Published in final edited form as:
J Pain. 2013 June ; 14(6): 628–637. doi:10.1016/j.jpain.2013.01.770.

Assessment of pain and itch behavior in a mouse model of Neurofibromatosis type 1

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Abstract

Neurofibromatosis type 1 (NF1) is characterized primarily by tumor formation in the nervous system, but patients report other neurological complications including pain and itch. Individuals with NF1 harbor one mutated *NF1* allele causing heterozygous expression in all of their cells. In mice, *Nf1* heterozygosity led to hyperexcitability of sensory neurons and hyperproliferation of mast cells, both of which could lead to increased hypersensitivity and scratching in response to noxious and pruritic stimuli. To determine whether *Nf1* heterozygosity may increase pain and itch behaviors independent of secondary effects of tumor formation, we used mice with a targeted, heterozygous *Nf1* gene deletion (*Nf1*+/−) that lack tumors. *Nf1*+/− mice exhibited normal baseline responses to thermal and mechanical stimuli. Moreover, with similar to wild-type littermates, *Nf1*+/− mice developed inflammation-induced heat and mechanical hypersensitivity, capsaicin-induced nocifensive behavior, histamine-dependent or –independent scratching, and chronic constriction injury-induced cold allodynia. However, *Nf1*+/− mice exhibited an attenuated first phase of formalin-induced spontaneous behavior and expedited resolution of formalin-induced heat hypersensitivity. These results are not consistent with the hypothesis that *Nf1* heterozygosity alone is sufficient to increase pain and itch sensation in mice, and suggest that additional mechanisms may underlie reports of increased pain and itch in NF1 patients.

Perspective—This study assessed whether *Nf1* heterozygosity in mice increased hypersensitivity and scratching following noxious and pruritic stimuli. Using *Nf1*+/− mice lacking tumors, this study finds no increases in pain or itch behavior, suggesting that there is no predisposition for either clinical symptom solely due to *Nf1* heterozygosity.

Keywords

Neurofibromatosis; pain; itch; Ras-GAP

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Disclosures

This work was supported by funding from National Institutes of Health grants R01NS48602 and R01DE022000 to RWG, NSF grant DGE-1143954 to DEO, and NCI grant U01-CA141549 to DHG. The authors have no conflicts of interest.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disease that affects approximately 1 in 3000 people worldwide. The disease results from a germline mutation in the *NF1* tumor suppressor gene encoding a Ras-GTPase activating protein (Ras-GAP), neurofibromin, which is expressed throughout the nervous system[11, 19]. NF1 is clinically characterized by tumor formation leading to generation of optic gliomas, neurofibromas, malignant peripheral nerve sheath tumors, and an increased incidence of rare cancers[10]. In addition, individuals with NF1 report neurological complications, including pain and itch. In this regard, there is a significant effect of body pain on their quality of life, which increases with disease severity[7, 14, 24, 26, 36, 42]. Likewise, itch is a neurological symptom that closely correlates with quality of life in children with NF1[23]. However, in these clinical reports on NF1 patients, clinicians often ascribe the pain and itch as either a direct or an indirect consequence of tumor burden[7, 9].

Recent studies in animal models suggest that the clinicians may mistakenly identify tumor load as causative for increased pain and/or itch in NF1 patients. In an *in vitro* study, heterozygous deletion of the *Nf1* gene (*Nf1*+/−) in mice leads to increased excitability of capsaicin-sensitive sensory neurons[40, 41]. Moreover, *Nf1*+/− capsaicin-sensitive, sensory neurons exhibit increased stimulus-evoked release of the peptide transmitter, calcitonin gene releasing peptide (CGRP)[18]. These capsaicin-sensitive neurons comprise a subpopulation of sensory neurons known to transmit pain and itch stimuli[39, 43] and are necessary for both inflammatory pain and itch[32]. This increased transmission through neurons involved in pain and itch could conceivably cause *Nf1*+/− mice to be hypersensitive to nociceptive and pruriceptive stimuli.

Besides increasing neuronal excitability, the *Nf1+/−* microenvironment may exacerbate behavioral responses to inflammatory mediators and pruritogens. In *Nf1* mouse models, *Nf1+/−* mast cells exhibit increased proliferation and secretion of cytokines[20, 21, 45]. Moreover, *Nf1+/−* mast cells are crucial for tumor initiation, highlighting the importance of this *Nf1+/−* mast cell phenotype in the disease[27, 46]. Mast cells are known to contribute to inflammatory pain and histamine-dependent itch through cytokine release and subsequent nociceptor sensitization[12, 28, 31]. Therefore, this *Nf1+/−* mast cell phenotype likely would increase both inflammatory pain and pruritogen-induced itch through heightened cytokine release and subsequent nociceptor sensitization.

The purpose of this study is to determine whether *Nf1* heterozygosity in mice increases pain and itch behavior. We sought to determine whether there is increased pain and itch behavior in *Nf1*+/− mice, which lack tumors, using assessments of somatosensation, itch, chronic inflammatory pain and neuropathic pain. Herein, we demonstrate that *Nf1+/−* mice exhibit no hypersensitivity to acute noxious or pruritic stimuli, and have unaltered hypersensitivity in the context of inflammatory and neuropathic pain models. Moreover, contrary to our original hypothesis, formalin-induced nocifensive responses are attenuated. Overall, *Nf1* heterozygosity in mice does not increase pain or itch behaviors, arguing that reduced *Nf1* expression is unlikely to be the primary etiology for the increased pain and itch observed in individuals with NF1.

Materials and Methods

Animal husbandry

All experiments were conducted in accordance with the National Institute of Health guidelines and received the approval of the Animal Care and Use Committee of Washington University School of Medicine. Mice were kept on a 12-hour light/dark cycle and allowed

ad libitum access to food and water. Targeted deletion of the *Nf1* gene was achieved by homologous recombination at the *Nf1* locus as previously described[4]. *Nf1+/−* and wild-type mice were bred and maintained on an inbred C57/Bl6 background. Experiments were all performed on seven-to-ten week old male *Nf1+/−* and wild-type littermates. Mice were genotyped using the protocol described previously[4]. For all experiments, the experimenter was blind to genotype.

Measurement of thermal and mechanical thresholds

All mice were tested in isolated behavior rooms at ~25°C with white noise generators to reduce the influence of outside sounds on testing. Each mouse was placed in an individual, plexiglass behavioral chamber (width, 10 cm; length, 10 cm; height, 15 cm) separated by opaque dividers to prevent mice from seeing each other during testing. Prior to all behavior tests, mice were allowed to acclimate in these chambers for 2–3 hours. Following acclimation, behavioral responses to heat, mechanical, or cold stimuli were assessed as described below.

To measure withdrawal thresholds to heat stimuli, a modified Hargreaves' test was performed[1, 17]. Briefly, mice were placed into behavioral chambers on a pre-warmed (~30°C) glass plate (390G Plantar Test Appar atus, IITC Life Science). Following acclimation, a radiant heat source was applied to the footpad of the hindlimb with an active intensity of 17% maximal, an inactive intensity of 3% maximal, and a cutoff of 20 seconds. Only awake, still mice were tested. Five independent measures were performed for each paw on all mice to establish baseline. Following injection of an inflammatory mediator the next day, withdrawal latency at hourly time points was obtained by averaging two measurements per paw performed during that hour. Each subsequent day's mean value consisted of three independent measures per paw for each mouse. All paw withdrawal latencies from the injection day and subsequent days were converted into and graphed as percent baseline.

For the responses to mechanical stimuli, mice were placed in behavioral chambers on a wire mesh. Modified, calibrated filaments (North Coast Medical) were used to assess mechanical thresholds using the up-down method[6]. Briefly, a filament was applied between the anterior and posterior hind paw footpads of awake, still mice. Filaments were bent slightly when applied and held for ~2 seconds prior to removal. Withdrawal to this stimulus was scored as a response to the filament. For baselines, mice were tested three times per paw and withdrawal thresholds were averaged. On the injection day of an inflammatory mediator, hourly time points represented one measurement per paw for each mouse. Subsequent days used the average of three independent measurements as on the baseline day.

For responses to cold stimuli, two different tests were used to assess either ongoing responses or paw withdrawal latency to a cold stimulus. Responses to cold stimuli were assessed using a drop of acetone (Sigma Aldrich) applied to the plantar surface of the hind paw as described previously[15]. The mice were observed for 5 minutes following this stimulus for signs of ongoing behaviors related to this cold stimulus including licking, lifting, and flicking of the tested hind paw. A positive response was defined as any of these behaviors occurring after the first 15 seconds following the stimulus since all mice responded during this period[15]. Likewise, time spent in these behaviors was recorded in the 5-minute period again excluding any time during the first 15 seconds. Additionally, paw withdrawal latency to a cold stimulus was measured using the cold plantar assay[5]. Briefly, mice were placed into behavioral chambers on ¼ inch glass. Powdered dry ice packed into a modified 3 ml syringe was applied to the glass surface underneath the plantar surface of the paw. Targeting was achieved using mirrors underneath the testing platform. Paw withdrawal latency was assessed every 7 minutes alternating between paws for each mouse. Five

measurements were taken per paw and all were averaged together to obtain the paw withdrawal latency for each mouse.

Thermotaxis and Temperature Preference

Thermotaxis behavior was assessed using a thermal gradient assay on an apparatus (GNF Systems Gradient Plate Version 1.0, San Diego, CA) ranging in temperature from ~12°C to ~47°C as described previo usly[13]. Briefly, mice were acclimated to the room in their home cage for 2–3 hours prior to testing. On the day prior to performing the gradient assay, mice were then acclimated for 2 hours on the apparatus with no temperature gradient. On the day of testing, the gradient was turned on during the mouse acclimation to the room in their home cage. Immediately prior to testing, the temperature of 20 equal-sized zones was determined by averaging the temperatures at the zone's edges. Mice were then placed in the apparatus and tested for 2 hours. Anymaze software (Stoelting Co., Wood Dale, IL) was used to track the center of each mouse and determine the mouse's zone location for time in zone calculations on both days. Temperature preference was defined as the temperature of the zone in which a mouse spent the most time for the entire test.

Formalin-induced spontaneous behaviors and heat hypersensitivity

On the day prior to testing mice for spontaneous behaviors associated with formalin intraplantar injection, mice were tested for their baseline responses to a radiant heat source. The following day, mice were acclimated on a Plexiglass platform in behavioral chambers for at least 2 hours. Following acclimation, mice were injected with a 10 μl intraplantar, subcutaneous dose of 2% formalin (Sigma-Aldrich) dissolved in a sterile 0.9% NaCl solution and were immediately returned to their assigned behavioral chamber[1]. Once all animals were injected, the experimenter exited the room quietly, and a video recording was taken for 1 hour using a webcam (Logitech, Newark, CA) with 960 x 720 video resolution. Spontaneous nocifensive behaviors of licking, lifting, and flicking were measured in 5-minute bins from these videos. During data analysis, the test was also divided into two distinct phases: the first phase (0–10 minutes) and the second phase (10–60 minutes) to assess both peripheral and central sensitization respectively. For responses to the radiant heat source using the modified Hargreaves' test, mice were transported to the Hargreaves' apparatus immediately following the hour of spontaneous behavior. These mice were allowed to acclimate for an hour after which 2 and 3-hour paw withdrawal thresholds following the initial injection were measured as described above. On subsequent days, three independent measures of paw withdrawal thresholds were performed and averaged.

Capsaicin-induced spontaneous behavior

Following acclimation, mice were injected with an intraplantar, subcutaneous dose of 10 μl of capsaicin (1μg/10 μl; Sigma-Aldrich). A video recording was taken for 5 minutes post-capsaicin injection using a webcam (Logitech, Newark, CA) with 960 x 720 video resolution. Spontaneous nocifensive behaviors of licking, lifting, and flicking were measured for 5 minutes after injection of capsaicin from these videos.

Inflammation-induced thermal and mechanical hypersensitivity

Complete Freund's adjuvant (CFA; 10 μl, 1.0 mg/mL; Sigma-Aldrich) was injected into the hind paw using an intraplantar, subcutaneous injection in order to induce a model of inflammatory pain known to be dependent on nerve growth factor (NGF)[44]. Mechanical hypersensitivity following CFA was assessed as described above. Following intraplantar injection of CFA, average paw withdrawal latencies were obtained from one measurement per paw for each hour after injection. For the subsequent day(s), three measurements were taken per paw and averaged to obtain a day mean value for both tests. In separate cohorts of

mice, NGF (10 μl, 20 ng/μl; Harlan Laboratories) was injected subcutaneously on the intraplantar surface of the hind paw and heat sensitivity was evaluated as described above. Average paw withdrawal latencies were obtained from two measurements per paw for each hour after injection and three measurements per paw on subsequent days following injection.

Pruritogen-induced scratching behaviors

For itch experiments, mice were placed into behavioral chambers on a Plexiglass platform and allowed to acclimate for at least 2 hours. To assess histamine-dependent scratching, histamine was injected subcutaneously into the nape of the neck. Briefly, for all itch experiments, the nape of the neck was shaved the day prior to testing. On the day of testing, histamine (50 μg/10 μl; Sigma-Aldrich) was injected into the nape of the neck while restraining the mouse. Likewise, to assess effects of mast cell degranulation and subsequent histamine-dependent and –independent scratching, the potent mast cell degranulator, compound 48/80 (60 μg/30 μl; Sigma-Aldrich) was injected into the nape of the neck in separate experiments. To assess histamine-independent scratching, separate experiments used the anti-malaria drug, chloroquine (200 μg/50 μl; Sigma-Aldrich). For all these experiments, mice were immediately placed back in their assigned behavioral chambers and bouts of scratching were measured live in 5-minute bins for 30 minutes following injection.

Chronic constriction injury (CCI) model

As a model of neuropathic injury, chronic constriction injury (CCI) was conducted using a modified version of the rat CCI model[3]. Briefly, the day prior to surgery, mice were tested for their acute responses to acetone as described above. Then, on the day of surgery, mice were anesthesized using an anesthetic mixture of ketamine (38 mg/mL, Fort Dodge Animal Health, Fort Dodge, IA), xylazine (1.92 mg/mL, Akorn Inc. Decatur, IL), and acepromazine (0.38 mg/mL, Butler Animal Health Supply, Dublin, OH). Upon loss of response to tail pinch, an incision was made into the upper thigh and the leg muscles were separated by blunt dissection to expose the sciatic nerve proximal to its branching into the three main branches. Using chromic gut, two loose ligatures were placed around the sciatic nerve approximately 1 mm apart. The skin was then sutured using 6-0 silk. Aseptic techniques were used throughout the ~1 hour-long surgery. Mice were allowed to recover on a warmed surgical pad until they were able to move freely, at which time they were returned to their home cage. Responses to acetone were tested as outlined above 1 day, 4 days, 7 days, 14 days, and 28 days after surgery in order to assess development and resolution of cold allodynia.

Statistical Analyses

Excel (Microsoft) and Prism (GraphPad) software were used to analyze all data. Data in all graphs are reported as the mean +/− the standard error of the mean (SEM). Statistical analyses are described in the Results section for each experiment. For all tests, p-values less than 0.05 were considered significant.

Results

Nf1 heterozygosity did not alter baseline responses to thermal and mechanical stimuli

To test whether there is predisposition for increased pain and itch behavior due to *Nf1* heterozygosity, we used *Nf1*+/− male mice[4] to measure responses to thermal and mechanical stimuli. Thermotaxis behavior and temperature preferences were similar in *Nf1+/−* and wild-type littermate mice (Figure 1A, two-way ANOVA and t-test respectively). Likewise, there was no difference in either the latency to paw withdrawal from a radiant heat source or paw withdrawal threshold from a mechanical stimulus between *Nf1+/−* and

wild-type littermate mice (Figure 1B–C; t-tests). Responses to evaporative cooling of acetone did not differ between groups either in percent of trials responding or total time responding in naïve mice (Figure 1D; t-tests). Likewise, there was no difference in paw withdrawal latency between *Nf1+/−* and wild-type littermate mice as measured by the cold plantar assay (Figure 1E; t-test). Overall, these data indicate that *Nf1* heterozygosity does not alter baseline sensitivity to thermal and mechanical stimuli.

Nf1 heterozygosity decreased formalin-induced nocifensive responses

To assess whether there are alterations in peripheral or central mechanisms of processing nociceptive stimuli in *Nf1*+/− mice, we compared formalin-induced spontaneous behaviors and heat hypersensitivity induced by formalin injection between *Nf1+/−* and wild-type littermate mice. Despite the evidence for hyperexcitability of a subpopulation of nociceptors[18, 40, 41], there was no statistically significant difference in the time course of the formalin-induced spontaneous nocifensive behavior between groups (Figure 2A; two-way repeated measures (RM) ANOVA). Moreover, when the test was parsed into its two distinct phases, there was a decrease in the first phase (0–10 minutes) for the *Nf1+/−* mice compared to their wild-type littermates (Figure 2B; t-test **p<0.01), but no difference in the second phase (10–60 minutes) between groups (Figure 2B; t-test). Similarly, formalin-induced heat hypersensitivity was attenuated in *Nf1+/−* mice compared to their wild-type littermates (Figure 2C; two-way RM ANOVA; main effect of genotype *p<0.05). A Bonferroni *post-hoc* test revealed a difference in hypersensitivity between groups at 1 day post-formalin, wherein the *Nf1+/−* male mice are less hypersensitive (Figure 2C; two-way RM ANOVA **p<0.01). Overall, these data suggest that *Nf1* heterozygosity decreases behavioral sensitization to formalin and thus, this result contradicts the hypothesis that *Nf1* heterozygosity may increase pain behaviors.

Nf1 heterozygosity did not alter capsaicin-induced nocifensive behavior

In vitro, *Nf1+/−* capsaicin-sensitive neurons exhibit hyper-responsiveness to stimuli[18, 40, 41] that may lead to increased behavioral hypersensitivity to capsaicin in the *Nf1+/−* mice. To assess whether this *Nf1+/−* capsaicin-sensitive neuron hyperexcitability[40, 41] and their increased stimulus-evoked CGRP release *in vitro*[18] lead to an exacerbated nocifensive response to capsaicin, we compared capsaicin-induced spontaneous nocifensive behavior in *Nf1+/−* and wild-type littermates. Following intraplantar, subcutaneous injection of 1μg/10 μl capsaicin, *Nf1+/−* and wild-type littermates exhibited similar time spent responding in the initial five minutes post-capsaicin (Figure 2D, unpaired t-test). Therefore, despite *in vitro* reports of hyper-responsiveness of this sensory neuron population in *Nf1+/−*, *Nf1* heterozygosity does not alter the behavioral response to capsaicin.

Nf1 heterozygosity did not alter NGF-dependent nocifensive responses to heat or mechanical stimuli

Previous studies have shown that NGF can initiate peripheral sensitization both directly via activation of signaling cascades in nociceptors and indirectly via activation of mast cells causing cytokine release[12, 28, 31]. In light of previous reports of hyperexcitability of *Nf1+/−* nociceptors[18, 40, 41] and the increased activity of *Nf1+/−* mast cells[20, 21, 45], we hypothesized that *Nf1+/−* mice might develop more robust NGF-induced heat hypersensitivity relative to wild type mice. To assess whether *Nf1*+/− mice exhibit increased NGF-dependent hypersensitvity, we tested both NGF-induced heat hypersensitivity and CFA-induced mechanical hypersensitivity in *Nf1+/−* mice and their wild type littermates. Following injection of 0.2 μg NGF, *Nf1+/−* and wild-type littermate mice develop similar thermal hypersensitivity that resolved 48 hours later (Figure 3A, two-way RM ANOVA). Both genotypes also develop similar mechanical hypersensitivity following 10 μl injection of

CFA (Figure 3A, two-way RM ANOVA). Overall, these data suggest that there is no alteration in NGF-dependent hypersensitivity as a result of *Nf1* heterozygosity.

Nf1 heterozygosity does not alter histamine-dependent or histamine–independent scratching

Although NGF-dependent hypersensitivity was unchanged, hyperactivity of *Nf1+/−* nociceptors[18, 40, 41] and mast cells[20, 21, 45] could increase histamine-dependent scratching. Therefore, to determine whether histamine-dependent itch was altered by *Nf1* heterozygosity, we quantified bouts of scratching behavior following injection of histamine. *Nf1+/−* mice were not statistically different compared to wild-type littermates in either the time course of scratching bouts (Figure 4A; two-way RM ANOVA) or the total number of scratching bouts following histamine (*Nf1+/−*: 91.86±13.61; WT: 109±25.59; unpaired t-test). To determine if mast cell degranulation caused increased scratching in *Nf1+/−* mice, we quantified scratching after administration of a potent mast cell degranulator, compound 48/80. Again, there was no difference between genotypes in the time course of scratching bouts (Figure 4B, two-way RM ANOVA) or the total number of scratching bouts following compound 48/80 (*Nf1+/−*: 397.17±48.61; WT: 404.44±29.27; unpaired t-test). While the itch phenotype in individuals with NF1 is currently not well characterized, clinical complaints of itch in the context of NF1 may result from histamine-independent mechanisms of itch. To assess whether histamine-independent itch is altered in *Nf1+/−* mice, we measured bouts of scratching following injection of the pruritic anti-malaria drug, chloroquine. *Nf1+/−* mice were not statistically different compared to wild-type littermates in either the time course for scratching bouts (Figure 4C; two-way RM ANOVA) or the total number of scratching bouts (*Nf1+/−*: 292.83± 40.91;WT: 323.5±36.75; unpaired t-test). Overall, these results suggest that there is no difference between these groups in either histamine-dependent or histamine-independent scratching.

Nf1 heterozygosity did not alter the development or maintenance of cold allodynia after CCI

Previous report show that there is increased excitability of DRG sensory neurons after the dissociation injury necessary for *in vitro* electrophysiological studies[48]. Therefore, since the reported hyperexcitability of *Nf1+/−* sensory neurons[40, 41] did not lead to increased acute pain, inflammatory pain, or itch, it is conceivable that *Nf1* heterozygosity may exacerbate nerve injury-induced excitability and thus, may predispose *Nf1+/−* mice to increased nerve injury-induced pain. To test this hypothesis, we assessed the extent of cold allodynia following CCI in both *Nf1+/−* and wild-type male littermates. *Nf1+/−* mice and wild-type littermates did not differ in percent response to acetone following CCI (Figure 5A; RM 2-way ANOVA). Likewise, both genotypes exhibited similar time responding to the acetone stimuli after CCI (Figure 5A; RM 2-way ANOVA). Overall, these data suggest that there is no change in the development or maintenance of cold allodynia following CCI due to *Nf1* heterozygosity.

Discussion

Although reported pain and itch in individuals with NF1 may result directly from *NF1* heterozygosity, studies have yet to address this question. In this present study, we directly assess whether *Nf1* heterozygosity alone increases responses to nociceptive and pruriceptive stimuli using *Nf1+/−* mice lacking nerve sheath tumors. We found that *Nf1*+/− mice did not exhibit increased sensitivity to acute, inflammatory, or neuropathic pain, or to pruritogens. Therefore, *Nf1* heterozygosity alone does not result in increased pain and itch.

Unexpectedly, formalin-induced nocifensive behaviors were attenuated in *Nf1*+/− mice, suggesting that peripheral afferent activation may be blunted in *Nf1*+/− DRG neurons. Since the first phase of spontaneous behavior elicited by formalin is thought to be dependent on peripheral afferent activation[30], a decrease in formalin-induced spontaneous behavior for only the first phase is consistent with decreased afferent activation *in vivo* following formalin injection in the paw. This result directly contradicts reports of *in vitro* hyperexcitability of *Nf1+/−* sensory neurons and increased CGRP release by nociceptors[18, 40, 41]. Unaltered nocifensive responses to capsaicin in *Nf1+/−* mice provides further evidence against the *in vivo* relevance of the reported, *in vitro Nf1+/−* sensory neuron hyperexcitability. As such, the behavioral data are consistent with the hypothesis that *in vivo* primary afferent nociceptors are not hyperexcitable to stimuli, but may actually be hypoexcitable, contrary to *in vitro* results. Future experiments should address this question using electrophysiological recordings, such as the skin-nerve preparation that more closely represents the situation *in vivo* in order to determine whether there is altered excitability of nociceptors *in vivo.* Alternatively, this blunted nocifensive behavior in the first phase of the formalin test may indicate a central mechanism of inhibition or may highlight the importance of other variables such as gender, depression and tumor burden in the pain and itch phenotypes observed in individuals with NF1.

In *Nf1+/−* mice, there is evidence to suggest that activity-dependent GABAergic inhibition may be increased, which could suppress pain and itch behaviors. Previous studies have shown that *Nf1* heterozygosity in forebrain inhibitory neurons increases GABA release following high frequency stimulation and thus adversely affects spatial learning[8]. If GABAergic inhibition is increased in the spinal cord or midbrain in *Nf1*+/− mice, the hypothesized pathological pain in *Nf1*+/− mice may be prevented since $GABA_A$ receptor activation in spinal and midbrain structures relieves hyperalgesia in mice[25, 35, 47]. Similarly, since inhibition of itch requires spinal cord dorsal horn inhibitory neurons including GABAergic neurons, increased GABA release in the spinal cords of *Nf1*+/− mice could prevent behavioral sensitization to pruritogens[37]. Future studies should test whether there is an increase in GABAergic inhibition throughout the pain neuraxis in *Nf1*+/− mice that could compensate for hyperexcitability of nociceptors and prevent behavioral sensitization to nociceptive and pruriceptive stimuli.

Previous studies suggest that there may be sex differences in the effect of *Nf1* heterozygosity on pain behavior in mice. In a recent preliminary report, increased hyperalgesia was observed in *Nf1+/−* females, but not *Nf1+/−* males, consistent with our data[29]. Sex differences in pain thresholds have been previously noted, with females being more sensitive to pain in general[34]. Compelling data from a recent post-hoc analysis of pain scores from de-identified subjects found that females rate their pain higher than males in a majority of diseases with co-morbid pain[38]. Furthermore, sex differences have been observed with respect to the effects of δ-opioid receptor involvement in thermal pain and GIRK2 involvement in pain and analgesia[33]. This group has also demonstrated that female rodents are more sensitive to pruritigens than males, providing a basis for sex differences in itch[16]. While there is a strong basis for sex differences in pain and itch, there are no data in the NF1 patient population to support gender-specific increases in either pain or itch. Future population studies using questionnaires and quantitative sensory testing (QST) will be required to determine whether sex differences in pain and itch exist in the NF1 patient population.

Our results may also highlight the importance of co-morbidities in NF1 and their role in pain. Among other neurological complaints, individuals with NF1 have an increased incidence of depression,[22] and studies show a higher prevalence and severity of pain in depressed patients[2]. Therefore, the increased effect of bodily pain on quality of life could

partially be a result of depression and not solely dependent on *Nf1* heterozygosity. As a result, future analyses should include an assessment of depression along with one of pain to determine whether there is any correlation between these co-morbidities in NF1. In addition, pain and itch in NF1 may be secondary effects of tumor formation, as suggested by prior clinical studies[7, 9, 14]. Although these reports suggest a prominent role for tumors in development of pain and itch in NF1, further study is necessary in order to better assess whether tumor growth correlates with pain and itch.

In summary, our results are consistent with the hypothesis that *Nf1* heterozygosity does not increase pain or itch behavior in mice. Coupled with previous patient studies, additional *Nf1* mouse models should be employed to more completely understand the molecular and cellular bases for the reported prevalence of pain and itch in NF1 patients.

Acknowledgments

The authors thank Dr. Gina Story for assistance with the thermotaxis experiment.

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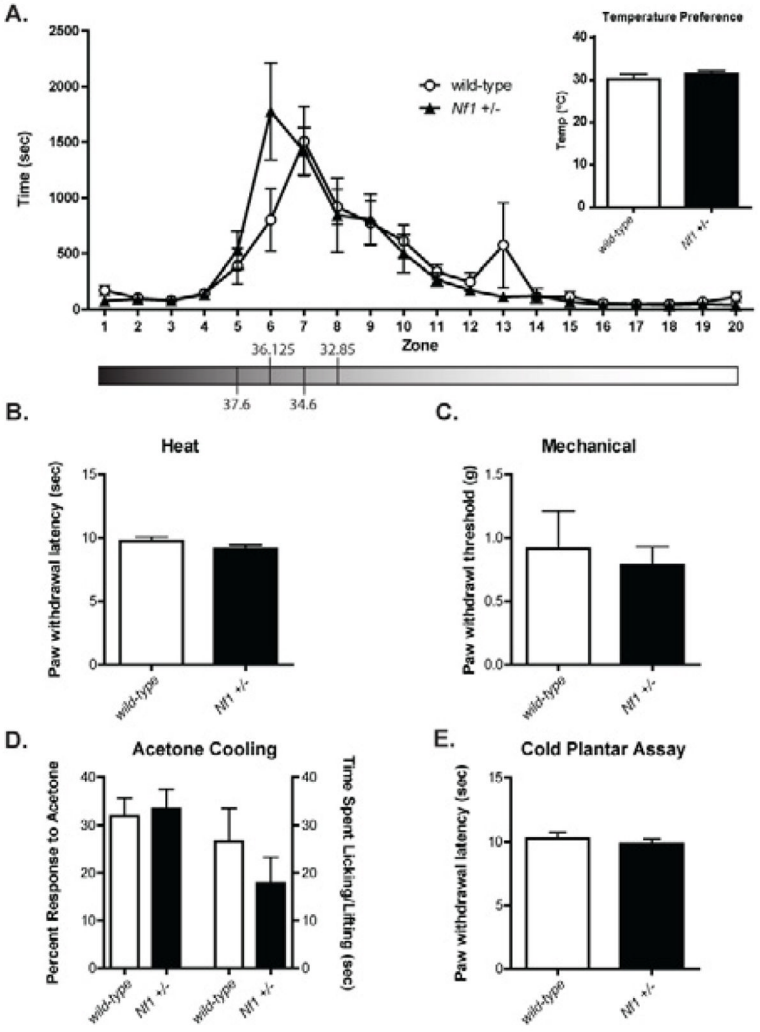

Figure 1. Nf1 heterozygosity did not alter baseline sensitivity to thermal or mechanical stimuli

A. To assess temperature preference, mice were subjected to a gradient ranging from ~12°C to ~47°C. Thermotaxis behavior over 2 hours was similar between WT (n=9) and *Nf1+/−* (n=9) littermates. The temperature of the zone in which they spent the most time, or their temperature preference was not different between WT (n=9) and *Nf1+/−* (n=9) littermates (inset). B. Sensitivity to acute, noxious thermal stimuli in the Hargreaves' test was similar between WT (n=21) and *Nf1+/−* (n=19) littermates. C. Sensitivity to acute, noxious mechanical stimuli in the up-down Von Frey method was not changed in the *Nf1+/−* (n = 6) compared to wild-type (n = 6) littermates. D. Responses to the cooling stimulus, acetone, were not different in either percent trials responding and total time spent responding between WT (n = 21) and *Nf1+/−* (n = 26) littermates. E. Paw withdrawal latency to a ramping cold stimulus in the cold plantar assay was similar between *Nf1+/−* (n = 12) and wild-type (n = 12) littermates.

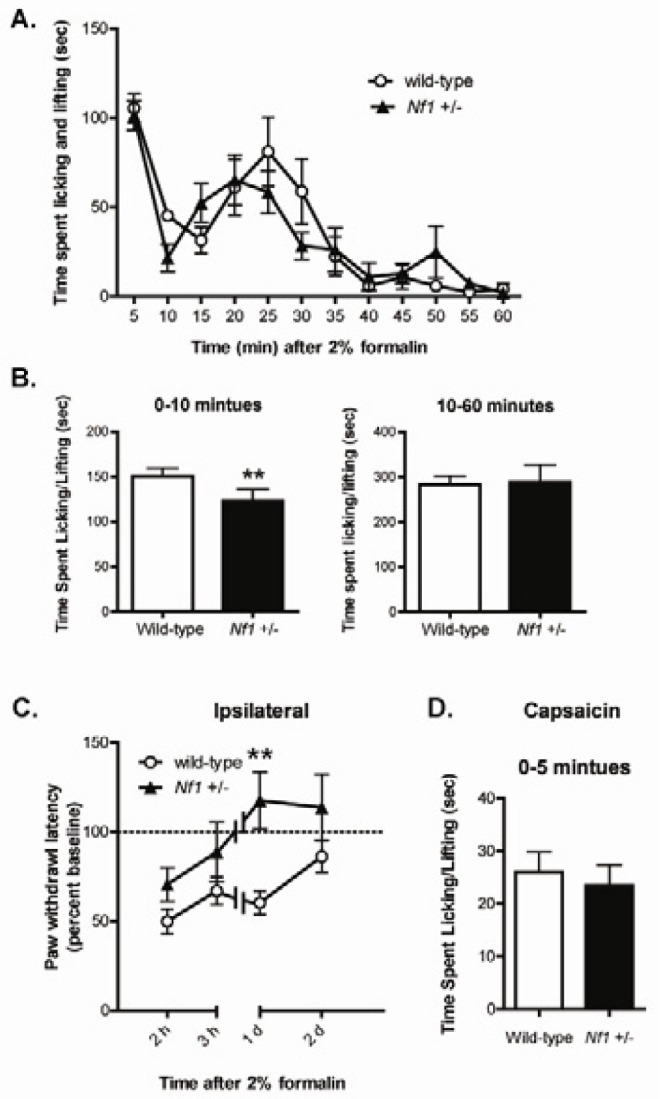

Figure 2. Nf1 heterozygosity attenuated formalin-induced spontaneous behaviors and heat hypersensitivity

A. Over the entire duration of the formalin test, WT (n = 6) and *Nf1+/−* (n = 8) littermates exhibited a comparable time course of spontaneous nocifensive responses following a 10 μl intraplantar injection of 2% formalin. B. *Nf1+/−* littermates showed decreased spontaneous nocifensive behavior in the phase 1 (0–10 minutes), but not phase 2, (10–60 minutes), compared to wild-type littermates (t-test; **p < 0.01). C. Similarly, formalin-induced heat hypersensitivity was blunted in *Nf1+/−* (n = 7) compared to wild-type (n = 7) littermates (RM 2-way ANOVA; main effect of genotype; *p < 0.05). At 1 day post-formalin, *Nf1+/−* (n = 7) littermates showed a difference compared to their wild-type littermates on 1 day post-formalin (two-way RM ANOVA with Bonferroni post-hoc test; **p < 0.01). D. Capsaicin-induced spontaneous behavior (0–5 minutes after intraplantar, subcutaneous injection) was unchanged in *Nf1+/−* (n = 9) compared to wild-type (n = 7) littermates (unpaired t-test).

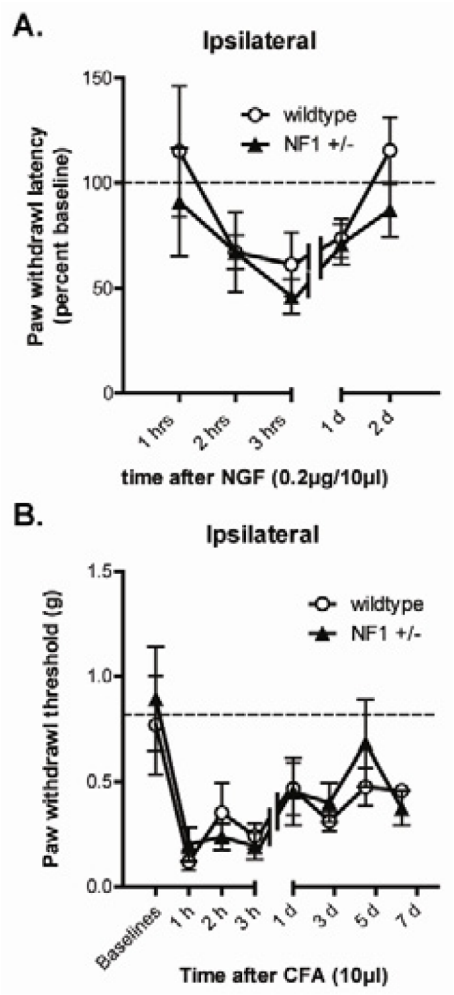

Figure 3. Nf1 heterozygosity did not affect NGF-induced hypersensitivity to heat or mechanical stimuli

A. In a model of chronic inflammatory pain, WT and *Nf1+/−* (n = 5 for each) littermates showed similar development and resolution of heat hyperalgesia following intraplantar injection of NGF (0.2μg/10μl). B. Likewise, for another NGF-dependent inflammatory mediator, WT and *Nf1+/−* (n = 6 for each) littermates showed similar development and resolution of mechanical allodynia following intraplantar injection of CFA (10μl).

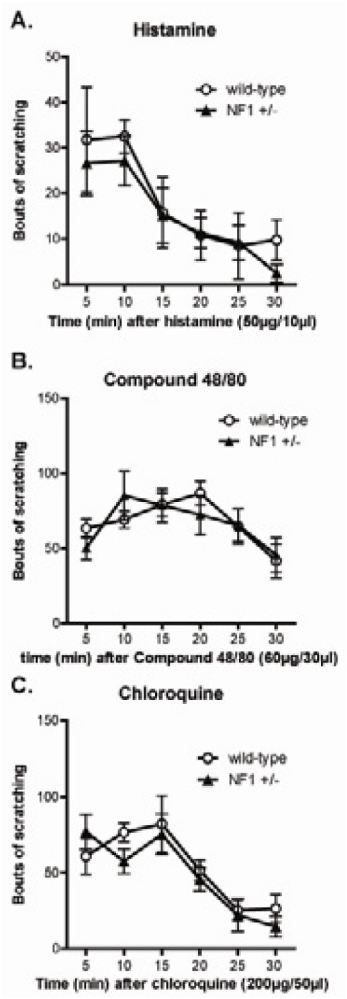

Figure 4. Nf1 heterozygosity does not alter histamine-dependent or –independent scratching behavior

A. WT (n = 4) and *Nf1+/−* (n = 7) littermates showed similar histamine-dependent scratching as demonstrated by the time course of scratching following histamine (50μg/10μl) injection into the nape of the neck. B. Potent mast cell degranulation by compound 48/80 (60μg/30μl) injection into the nape of the neck caused a similar time course of scratching in WT (n = 9) and *Nf1+/−* (n = 6) littermates. C. WT (n = 4) and *Nf1+/−* (n = 6) littermates exhibited a comparable time course of histamine-independent scratching induced by chloroquine (200μg/50μl) injection into the nape of the neck.

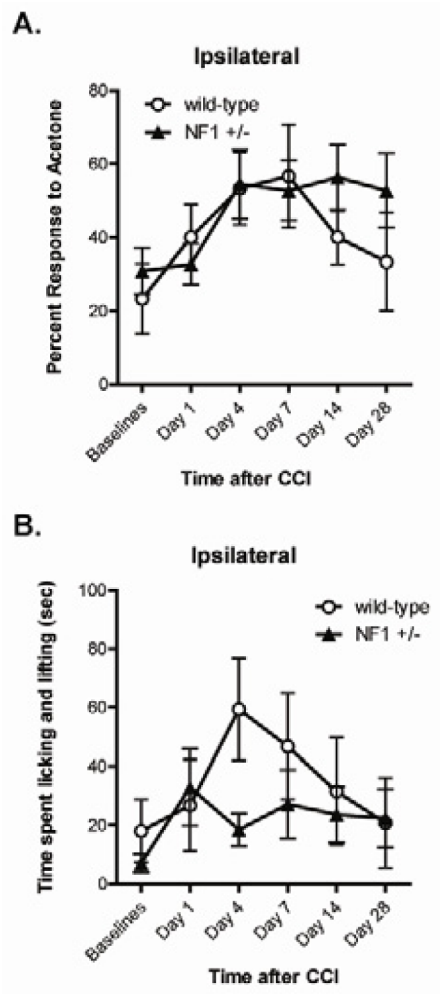

Figure 5. Nf1 heterozygosity did not alter CCI-induced cold allodynia

A. Cold allodynia was assessed using acetone following the CCI in both WT and *Nf1+/−* littermates (n = 6–11). The time course for percent of trials responding after injury did not differ between the WT and *Nf1+/−* littermates. B. These WT and *Nf1+/−* littermates did not differ in the time course for time spent responding to acetone stimuli after CCI (two-way RM ANOVA).