absence or presence of various concentrations of NGF. In preparations from both genotypes, the total content of iCGRP was enhanced by growth in increasing concentrations of NGF, although there was no difference in the content of iCGRP between the genotypes treated with the same concentration of NGF (data not shown). Because exposure to NGF increased the content of iCGRP in the isolated neuronal preparation, the release data in this series of experiments are expressed as percent

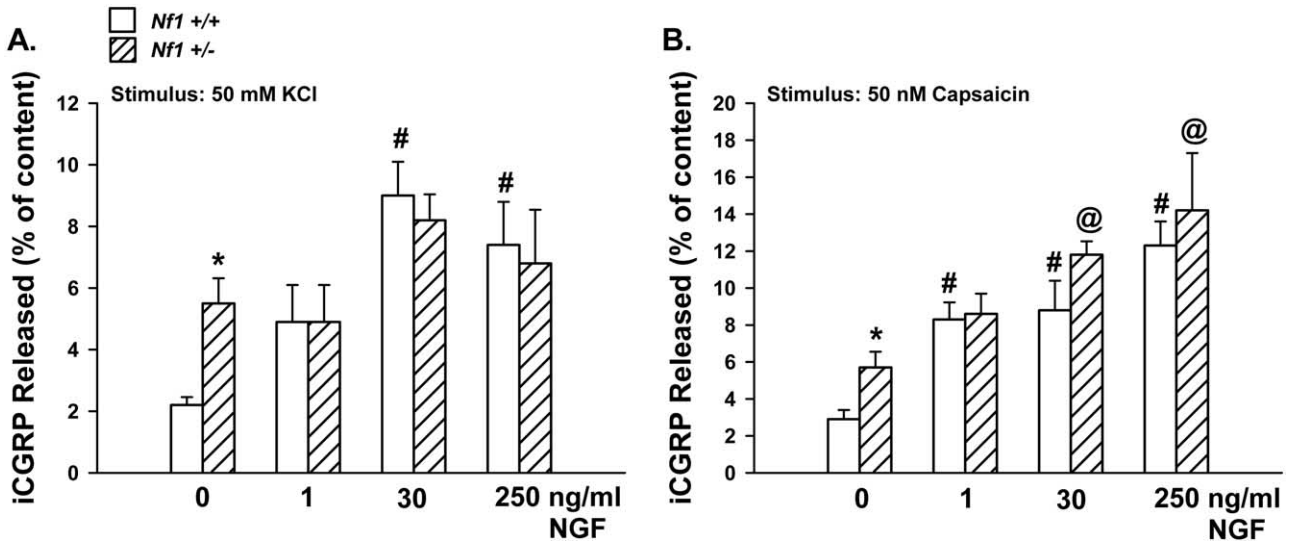

Fig. 3. The effect of continuous treatment of isolated sensory neurons with NGF on stimulus-evoked release of iCGRP. Adult mouse sensory neurons receiving NGF treatment were grown in the absence or presence of NGF for 5–7 days. Release stimulated by a 10 min exposure to HEPES buffer containing 50 mM KCl (K) or release stimulated by HEPES buffer containing 50 nM capsaicin (C) is expressed as the percent of total iCGRP content per well of cells. The columns represent the mean±S.E.M. peptide release as a percent of total iCGRP content from neurons from at least three separate preparations (n=6–22 wells per condition) with the open columns representing release from wildtype neurons and hatched columns representing release from *Nf1*+/− neurons. Asterisks (*) indicate statistically significant differences between genotypes receiving the same treatment using a t-test (P<0.05). The # symbol indicates a significant difference in iCGRP release from wildtype neurons with NGF treatment compared with release from wildtype neurons that did not receive NGF treatment (ANOVA with Dunnet's post hoc analysis, P<0.05). @ Indicates a significant difference in iCGRP release from *Nf1*+/− neurons with NGF treatment compared with release from *Nf1*+/− neurons that did not receive NGF treatment (ANOVA with Dunnet's post hoc analysis, P<0.05).

of total content. Treatment with NGF did not cause a significant difference in basal or resting release expressed as either absolute amounts or as a percent of total iCGRP content (data not shown).

When cells were maintained in NGF for the entire 5–7 days, both the potassium and capsaicin-stimulated release of iCGRP were significantly enhanced in wildtype sensory neurons. As seen in Fig. 3, potassium-evoked release from wildtype sensory neurons maintained in either 30 or 250 ng/ml NGF was significantly higher than from neurons that were not exposed to NGF, whereas capsaicin-stimulated release was significantly elevated at 1, 30, or 250 ng/ml NGF (n=6–22 wells for each genotype at each treatment, $P<0.05$ using a one-way ANOVA with Dunnet's post hoc testing using absence of NGF as the control value). Treatment with NGF did not significantly alter potassium-stimulated iCGRP release from *Nf1*+/− sensory neurons, and so the difference in potassium-stimulated peptide release between genotypes that was observed in the absence of NGF was not observed when cells were grown in media with added NGF. When *Nf1*+/− neurons were treated with 30 or 250 ng/ml NGF, the capsaicin-stimulated release was higher than that in the absence of NGF (n=6–22 wells for each genotype at each treatment, $P<0.05$ using a one-way ANOVA with Dunnet's post hoc testing using absence of NGF as the control value). These data demonstrate that NGF treatment mimics the effect of the *Nf1* mutation on potassium-stimulated release of iCGRP, but that the capsaicin-stimulated release can be enhanced by a similar degree in sensory neurons from either genotype.

In another series of experiments, isolated sensory neurons were maintained in exogenous NGF (1–250 ng/ml) for 3 days only. After this treatment period, the neurons were maintained in media without added NGF for 48 h prior to the release experiments. Although NGF treatment again caused an increase in stimulus-evoked release of iCGRP from wildtype sensory neurons, there was also a pronounced concentration-related enhancement of iCGRP release from *Nf1*+/− sensory neurons (see Fig. 4). This difference is most clear in the response to capsaicin where, for example, the stimulated release of iCGRP was 5.7±0.85% of total content in the absence of added NGF, 14±3.1% in the presence of 250 ng/ml NGF for 5–7 days (Fig. 3b), but was 43±13% from neurons treated with 250 ng/ml NGF for only 3 days.

Taken together, these data clearly show that there is an enhanced sensitivity of *Nf1*+/− sensory neurons to stimulus-evoked release of neuropeptides, either from spinal cord slices or isolated sensory neurons maintained in culture. This sensitization can be mimicked in wildtype sensory neurons by growing the cells in NGF. In addition, removal of added NGF 48 h before the release experiment allowed even greater levels of stimulus-evoked release from *Nf1*+/− preparations in comparison to neurons from *Nf1*+/− mice that received treatment with NGF throughout. This result suggests that continuous treatment with NGF causes the initiation of compensatory mechanisms

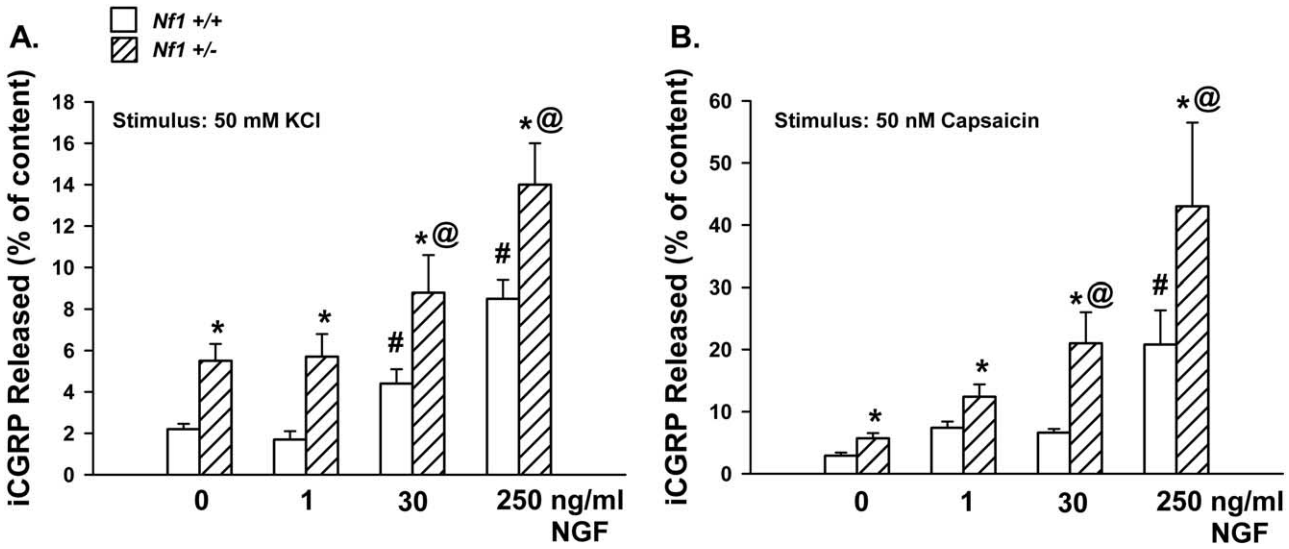

Fig. 4. The effect of removing NGF 48 h before measuring stimulus-evoked release of iCGRP. Adult mouse sensory neurons receiving NGF treatment were grown in the presence of absence or presence of NGF for 3 days. The medium was changed to that containing no added NGF 48 h before release experiments were performed. Neurons that did not receive NGF treatment were maintained in culture for 7 days. Release stimulated by a 10 min exposure to HEPES buffer containing 50 mM KCl (K) or release stimulated by HEPES buffer containing 50 nM capsaicin (C) is expressed as the percent of total iCGRP content per well of cells. The columns represent the mean±S.E.M. peptide release as a percent of total iCGRP content from neurons from at least three separate preparations (n=6–22 wells per condition) with the open columns representing release from wildtype neurons and hatched columns representing release from *Nf1*+/− neurons. Asterisks (*) indicate statistically significant differences between genotypes receiving the same treatment using a t-test ($P<0.05$). The # symbol indicates a significant difference in iCGRP release from wildtype neurons with NGF treatment compared with release from wildtype neurons that did not receive NGF treatment (ANOVA with Dunnet's post hoc analysis, $P<0.05$). @ Indicates a significant difference in iCGRP release from *Nf1*+/− neurons with NGF treatment compared with release from *Nf1*+/− neurons that did not receive NGF treatment (ANOVA with Dunnet's post hoc analysis, $P<0.05$).

which reduce the level of sensitization that can be achieved in the *Nf1*+/− neurons.

DISCUSSION

As shown above, primary sensory neurons that have a mutation of the *Nf1* gene, exhibit enhanced stimulus-evoked release of neuropeptides. In this mouse model of the human disorder, NF1, there is reduced production of neurofibromin, a GAP for Ras (Jacks et al., 1994; Vogel et al., 1995; Klesse and Parada, 1998). To date, there has been little direct investigation of Ras activation in peripheral sensitization and no examination of the role of GAPs in this process. Since the Ras transduction cascade can be critical to the signaling of numerous inflammatory growth factors and cytokines, better understanding of the regulation of this pathway is important in elucidating the mechanisms of peripheral sensitization. For example, pERK levels are elevated in cells of the DRG after capsaicin treatment or electrical stimulation in the periphery (Dai et al., 2002). Similar increases in pERK were seen in DRG that were treated with complete Freund's adjuvant to cause peripheral inflammation (Obata et al., 2003). In addition, spinal nerve ligation in the rat increases levels of phosphorylated p38 (another member of the MAPK pathway) in DRG five hours post-procedure (Schafers et al., 2003). Thus, these studies suggest that downstream effectors of the Ras transduction pathway are activated during injury and inflammation.

Regulation of the levels of Ras-GTP by GAPs, such as neurofibromin, is an important point of modulation of these cascades and, as we have shown, a mutation that causes a reduction of functional neurofibromin results in the sensitization of sensory neurons. This sensitization was observed in spinal cord slices that contain the central terminals of primary sensory neurons. The dorsal horn of the spinal cord contains the central terminals of primary sensory neurons that release peptide neurotransmitters when stimulated. Capsaicin selectively activates small diameter nociceptive sensory neurons (Bevan and Szolcsányi, 1990; Holzer, 1991), and so is a useful agent to target the primary sensory nerve endings involved in pain signaling in the spinal cord slices which contains many types of neuronal and non-neuronal cells. An enhanced response to stimulation also was observed in isolated *Nf1*+/− sensory neurons maintained in culture. The observation of increased stimulus-evoked neuropeptide release in both preparations demonstrates that the enhanced response was not an artifact of culturing the neurons, nor a result of actions by non-neuronal cells or interneurons in the spinal cord. The enhanced release of iCGRP from *Nf1*+/− neurons was observed using either high extracellular potassium or capsaicin as a stimulus. This observation suggests that the mechanisms for sensitization in *Nf1*+/− neurons are not restricted to modulation of TRPV1 receptors, but involve a more general modulation of ion channels or peptide release mechanisms in nociceptive sensory neurons. Elucidating these targets will be critical for further understanding of the mechanisms underlying the sensitization observed with the *Nf1*+/− neurons.

NF1 is a common human genetic disease characterized by numerous abnormalities that include benign and malignant tumors. However, people with NF1 often experience abnormal painful sensations, such as hyperalgesia, dysesthesias and allodynia, and painful itching or pruritus, particularly in response to injury or minor trauma (Riccardi and Eichner, 1992; Creange et al., 1999; Wolkenstein et al., 2001). The enhanced release of iCGRP from *Nf1*+/− spinal cord slices and isolated sensory neurons is consistent with the enhanced sensory symptoms in those afflicted with NF1. In addition to nociceptive sensory neurons, other cell types may be affected. For example, the function of mast cells is altered by mutation of the *Nf1* gene wherein mast cell progenitors from *Nf1*+/− mice have increased activity of the Ras pathway in response to stem cell factor as well as a gain of function for a variety of cellular processes including proliferation, survival, chemotaxis and degranulation (Ingram et al., 2000, 2001). Although, our studies have focused on the direct effects of the *Nf1* mutation on the sensitization of sensory neurons by assessing stimulus-evoked neuropeptide release, we cannot exclude a role for non-neuronal tissues in contributing to the responses of *Nf1*+/− spinal cord tissue and DRG neurons in the absence or presence of NGF. Certainly, mast cells, Schwann cells, fibroblasts and other cells are likely to play a role in the enhanced sensory responses of people with NF1 who experience injury.

Our observation that the stimulus-evoked release of iCGRP was enhanced in wildtype mouse sensory neurons treated with NGF was not surprising and such findings have been observed before in similar rodent preparations (Southall and Vasko, 2000). The NGF-induced sensitization of iCGRP release from wildtype sensory neurons was similar whether the neurons were treated continuously for 5–7 days or when the NGF was removed 48 h before release experiments were performed. In contrast, *Nf1*+/− sensory neurons continuously exposed to NGF did not exhibit an enhancement in potassium-stimulated iCGRP release and the effects of NGF treatment on capsaicin-stimulated release were small, albeit significant at 30 and 250 ng/ml. Because of the difference in the responsiveness of the sensory neurons from the two genotypes to NGF, the significant difference in iCGRP release between genotypes that was observed in the absence of added NGF was negated. However, when NGF was removed 48 h before release experiments were performed, the augmentation of stimulus-evoked release of iCGRP from *Nf1*+/− neurons compared with wildtype neurons was observed. In fact, the magnitude of the capsaicin-stimulated release in preparations treated with NGF for three days was close to double that from those receiving continuous NGF treatment for 5–7 days. One component of the enhanced capsaicin-stimulated response is likely secondary to the increased expression of TRPV1 in response to NGF. Treatment with constitutively active Ras mimics the action of NGF to increase TRPV1 expression on isolated sensory neurons (Bron et al., 2003). NGF-induced in-

creases in the expression of the TRPV1 receptor in DRG neurons have been associated with activation of p38 (Ji et al., 2002), as well as increases in pERK and pAkt (Bron et al., 2003). In addition, the Mek inhibitor, PD98059, reduced capsaicin sensitivity of neurons that were treated with NGF for a week (Ganju et al., 1998). In the *Nf1*+/− sensory neurons, NGF-induced activation of the Ras transduction cascade may be augmented by decreased neurofibromin levels. The lack of a critical GAP could result in increased TRPV1 expression in the *Nf1*+/− neurons compared with wildtype neurons with normal GAP activity.

It seems unlikely that the lack of enhanced release in the cultures of *Nf1*+/− cells treated continuously with NGF was secondary to having reached a maximal level of stimulation or releasable peptide, since greater release was achieved in neurons receiving only three days of NGF treatment. Instead, our data suggest that there may be compensatory mechanisms that are modulating NGF-induced sensitization of the neurons with reduced neurofibromin, but that these mechanisms are reversible when NGF is removed. Indeed, the NGF-mediated changes in tetrodotoxin-resistant sodium and delayed-rectifier potassium currents in isolated rat sensory neurons resolve within 20 min after removing the NGF (Zhang et al., 2002). In *Nf1*+/− sensory neurons, some of NGF's actions to enhance peptide release may be blocked by the consequences of a constitutively active Ras transduction cascade; explaining the lack of a robust effect of continuous NGF treatment on peptide release from *Nf1*+/− neurons compared with that from wildtype neurons. Other actions of NGF to sensitize *Nf1*+/− sensory neurons may not be blocked by a constitutively active Ras transduction cascade and may persist after the removal of NGF for as long as 48 h; explaining the enhanced peptide release from neurons treated with NGF for only the first 3 days in culture. The *Nf1*+/− sensory neurons may serve as an excellent model to determine the potentially multiple cellular pathways mediating NGF-induced changes in peptide release.

It is clear from our data that GAPs, such as neurofibromin, are important in regulating the sensitization of sensory neurons. The response to an inflammatory mediator, such as NGF, is altered by mutation of the *Nf1* gene which may explain the abnormal painful responses to injury seen in people with NF1. Further study of the role of GAPs in the sensitization of sensory neurons may lead to better understanding of the chronic pain experienced by those with NF1 and may help elucidate key mechanisms of peripheral sensitization associated with other chronic pain conditions.

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