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Nociceptor sensitization depends on age and pain chronicity

Nociceptor firing depends on age & pain chronicity

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Abstract

Peripheral inflammation causes mechanical pain behavior and increased action potential firing. However, most studies examine inflammatory pain at acute, rather than chronic time points, despite chronic pain's greater burden on patient populations, especially aged individuals. Furthermore, there is disagreement in the field about whether primary afferents contribute to chronic pain. Therefore, we sought to evaluate the contribution of nociceptor activity to the generation of pain behaviors during the acute and chronic phases of inflammation in both young and aged mice.

We found that both young (2 months) and aged (> 18 months) mice exhibited prominent pain behaviors during both acute (2-day) and chronic (8-week) inflammation. However, young mice exhibited greater behavioral sensitization to mechanical stimuli than their aged counterparts.

Teased fiber recordings in young animals revealed a 2-fold mechanical sensitization in C fibers during acute inflammation, but an unexpected 2-fold reduction in firing during chronic inflammation. Responsiveness to capsaicin and mechanical responsiveness of AM fibers were also reduced chronically. Importantly, this lack of sensitization in afferent firing during chronic inflammation occurred even as these inflamed mice exhibited continued behavioral sensitization. Interestingly, C fibers from inflamed aged animals showed no change in mechanical firing compared to controls during either the acute or chronic inflammatory phases, despite strong behavioral sensitization to mechanical stimuli at these time points.

These results reveal two important findings: 1) nociceptor sensitization to mechanical stimulation depends on age and the chronicity of injury, and 2)

maintenance of chronic inflammatory pain does not rely on enhanced peripheral drive.

Significance Statement: Most peripheral pain research examines acute pain in young animals, with the assumption that peripheral pain mechanisms are similar during acute pain and chronic pain for animals of all ages. Our results indicate that peripheral nociceptors may contribute minimally to pain sensation at chronic inflammatory time points in young populations, and at either acute or chronic time points in aged populations. These findings have important implications for novel analgesic design, as drugs targeting peripheral pain mechanisms observed under acute inflammatory conditions may be unlikely to show efficacy under chronic inflammatory conditions. Additionally, since nociceptors from aged animals do not change their firing rates in response to acute or chronic pain, peripherally-acting analgesics may also be largely ineffective in aged populations.

Introduction

Chronic pain results in hundreds of billions of dollars in economic costs in the United States (Committee on Advancing Pain Research, Care, and Education, 2011), but, despite a massive research effort over the past few decades, the successful translation of novel analgesics from preclinical models to the clinic has dwindled (Percie du Sert and Rice, 2014). While the cause of this drought is multifactorial, one of the primary sources may be limitations in the animal models used to elucidate the mechanisms of pain at the molecular level (Berge, 2011). Specifically, a significant shortcoming for many pain models, especially those examining inflammatory pain, has been the brief time course over which pain behaviors and molecular mechanisms are examined (Berge, 2011). Because of pressures related to animal housing costs, planning, and time to complete experiments, most studies involving inflammatory pain examine relatively acute time points following injury instead of true chronic time points that are often most relevant clinically (Wilson et al., 2006; Berge, 2011).

As a result, researchers have long inferred that the mechanisms discovered during the acute inflammatory pain phase remain constant even as pain becomes chronic, and that any drug targets identified acutely will also be reliable targets chronically. However, this premise has rarely been tested in animal models of bona fide chronic inflammatory pain (Wilson et al., 2006).

As an extension of this uncertainty, there is long-standing disagreement in the field over whether chronic pain is mediated by a combination of peripheral (primary afferent) and central (spinal cord/brain) mechanisms, or just central mechanisms alone. However, because few studies have mechanistically examined pain sensation during chronic time points, this question is still unresolved. This is an important concern, as much research has focused on identifying potential drug targets in the peripheral nervous system in an effort to combat chronic pain (Cairns, 2009).

Although chronic pain affects individuals of all ages, one group it affects disproportionately is the elderly. Recent health surveys have found that greater than 50% of individuals over the age of 65 have complaints of pain, and that in 30% of these patients the pain is bad enough to interfere with the completion of activities of daily living (Thomas et al., 2004; Mottram et al., 2008; Patel et al., 2013, 2014). This pain is the result of a variety of pathologies that involve inflammatory mechanisms, including rheumatoid arthritis, osteoarthritis, gout, and musculoskeletal pain (Bruckenthal et al., 2009). However, a common thread amongst all of these is that the pain suffered by aged patients is often unresolved despite pharmacological treatment (Cavalieri, 2005; Tracy and Sean Morrison, 2013). Although this is becoming recognized as a considerable problem at the clinical level, comparatively little basic research has been conducted on pain mechanisms in aged animal models, and those studies that have examined pain responses in aged animals have shown conflicting results (Yezierski, 2012).

Therefore, using a mouse model of truly chronic inflammatory pain, we sought to determine whether mechanical pain sensation changes with age, and, furthermore, whether the peripheral nervous system contributes to mechanical pain sensation at chronic time points in both young and aged animals. Using a combination of behavioral, electrophysiological, and molecular approaches, here we show that age affects pain sensation under both basal and chronic inflammatory conditions, and, surprisingly, that peripheral afferent drive contributes minimally to the behavioral sensitization during the chronic phase of an inflammatory injury.

Methods

Animals: “Young” mice were 7-20 weeks of age ($\bar{x}$ = 13.6 ± 0.69 weeks) at the start of behavioral testing (and thus 15-28 weeks of age at the time of electrophysiological

experiments). “Aged” mice were all > 77 weeks of age ($\bar{x}$ = 94.4 ± 1.1 weeks) at the start of behavioral testing (85 - 108 weeks at the time of electrophysiological experiments). Mice that are 20 weeks of age correspond roughly to a 27 year old human, while mice 100 weeks of age correspond roughly to a 67 year old human (Flurkey et al., 2007). Animals used in these experiments were all male. Mice were predominantly from a mixed C57Bl/6 / outbred Swiss Webster / CBA background (https://www.jax.org/strain/004782); 3 aged animals were from a C57BL/6 only background, but no differences were observed between these animals and the mixed background animals. Animals were housed in a climate-controlled room with a 14:10 light:dark cycle and *ad libitum* access to food and water. All behavioral assays and research protocols involving animals were approved by the Institutional Animal Care and Use Committee at the author’s institution.

Behavior: Behavioral testing for mechanical sensitivity was performed in a dedicated behavioral suite at the author’s institution. Prior to testing, animals were placed in a small plastic chamber situated on a wire mesh that allowed access to mechanical probing of the plantar paw. Animals were habituated in these chambers for at least 1 hour prior to testing. After the habituation period, the experimenter utilized calibrated von Frey filaments (North Coast Medical, USA) to mechanically stimulate the glabrous skin of the hindpaw. The Up-Down method was utilized to determine paw withdrawal thresholds, as described (Chaplan et al., 1994). Additionally, a repeated, suprathreshold 3.61 mN von Frey filament was applied to the hindpaw 10 times and the number of responses to this stimulus were recorded. For both the Up-Down test and the suprathreshold test, sufficient time was given between each stimulus to avoid sensitization of the paw.

For the capsaicin behavioral tests, mice were habituated in a small cage on a wire mesh for at least 30 minutes. Animals were then lightly anesthetized with isoflurane and 30 μL of 100 μM capsaicin dissolved in 1% 1-methyl-2-pyrrolidone was injected into the left hindpaw. Animals were then videotaped for 5 minutes and the number of licking/biting behaviors during this time were then analyzed. Blinding was not possible for these experiments as a result of the significant swelling observed in animals injected with Complete Freud's Adjuvant (CFA).

Inflammation Induction: Following basal mechanical sensation testing, young or aged mice were lightly anesthetized via inhaled isoflurane and injected subcutaneously with 30 μL of either sterile PBS or CFA into the left hindpaw. CFA injection resulted in a significant circumferential swelling of the hindpaw coupled with redness and decreased weight bearing that was visually observable. Signs and symptoms of inflammation were noticeable for the duration of the study (at least 8 weeks after injection). We considered the acute inflammatory phase to last from injection of CFA through the first 2 weeks after injection, and the chronic inflammatory phase to include weeks 3-8 post-injection, in accord with previous studies examining the transition from acute to chronic pain (Schwartz et al., 2013; Garrison and Stucky, 2014).

Histology: To obtain examine immune infiltration of the whole paw, paws were fixed in 10% neutral buffered formalin. Specimens were then decalcified and embedded in paraffin blocks. Coronal sections were then made at the level of the metatarsal-phalangeal joint and were stained with hematoxylin and eosin for histologic analysis.

153 *Paw Metrics:* At the time of death, a digital caliper (VWR, USA) was used to measure the
154 width of the affected paw across the metatarsal-phalangeal joints and the height from
155 the plantar surface of the paw to the dorsal surface across the head of the 3rd
156 metatarsal.
157
158 *Teased Fiber Electrophysiology*: To assess primary afferent firing, we utilized saphenous
159 skin-nerve preparations, as described (Reeh, 1986). Briefly, animals were lightly
160 anesthetized and then sacrificed via cervical dislocation. The leg was then quickly
161 shaved with commercial clippers, and the hairy skin and innervating saphenous nerve
162 were quickly removed from the carcass and placed in a heated (32 ± 0.5 °C),
163 oxygenated bath consisting of (in mM): 123 NaCl, 3.5 KCl, 0.7 $MgSO_4$, 1.7 NaH_2PO_4, 2.0
164 $CaCl_2$, 9.5 sodium gluconate, 5.5 glucose, 7.5 sucrose and 10 HEPES. The buffer in the
165 bath was titrated to a pH of 7.45 ± 0.05. The skin was then pinned down and the
166 saphenous nerve was placed in a mineral oil-filled chamber and teased into small
167 fascicles. Nerve bundles were then placed on the recording electrode and a blunt glass
168 probe was used to mechanically stimulate the preparation to identify single unit receptive
169 fields. C fibers displayed conduction velocities <1.2 m/s and A-mechanonociceptors
170 (AM's) displayed conduction velocities between 1.2 and 10 m/s (Koltzenburg et al.,
171 1997). All fibers utilized for these experiments exhibited slow adaptation to a sustained
172 mechanical stimulus
173
174 Once identified, basal activity of each fiber was recorded for 30 -120 seconds. A
175 feedback-controlled mechanical simulation device was then placed over the receptive
176 field, and an increasing series of 15 mN, 35 mN, 70 mN, and 140 mN forces was applied
177 to the receptive field for 12 seconds each. A 1-minute interval was given between each
178 mechanical stimulus to prevent sensitization/desensitization of the fiber.

For another set of experiments, the responsiveness of C fibers to capsaicin was tested. Once the receptive field of a C fiber was identified, a metal ring was sealed around the receptive field using vacuum seal grease. Baseline recordings were then made for 2 minutes to establish a basal firing rate. The buffer within the metal ring was then evacuated and replaced with a solution containing 10 μM capsaicin dissolved in 0.1% 1-methyl-2-pyrrolidone for 2 minutes. Recordings were then analyzed offline and action potentials fired at baseline were subtracted from action potentials fired during capsaicin incubation. To be considered a "responder" to capsaicin, we required that a fiber fire a net of 3 action potentials over the duration of the 2 minute incubation.

Quantitative Real-Time Polymerase Chain Reaction (qPCR): qPCR was performed on L2-5 Dorsal Root Ganglia (DRG) taken from experimental animals at the time of death. Samples were stored in RNALater solution at -20 °C until the time of extraction. DRG samples were first manually homogenized in Trizol (Life Technologies, USA) and RNA was then extracted using the Purelink RNA Micro Scale kit (Life Technologies, USA). RNA samples were then reverse transcribed into cDNA using the Superscript Variable Input Linear Output (VILO) cDNA Synthesis Kit (Life Technologies, USA). qPCR was performed on a Mastercycler ep Realplex^2 thermal cycler (Eppendorf, Germany) using Taqman primers (Life Technologies, USA) according to the manufacturer's instructions. Context sequences and assay IDs can be found in Table 1. Three technical replicates were averaged to obtain a mean cycle time for a given transcript.

Data Analysis and Statistics: All statistical tests were performed using Prism software (Version 5, Graphpad Software, La Jolla, CA). For behavioral testing, paw withdrawal thresholds and percent responses were compared between groups over time using a 2-way repeated measures Analysis of Variance (ANOVA) with Bonferroni post-hoc test for

significance at individual time points. Comparisons of basal (prior to injection) mechanical sensitivity were made using a nonparametric Mann-Whitney test. Capsaicin behavior was compared between groups using a 1-way ANOVA.

For skin-nerve recordings, data was digitized using a PowerLab A/D converter (AD Instruments, USA) and analyzed offline using LabChart 7 software with the Spike Histogram extension (AD Instruments, USA). Recordings were only utilized if the recorded fiber was clearly distinguishable by action potential profile from background noise and other fibers firing during the mechanical stimulation. Comparisons between groups over the force series were made using a 2-way ANOVA with Bonferroni post-hoc analysis. Von Frey thresholds of individual C fibers were compared between CFA- and PBS-injected groups using a non-parametric Kruskal-Wallis test. Spontaneous firing of C fibers was performed using a contingency table with Fisher's exact test. Binned interspike intervals (ISIs) were compared through the use of a chi-square with Fisher's exact test. Coefficients of Variation (CV_2) were determined by the following equation: $((\sqrt{2})*\sigma)/\bar{x}$ where σ is the standard deviation of two adjacent ISIs and $\bar{x}$ is the average of those two ISIs (Holt et al., 1996). All CV_2 for a given spike train were then averaged to yield a single number that was compared between cohorts using a one-way ANOVA with a Bonferroni post-hoc analysis for specific comparisons. Percent responders to capsaicin were compared using a χ^2 test followed by Fisher's exact test. Number of action potentials fired in response to capsaicin incubation was compared using a 1-way ANOVA.

For qPCR, the change in cycle time between the gene of interest and control gene was compared between PBS-injected and CFA-injected groups using a student's t-test to determine significant changes in gene expression at a given time point for a specific group. Changes between groups were analyzed using a 1-way ANOVA of the fold changes for each group with Bonferroni post-hoc analysis.

Prior to study initiation, we set the alpha level to p = 0.05.

Results

Young mice exhibit greater inflammatory mechanical sensitization than aged mice

Few studies have examined mechanical sensation in preclinical studies using aged rodents, and those that have offer discordant results: increased sensitivity in aged rats (Kitagawa et al., 2005), decreased sensitivity in aged mice (Garrison and Stucky, 2014), or no change between young and aged rats (Taguchi et al., 2010) have all been reported. Therefore, we first assessed whether age affects mechanical sensation by measuring paw withdrawal thresholds in young (13 weeks) and aged (> 77 weeks) mice. We found that naïve aged mice exhibited lower mechanical thresholds than naïve young animals (mean 2.35 mN vs 3.22 mN for young animals), indicating an elevated basal sensitivity to mechanical stimuli with older age (Fig 1A[a], * $p < 0.05$, Mann-Whitney test, n = 19 animals for aged group and 14 for young group).

Past studies examining changes in pain perception during aging have found discordant results, with about half of published reports indicating that aged animals have increased pain sensitivity and the other half indicating that aged animals have diminished pain sensation or unaltered pain sensation compared to young animals (for review, see Yezierski, 2012). Therefore, we next considered the effect of a painful inflammatory insult on mechanical thresholds in these populations by injecting CFA subcutaneously into the plantar hindpaw. In comparison to mice injected with PBS, both young and aged animals injected with CFA showed a sharp decline in mechanical paw withdrawal thresholds from the acute inflammatory phase (2-day and 2-week time points) through the chronic inflammatory phase (3 week - 8 week time points) (Fig 1B[b] and 1C[c], **** $p < 0.0001$, 2-way repeated measures ANOVA; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ with Bonferroni post-hoc test for multiple comparisons, n =

6-12 animals as noted on figure). Although young and aged mice both displayed significant reductions in paw withdrawal thresholds following inflammation induction, the amount of sensitization was markedly different between these two age groups. From the end of the acute phase (2 weeks) through much of the chronic phase, aged inflamed mice displayed mechanical thresholds that were 4- to 10-fold higher (less sensitive) than young mice (Fig 1D[d], * $p < 0.05$ using a repeated measures 2-way ANOVA ## $p < 0.01$ and #### $p < 0.0001$ with Bonferroni post-hoc test, n = 8 and 7 animals). Compared to their baseline mechanical thresholds, aged inflamed mice showed reductions in mechanical paw withdrawal thresholds between 43 and 73 percent over the duration of testing, while young mice showed 91-97 percent reductions in paw withdrawal thresholds over the same period (Fig 1E[e], *** $p < 0.001$ with a 2-way repeated measures ANOVA, ## $p < 0.01$ with Bonferroni post-hoc analysis, n = 8 and 7 animals).

We further examined the responses of young and aged mice to a repeated 3.61 mN von Frey filament in order to test mechanical responsiveness to suprathreshold stimuli. While a reduction in mechanical thresholds is characteristic of allodynia, increased responsiveness to a suprathreshold stimulus may be an indication of hyperalgesia. In contrast to the age differences observed for mechanical thresholds, response frequencies to a suprathreshold mechanical stimulus were not different at baseline between young and aged mice (Fig 1F[f], $p > 0.05$, student's t-test, n = 14 and 19 animals). Following inflammation induction, both young and aged mice exhibited significant elevations in response frequencies to the suprathreshold stimulus, with each group ultimately responding approximately 80% of the time compared to 40% at baseline (Fig 1G[g] and 1H[h], **** $p < 0.0001$, 2-way repeated measures ANOVA; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, and #### $p < 0.0001$ with Bonferroni post-hoc test for multiple comparisons, n = 6-12 animals as noted on figure). Interestingly, however, the time course of the sensitization to suprathreshold stimuli was different between young and

aged mice. Whereas young mice injected with CFA responded 80% of the time to a suprathreshold stimulus within 2 days of inflammation induction, aged mice injected with CFA exhibited responses to suprathreshold stimuli that were similar to controls until 3 weeks after injection, in conjunction with the beginning of the chronic phase of pain (Fig 1I', * $p < 0.05$ with 2-way repeated measures ANOVA, # $p < 0.05$, ## $p < 0.01$ with Bonferroni post-hoc test for multiple comparisons, n = 8 and 7 animals). This complements previous reports from both animal models of pain and human studies indicating that aged subjects may develop experimental pain more slowly than young participants (Zheng et al., 2000; L. R. Cruce, John A. Lovell, Terria, 2001).

Also of note is that in our hands, von Frey thresholds and suprathreshold response frequencies never returned to baseline throughout the 8 weeks following CFA injection and instead exhibited quite pronounced sensitization at 8 weeks. This matches our observations of significant swelling and redness in the injected paw, which continued to be present at least 8 weeks after the initial injection (Fig 1J', ** $p < 0.01$,*** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA with Bonferroni post-hoc test, n = 7 animals for aged CFA, 5 animals for aged PBS, 5 animals for young CFA, and 8 animals for young PBS). Furthermore, H&E stained paw sections from naïve, acutely inflamed, and chronically inflamed young animals demonstrate consistent infiltration of immune cells at both 2 days and 8 weeks after CFA injection in accord with a recent report (Ghasemlou et al., 2015) (Fig 1K). Behavioral testing of the contralateral (uninjected) paw yielded no differences in mechanical sensitivity compared to controls (data not shown).

Collectively, these data suggest that, although both young and aged animals display significant pain behaviors during long-standing inflammation, aged animals have a blunted response to inflammatory pain.

Young, but not aged, C fiber nociceptors are sensitized during acute inflammation

Since behavioral pain responses were notably different between young and aged animals, we next wondered whether this was reflected in the firing of primary afferents from these animals. The presence of peripheral sensitization to mechanical stimuli following acute inflammatory injuries has been debated, with some research indicating that primary afferents are sensitized to mechanical stimuli following inflammation (Andrew and Greenspan, 1999; Potenzieri et al., 2008; Lennertz et al., 2012; Smith et al., 2013), while other research does not show an elevation in nociceptive firing following peripheral injury (Kocher et al., 1987; Koerber et al., 2010; Schmidt et al., 2012b). Although recent research has indicated that myelinated fibers may play an important role in mechanical hyperalgesia following CFA-mediated inflammation (Meyer et al., 1991; Andrew and Greenspan, 1999; Potenzieri et al., 2008; Weyer et al., 2015), we chose to first focus on unmyelinated C fibers, since this afferent class has traditionally been understood to transmit painful stimuli to the central nervous system.

We first examined the effect of acute inflammation on C fiber firing in young and aged animals using an *ex vivo* skin-nerve preparation (Fig 2A). We noted a significant 2-fold sensitization in action potential firing to a series of increasing mechanical forces in C fiber afferents from young animals when skin-nerve preparations were harvested 2 days after CFA injection (Fig 2B^{k}, **** $p < 0.0001$ with 2-way ANOVA, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#\#}$ $p < 0.0001$ with Bonferroni post-hoc test, n = 25 fibers for PBS and 28 fibers for CFA, data obtained from 3 animals in each group). In contrast, we found that C fibers from aged animals exhibited a strong trend toward sensitization to mechanical stimuli following acute CFA inflammation as compared to PBS controls, but this relationship was not statistically significant (Fig 2C^{l}, $p = 0.0505$ with 2-way ANOVA, n = 25 fibers for PBS and 32 fibers for CFA, data obtained from 3 animals in each group). The lack of a strong sensitization in aged animals following an acute inflammatory injury may reflect the fact that systemic inflammation increases with age (Singh and Newman, 2011): aged

animals may already have an elevated level of inflammation compared to young animals, such that an additional inflammatory load has limited effects. This hypothesis is supported by recordings of C fibers from uninjured young and aged mice, as action potential firing in response to a mechanical stimulus was significantly higher in uninjured aged animals as compared to uninjured young animals (Fig 2D[m], $p < 0.05$ with 2-way ANOVA, ### $p < 0.001$ with Bonferroni post-hoc test, n = 25 fibers for both aged and young, 3 animals each group). The age-dependent differences in baseline afferent firing also mirror our behavioral observations (Fig 1A), whereby aged control mice exhibited greater mechanical sensitivity at baseline compared to young control mice.

We also examined von Frey thresholds of isolated C fibers from acutely inflamed and control animals. Despite marked reductions in behavioral von Frey thresholds in both age groups after 2 days of CFA inflammation, von Frey thresholds of individual C fibers in the skin-nerve preparation were unchanged in either cohort following acute inflammation (Fig 2E[n], $p > 0.05$ with Kruskal-Wallis test, n = 25 fibers for young PBS, 28 fibers for young CFA, 25 fibers for aged PBS, and 32 fibers for aged CFA, 3 animals each group). In fact, von Frey thresholds of individual C fibers were similar between PBS-injected aged and young mice, despite the differences in mechanical paw withdrawal thresholds between these cohorts at baseline (Fig 1A). These seemingly disparate findings in von Frey threshold measures between single afferent fibers and behavioral responses may reflect the fact that mechanical stimulation on the behavioral level activates many different fiber types with overlapping receptive fields whose responses are all integrated at the spinal and brain levels, while skin-nerve preparations entail recordings from the receptive field of only one fiber at a time. Alternatively, these findings may also be the result of testing the glabrous skin behaviorally and recording from afferents innervating the hairy skin in the *ex vivo* skin-nerve preparation.

Additionally, we also examined ongoing discharge of C fibers from acutely inflamed animals, as this type of activity may partially mediate non-evoked pain (Bennett, 2012). Ongoing discharge was classified as a firing rate greater than 0.05 Hz (6 action potentials over a 2 minute interval). We found that a higher percentage of C fibers from inflamed preparations exhibited spontaneous activity in both young and aged animals (~60% of fibers in CFA-inflamed preps and ~30% in PBS-injected control preparations), although this relationship was not statistically significant (Fig 2F^{o}, $p > 0.05$ for both young and aged with Fisher's exact test, n = 25-32 fibers as noted previously, 3 animals each group). Conduction velocities were slightly different for C fibers from young, PBS-injected animals (0.46 ± 0.03 m/s) compared to C fibers from young, CFA-injected animals (0.62 ± 0.04 m/s) (** $p < 0.01$, student's t-test), but no differences were noted in the conduction velocities of aged C fibers from the CFA- and PBS-treated groups or when comparing the aged PBS group to the young PBS group (data not shown).

Young, but not aged, C fiber nociceptors are inhibited during chronic inflammation

Although skin-nerve recordings from acutely inflamed animals showed intriguing differences between young and aged animals, we were particularly interested in the responses of C fiber nociceptors during *bona fide* chronic pain, as this is a more pressing issue clinically than acute pain. Therefore, we also performed recordings from young and aged animals 8 weeks after CFA or PBS injection (Fig 3A).

Strikingly, C fibers from CFA-injected animals actually exhibited a reduction in firing rates as compared to PBS controls at the 8-week time point in young animals, with the reduction in firing most evident at the lowest forces (Fig 3B^{p}, *** $p < 0.001$ with 2-way ANOVA, $^{\#}$ $p < 0.05$ with Bonferroni post-hoc analysis, n = 26 and 29 fibers, 4 animals for

PBS group and 6 animals for CFA group). In aged animals, chronic CFA-mediated inflammation had no effect on C fiber firing in comparison to PBS-injected controls (3C[q], $p > 0.05$ with 2-way ANOVA, n = 24 and 32 fibers, 10 animals for PBS group and 7 animals for CFA group). Importantly, for both young and aged animals, firing from chronically-inflamed C fibers was significantly lower than the firing from acutely-inflamed C fibers throughout the force series (Fig 3D[r] and E[s], **** $p < 0.0001$ with 2-way ANOVA, ## $p < 0.01$ and #### $p < 0.0001$ with Bonferroni post-hoc test, n = 28-32 fibers as noted on figures). These findings were incredibly surprising since chronically-inflamed young and aged animals displayed continued, prominent behavioral sensitization to mechanical stimuli at this 8-week chronic time point (Fig 1). Interestingly, when we examined the firing rates of individual C fibers at each force, we noted that acute, 2-day CFA-mediated inflammation results in a population-wide shift towards elevated firing rates in both young and aged animals (Fig 3F and G). Recent research has indicated that C fiber sensitization following inflammation is mediated entirely by a population of C fibers that is responsive to both cold and mechanical, but not heat, stimulation (Lennertz et al., 2012). We did not test multiple modalities on individual C fibers in this study, but our finding that the entire population of C fibers responds with increased mechanical firing following acute inflammation argues that other populations of C fibers, including the C-mechano only, C-mechano-heat-cold, and C-mechano-heat subtypes, are also likely to be sensitized to mechanical force following inflammation.

Additionally, we examined von Frey thresholds of isolated C fibers from young and aged animals after 8 weeks of CFA-mediated inflammation. Although we found no differences in von Frey thresholds between CFA-injected animals and PBS-injected controls at the 2-day time point (Fig 2E), at 8 weeks we unexpectedly found significant elevations in von Frey thresholds of C fibers obtained from both young and aged inflamed mice (Fig 3H[t], $p < 0.0001$ overall with Kruskal-Wallis test, *** $p < 0.001$, * $p <$

0.05 with Bonferonni post-hoc analysis, n = 26-32 fibers as previously indicated, 4-10 animals as previously indicated). Together, the elevated von Frey thresholds and reduced suprathreshold firing of C fibers after 8 weeks of inflammation in young animals suggest that a previously unreported plasticity is occurring in nociceptors of chronically inflamed young animals. In a similar vein, the elevated von Frey thresholds and trend towards reduced suprathreshold firing for aged C fibers points toward a similar, albeit weakened, phenomenon in aged animals.

Interestingly, despite the apparent reduction in action potential firing in response to evoked mechanical stimuli at 8 weeks of chronic inflammation, we did observe a significant elevation in the number of C fiber afferents displaying spontaneous firing in young animals at this time point (Fig 3I^{U}, ** $p < 0.01$ for young and $p > 0.05$ for aged with Fisher's exact test, n = 26-32 fibers as previously indicated, 4-10 animals as previously indicated). Thus, at least in young animals, spontaneous chronic pain may still be mediated by ongoing discharge of peripheral afferents. Additionally, no differences in conduction velocity were noted between any of the cohorts.

Firing patterns in C fibers are unchanged during chronic inflammation

Given the continued behavioral sensitization to mechanical stimuli, it was surprising that nociceptor firing would be so strongly reduced in both young and aged animals 8 weeks after CFA injection as compared to 2 days post-CFA injection (Fig 3D and 3E). In our view, three leading possibilities could explain this phenomenon: a) the pain behaviors displayed by chronically-inflamed mice were solely dependent on plasticity in the central nervous system (central sensitization), b) painful information during chronic inflammation is propagated to the central nervous system along a different type of peripheral afferent, or c) peripheral afferent communication of painful

437 information to the spinal cord depends on a mechanism other than the absolute number
438 of action potentials propagated, such as firing patterns or spike timing.

439 How different signals are communicated to the central nervous system has not
440 yet been fully resolved, but some studies have indicated that spike-timing of action
441 potentials is an important component of pain sensation (Wan et al., 2000; Tanner et al.,
442 2003). To explore the possibility that sensations of pain are communicated to the central
443 nervous system via spike patterns during situations of chronic pain, rather than just the
444 overall firing rate, we first examined plots of instantaneous firing frequency over time (Fig
445 4A-D). For young animals during acute inflammation, we observed elevated
446 instantaneous firing rates compared to PBS controls throughout the 12-second duration
447 of the mechanical stimulus; additionally, there appeared to be a lack of adaptation by C
448 fibers from acutely inflamed preparations as compared to controls (Fig 4A). C fibers from
449 acutely inflamed aged animals behaved similarly in that firing was elevated throughout
450 the stimulus, but fibers from inflamed and control preparations seemed to adapt
451 equivalently (Fig 4B). When examining chronic time points for C fibers from young and
452 aged animals, we saw much of the same phenomenon: although C fibers fired fewer
453 action potentials than during acute inflammation, these recordings showed similar
454 adaptation and firing throughout the stimulus as PBS controls for both age groups (Fig
455 4C and 4D).

456 Since there were no consistent differences in firing adaptation during the
457 mechanical stimulus, we next decided to examine whether fibers exposed to chronic
458 inflammation fired with shorter interspike intervals (ISIs). Some studies examining action
459 potential firing in a variety of pain models found that subsets of C fibers fired more action
460 potentials with short, 100-200 msec intervals between successive spikes (Chen and
461 Levine, 2003, 2007; Tanner et al., 2003). The specific timing of action potentials within a
462 train has also been shown to be important in systems such as the whisker barrel column

of the somatosensory cortex in rats (Panzeri et al., 2001). When we examined the responses of C fibers to a 140 mN stimulus, we found that while acute inflammation resulted in a significantly higher percentage of ISIs in the 0-99 ms range (65.6% vs 45.9% for young and 68.06% vs 59.7% for aged), there was no difference between the CFA and PBS groups 8 weeks after injection in either young or aged mice (Fig 4E^{v}, **** $p < 0.0001$, χ^2 test with subsequent Fisher's exact test for individual comparisons, n = 1082-2971 total ISIs per group). Thus, chronic inflammatory pain is unlikely to be communicated based on the rapidity with which C fiber nociceptors fire within a given spike train.

We next reasoned that a message of pain could conceivably be communicated to the central nervous system by the timing or variability in the timing of action potentials within the spike train. Indeed, some researchers have postulated that the brain actually uses variability in action potential timing to alter the probability that neurotransmitters are released at a given synapse (Smetters and Zador, 1996). Models from computational studies have shown that seemingly variable action potential firing patterns may contain important contextual information that other neurons are able to decode (Softky, 1995). Furthermore, the central nervous system may differentiate input from different end organs in the skin based on the variability of firing within their action potential trains (Wellnitz et al., 2010). Therefore, we measured the Coefficient of Variation (C_{V2}, see methods) (Chen and Levine, 2003, 2007; Tanner et al., 2003) for every interspike interval within a given cohort, with higher values indicating more variability in the spike-timing for a given action potential train (Fig 4F^{x}, **** $p < 0.0001$ with one-way ANOVA, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#\#}$ $p < 0.0001$ with Bonferroni post-hoc test, n = 808-2001 ISIs). Although we found differences between fibers from CFA-inflamed preparations and their controls for 3 of the 4 cohorts in response to a 140 mN stimulus, the changes we observed were not consistent. For instance, C fibers from inflamed young animals at the acute time

point exhibited less variability (0.61) than their PBS controls (0.68), while the opposite was true for C fibers from inflamed aged animals at the acute time point (0.70 vs 0.61 for aged PBS controls). However, by 8 weeks C fibers from chronically inflamed young animals exhibited more variability than their PBS controls, and no difference was found between the PBS and CFA groups for aged animals. Thus, the variability in action potential firing, which could conceivably code messages of pain due to mild oscillating or bursting behavior, also cannot explain how chronically inflamed animals are able to exhibit pain behaviors in spite of the markedly reduced action potential firing rates in primary afferent fibers.

Finally, we decided to examine the time from onset of our mechanical stimulus to firing of the first action potential in the train, since other somatosensory research has found that the time from mechanical stimulus onset to first spike generation by low-threshold mechanoreceptive afferents is critical for encoding tactile information (Johansson and Birznieks, 2004). Again, we found no difference between specific groups for this measure (Fig 4G^{y}, $p < 0.05$ overall with one-way ANOVA, n = 23-31 fibers, no specific differences with Bonferroni post-hoc), making it unlikely that pain is simply coded by the timing of the first action potential in response to a stimulus.

Collectively, this data, coupled with our recordings from primary afferents showing reduced firing during chronic inflammation, suggest that alterations in C fiber activity patterns or timing of impulses do not contribute to pain sensation during a chronic inflammatory state in either young or aged animals.

C fiber responses to chemical agonists are also reduced after 8 weeks of chronic inflammation.

Because our data strongly suggested that chronic inflammation causes reduced afferent drive to the central nervous system in response to mechanical stimuli in young

animals, we next asked whether this phenomenon could be generalized to other types of somatosensory stimuli. Therefore, we decided to test the responsiveness of C fibers to the potent TRPV1 agonist capsaicin. TRPV1 is located on 33-45% of small diameter neurons (Breese et al., 2005; Cavanaugh et al., 2011), and capsaicin generates a robust calcium influx and action potential trains when applied to the cell body or afferent terminals, respectively (Caterina et al., 1997; Seabrook et al., 2002; Carlton et al., 2004; Correll et al., 2004; Barabas and Stucky, 2013). Importantly, in an effort to record from the same population of C fibers, these experiments utilized only C fibers that were responsive to mechanical stimuli and excluded mechanically insensitive fibers.

In young naïve animals, we found that 41.7% of C fibers fired at least 3 action potentials in response to incubation with 10 μM capsaicin for 2 minutes, with an average of 38.3 ± 10.6 action potentials generated (Fig 5A and B, n = 10 of 24 fibers, data from 4 animals). After 2 days of acute inflammation, we found that a similar percentage of C fibers from young animals responded to capsaicin with comparable firing rates (38.09% responders, 30.88 ± 14.4 action potentials, Fig 5A and B, n = 8 of 21 fibers, data from 3 animals). Although we could not find any other studies that had tested the responsiveness of C fibers to capsaicin in the skin-nerve preparation after CFA-mediated inflammation, the lack of sensitization (either in percent responders or magnitude of firing rate) was surprising in light of studies demonstrating sensitization of the cell body to capsaicin after acute inflammation (Breese et al., 2005; De Souza et al., 2013). However, after 8 weeks of chronic inflammation, we observed a strong reduction in responses to capsaicin that was reminiscent of the reduced mechanically-induced firing observed at this time point (11.1% responders, 6.33 ± 1.20 action potentials, Fig 5A^{z} and B^{aa}, 3 of 27 fibers, data from 4 animals; $p < 0.05$ overall with χ^2 test, * $p < 0.05$ for naïve vs 8-weeks and for 2-days vs 8-weeks with Fisher's exact test). Additionally, no differences in conduction velocity were noted between any cohort.

Similar teased fiber experiments were also performed in aged animals. At baseline, 37.5% of C fibers from aged animals responded to capsaicin incubation with action potential firing (Fig 5C[bb], 3 of 8 fibers, data from 2 animals). After 2 days of acute inflammation, 14.3% of fibers responded, while after 8 weeks of chronic inflammation, 28.6% of C fibers responded to capsaicin (Fig 5C, 1 of 7 fibers for acute and 2 of 7 fibers for chronic groups, data from 2 animals). We advise caution in interpreting these findings from aged animals, as they are derived from low n's (7-8 fibers per group) due to limitations in the availability of animals > 18 months of age in our animal colony. However, it is interesting to note the number of C fibers responsive to capsaicin after 8 weeks of chronic inflammation in aged animals (2/7) as compared to C fibers from young animals at the same time point (3/27). Grossly, the percent responders to capsaicin reflects the responsiveness of C fibers to mechanical stimulation at the chronic time point: in young animals, there is a generalized reduction in responsiveness, while in aged animals there is only a slight, non-significant reduction in responsiveness to somatosensory stimuli. Additionally, no differences in conduction velocity were noted between any of the cohorts.

Importantly, we also tested the behavioral responses to capsaicin for another cohort of animals at the naïve, acute inflammatory, and chronic inflammatory time points. As expected, young animals experiencing both acute 2-day inflammation and chronic 8-week inflammation exhibited sensitized responses to 100 μM capsaicin injection as compared to naïve animals (Fig 5E[cc], * $p < 0.05$ overall with 1-way ANOVA, 4 animals per group). This corresponds well with our mechanical data at the behavioral and afferent levels, as chronically-inflamed animals continued to show strongly sensitized pain behaviors despite reduced afferent responsiveness. Thus, we conclude that chronic inflammation mediates a global reduction in afferent drive in nociceptive C fibers that is not modality-specific.

AM fibers also exhibit reduced drive after 8 weeks of chronic inflammation

Our data convincingly provides evidence that C fibers are desensitized to multiple modalities as a result of chronic inflammation, in spite of continued behavioral sensitization to these modalities. Although C fibers have been the most-studied class of afferents with regard to pain, we wondered whether chronic pain could be mediated by Aδ nociceptors, since this population of afferents also transmits sensations of mechanical pain. Therefore, we decided to examine the responsiveness of Aδ nociceptors (A-mechanonociceptors, AM's) to mechanical stimuli under naïve, acute inflammatory, and chronic inflammatory conditions in young animals (these experiments could not be performed in aged animals due to a lack of aged animals in our colony).

Similar to chronically-inflamed C fibers, we found that chronically-inflamed AM fibers from young animals also exhibited a significant reduction in firing rates in response to a series of increasing mechanical forces (Fig 6A^{dd}, *** $p < 0.001$, 2-way ANOVA overall, n = 14-25 fibers as indicated on graph, 5 animals for naïve and 2-day groups, 4 animals for 8-week group). Surprisingly, we also observed a reduction in the firing of AM fibers after a 2-day acute inflammatory injury (Fig 6A). Other studies have shown either a sensitization of A fibers (Andrew and Greenspan, 1999; Potenzieri et al., 2008; Moshourab and Stein, 2012) or no change in the firing rates of Aδ fibers (Lennertz et al., 2012) after acute CFA-mediated inflammation. Interestingly, the results obtained from many of those studies examined A fibers in the glabrous skin of the hind paw (Andrew and Greenspan, 1999; Potenzieri et al., 2008; Lennertz et al., 2012), while this study utilized inflamed hairy skin innervated by the saphenous nerve. We therefore cannot rule out the possibility that the responsiveness of A fibers is dependent on the type of skin (hairy or glabrous) that is innervated; indeed, a recent report has demonstrated that the target of innervation is critical for the mechanical responses of myelinated neurons to

inflammatory stimuli (Weyer et al., 2015). However, another report examining AM fibers from the rat hairy skin after acute (3-4 day) CFA-mediated inflammation also found sensitization to mechanical stimuli (Moshourab and Stein, 2012). Future AM recordings following inflammation must be performed to sort out this discrepancy.

When we plotted the responses of individual AM fibers to increasing force for each group, we noted that the difference between cohorts was really due to a selective loss of a population of AM fibers from the inflamed groups with extremely high response rates to mechanical stimuli that are present in the naïve group (Fig 6B). At this point, our results cannot determine whether this subpopulation of AM fibers is rendered silent by inflammation, or whether the inflammatory process simply reduces this population's firing to a level similar to other moderate-firing AM fibers. However, this finding is striking when compared to C fibers, which displayed a population-wide shift towards higher firing frequencies following inflammation (Fig 3F).

Also in accord with the findings from C fibers, AM fibers exhibited no change in von Frey thresholds after an acute inflammatory injury, but displayed significantly elevated von Frey thresholds after 8 weeks of chronic inflammation (Fig 6C^{ee}, *** $p < 0.001$ with Kruskal-Wallis test, n = 14-25 fibers, 5 animals for naïve and 2-day groups, 4 animals for 8-week group). Additionally, no differences in conduction velocity were noted between any of the cohorts.

Collectively, these data demonstrate two important points. First, our data suggests that the behavioral hyperalgesia observed in response to mechanical stimulation during acute inflammation is dependent primarily on C fibers, and not Aδ fibers, in the peripheral nervous system. Secondly, the continued behavioral sensitization during chronic inflammation is not dependent on elevated nociceptive afferent drive to the central nervous system, as both C fibers and AM fibers display

elevations in their thresholds and reductions in suprathreshold firing rates at chronic time points.

Changes in gene expression do not explain the reduced afferent firing during chronic inflammation

We next wondered what mechanisms underlie the changes in action potential firing at 2 days post-CFA injection and 8 weeks post-CFA injection. We reasoned that changes in gene expression of key mechanosensitive and voltage-gated ion channels in sensory neurons could cause the amplification of afferent firing we observed at 2 days and the reduction in firing at 8 weeks. Therefore, we began by examining the effects of acute and chronic inflammation on the expression of voltage-gated sodium channels specific to nociceptors (Cummins et al., 2007) in the left lumbar 2-5 Dorsal Root Ganglia (DRG), which innervate the left hindpaw.

Previous research has demonstrated significant dysregulation of voltage-gated sodium channels in sensory neurons in a variety of pain models (Waxman et al., 2000; Craner et al., 2002). When compared to the cognate L2-5 DRGs from PBS-injected controls (Fig 5^{ff,gg}, red lines), we found that *SCN9A* ($Na_V1.7$) transcripts were significantly elevated by 1.5 fold in young mice 2 days after CFA injection, but found no differences in *SCN9A* expression in the DRGs of young mice after 8 weeks of inflammation or aged mice after 2 days or 8 weeks of inflammation compared to controls (Fig 7A, left, * $p < 0.05$ with student's t-test - CFA vs PBS samples^{ff}, $p < 0.001$ with one-way ANOVA for fold changes between group^{gg}, $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ with Bonferroni post-hoc test, n = 3 animals for aging groups, 6 animals for young groups). We saw a similar trend for *SCN10A* ($Na_V1.8$), with elevated expression of these transcripts compared to controls during acute inflammation in young mice, although these changes were not statistically significant due to increased variability (Fig 7A, middle and right). Furthermore, we again

found no differences in the expression of these channels in aged animals or in young animals after 8 weeks of inflammation.

Interestingly, the expression of all three voltage-gated sodium channels was unchanged in aged animals following acute inflammation as compared to PBS controls, which perhaps contributes to the lack of strong afferent sensitization to mechanical stimuli observed with teased fiber recordings at the 2-day time point in aged mice (Fig 2C). Perhaps most importantly, however, was that expression of *SCN9A*, *SCN10A*, and *SCN11A* was not different in chronically-inflamed young and aged animals compared to PBS controls at the 8-week time point. This suggests that the reduced action potential firing at chronic time points is not due to a decrease in the expression of these voltage-gated sodium channels.

Interestingly, although changes in voltage-gated sodium channels do not seem to underlie the reduced firing we observed in young animals after 8 weeks of chronic inflammation, the elevated expression we observed in these channels after two days of inflammation may explain why C fibers from this cohort exhibited elevated conduction velocities. Likewise, the lack of change in Na_V channel gene expression in aged animals after two days mirrors the lack of change in conduction velocity when recording from aged, acutely inflamed C fibers.

We also examined channels that have been linked to mechanotransduction, as alterations in the channels that sense the initial mechanical stimulus could have a large impact on the number of action potentials propagated in response to a given mechanical stimulus (Fig 7B). Piezo2, which is the major mechanotransducer in myelinated low-threshold mechanoreceptors (Ranade et al., 2014), had unaltered gene expression in the 4 cohorts (Fig 7B^{ff,gg}, upper left, $p > 0.05$ with student's test for CFA vs PBS for each time point, n = 3 animals for aged groups and 6 animals for young groups). In contrast, Transient Receptor Potential Ankyrin 1 (TRPA1), which has shown to be integral to the

mechanical sensitization observed after an acute inflammatory insult (Lennertz et al., 2012), was found to be elevated 3-fold in both young and aged DRGs 2 days after CFA injection (Fig 7B^{ff,gg}, upper middle, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 3 animals for aging, 6 animals for young). Interestingly, TRPA1 transcript levels remained elevated during chronic inflammation in aged animals, but not for young animals ($p < 0.01$ with one-way ANOVA for TRPA1 expression levels, $^{\#\#}p < 0.01$ with Bonferroni post hoc test). This mirrors recent behavioral findings indicating that TRPA1 is critical for chronic pain in aged animals, but only for acute pain in young animals (Garrison and Stucky, 2014), .

Transient Receptor Potential Vanilloid 1 (TRPV1), which has widely been shown to be involved in pain sensation and may be activated by mechanical stimuli under some circumstances (Hillery et al., 2011; Julius, 2013), showed a small (33%), but significant, elevation 8 weeks after CFA injection in aged animals (Fig 7B^{ff,gg}, upper right, * $p < 0.05$, student's t-test, n = 3). Our data showed no change in TRPV1 gene expression in young animals after 8 weeks of inflammation compared to controls, suggesting that the reduced afferent responsiveness to capsaicin (Fig 5A and B) is not due to a reduction in TRPV1 transcript expression. Transient Receptor Potential Canonical (TRPC) 3, along with its family member TRPC6, has been linked to normal mechanotransduction in subsets of small diameter neurons (Quick et al., 2012). TRPC3 was reduced 28% in aged mice during acute inflammation, but levels were normalized by 8 weeks of inflammation (Fig 7B^{ff,gg}, lower left, * $p < 0.05$ with student's t-test, n = 3). TRPC6 levels were reduced by one-third in young mice after 8 weeks of chronic inflammation (Fig 7B^{ff,gg}, lower middle, * $p < 0.05$ with student's t-test, n = 6).

Although some changes were noted in channels linked to mechanotransduction, none of the changes pointed to a clear explanation for the reduced firing observed after 8 weeks of chronic inflammation. We therefore examined whether potassium channels, which help to control the firing rates of nociceptors and may be dysregulated during

painful injuries (Tsantoulas and McMahon, 2014), could have altered expression to account for the observed physiology. Transcript levels for *KCNA1* ($K_V1.1$), which has recently been found to serve as a "brake" for mechanically-gated currents in nociceptors (Hao et al., 2013), were unaltered in any of the four cohorts (Fig $7C^{ff,gg}$, left). We also chose to examine the expression of *KCNQ2* ($K_V7.2$) and *KCNQ3* ($K_V7.3$), which together mediate the "M" current in sensory neurons that constitutes the major subthreshold K^+ current and may limit inflammatory pain when activated (Passmore et al., 2003). *KCNQ2* transcript levels were unaltered in any group, and *KCNQ3* transcript levels were elevated 2-fold only in young animals after 2 days of acute inflammation (Fig $7C^{ff,gg}$, middle and left, * $p < 0.05$ with student's t-test, n = 6 animals).

Cumulatively, these results argue against the hypothesis that alterations in *SCN9A, SCN10A, SCN11A, TRPA1, Piezo2, TRPV1, TRPC3, TRPC6, KCNA1, KCNQ2,* or *KCNQ3* gene expression underlie the reduced peripheral drive observed after 8 weeks of chronic inflammation as compared to 2 days of acute inflammation in both young and aged animals.

Discussion

These data highlight the novel finding that C fiber nociceptors in young animals exhibit enhanced mechanical firing following an acute inflammatory injury, but reduced firing during the chronic inflammatory phase. Importantly, the reduced nociceptor firing observed chronically in response to both mechanical and chemical stimulation occurs in spite of continued prominent behavioral sensitization, suggesting that increased peripheral drive is necessary for the installation, but not the maintenance, of central sensitization in young animals. Additionally, reduced firing in AM afferents after 8 weeks of chronic inflammation suggests that reduced afferent drive during chronic pain is not C fiber-specific, but rather a global mechanism in nociceptive afferents. In contrast to data

722 from young animals, our results also suggest that aged animals are less malleable in
723 response to an inflammatory injury: they exhibit less behavioral sensitization, and their C
724 fibers fire at rates similar to controls during both acute and chronic inflammation.
725
726 ***Rationale for reduced afferent firing after chronic inflammation***
727 These findings are no doubt surprising given the large body of evidence
728 examining peripheral mechanisms of pain under the assumption that input from
729 peripheral afferents mediates and/or maintains chronic pain states. However, this finding
730 is not unprecedented, as nociceptive afferent firing in response to mechanical
731 stimulation has been shown to be reduced following a chronic constriction injury
732 (Schmidt et al., 2012a).
733 We initially speculated that the desensitization of C fibers and AM fibers in
734 response to mechanical stimuli as a result of chronic inflammation was due to changes
735 in the gene expression of voltage-gated or mechanosensitive ion channels. However,
736 none of the channels we examined displayed changes in gene expression that could
737 account for the reduction in firing. This does not, of course, preclude the possibility that
738 unexamined channels are responsible for the changes, or that protein levels or channel
739 functionality are altered following chronic inflammation. Additionally, an alternative
740 explanation is that low-threshold mechanoreceptors may be sensitized during chronic
741 pain states and are responsible for the majority of the pain phenotype observed. Some
742 evidence suggests that this occurs in nerve injury models, where myelinated afferents
743 have been shown to be critically important for tactile allodynia and hyperalgesia
744 (Campbell et al., 1988; Sun et al., 2001; King et al., 2011; Boada et al., 2014).
745 Altogether, our data illustrating reduced nociceptive afferent firing points to a
746 novel plasticity in C and AM fibers that has not been previously documented in chronic
747 inflammatory pain. Therefore, we propose that the reduced peripheral drive at this time

748 point serves to limit the amount of painful afferent information carried to the central
749 nervous system. It is well-documented that central sensitization, a form of central
750 plasticity at nociceptive synapses, is a crucial component of chronic pain (Woolf, 2011).
751 Since this plasticity can result in an increased probability of synaptic vesicle release per
752 action potential volley (Schulz, 1997), it follows that the body's attempt to limit pain
753 transmission would occur via a reduction in the number of action potentials reaching the
754 central synapse.
755
756 ***Alterations in C fiber firing during chronic pain depend on disease pathology***
757 It is also interesting to contrast this work with primary afferent recordings
758 performed in other models of chronic pain. In another model with a persistent
759 inflammatory component, a mouse model of sickle cell disease, animals experience
760 chronic pain throughout their lives as a result of frequent hypoxic events, but C fiber
761 recordings exhibit consistent mechanical sensitization compared to controls (Hillery et
762 al., 2011). In studies of neuropathic pain employing the spared nerve injury model or
763 spinal nerve ligation model, recordings of C fibers at chronic time points demonstrated
764 significant sensitization to mechanical stimuli (Shim et al., 2005; Smith et al., 2013). In
765 contrast, another study examining the mechanical sensitivity of C fibers following a
766 chronic constriction injury found that afferent firing was reduced in response to
767 mechanical stimuli (Schmidt et al., 2012a). Thus, the response of C fibers to pain
768 critically relies on both the time since injury induction and the etiology of the injury.
769 It is important to note that a recent study examined the role of TRPA1 in chronic
770 inflammatory pain in an aging model (Garrison and Stucky, 2014). Interestingly, that
771 study found that C fibers from both young and aged mice exhibited *sensitization* to
772 mechanical stimuli at 8 weeks after CFA inflammation, and that this sensitization was
773 dependent on TRPA1 in aged animals. These findings are contrary to those presented in

774 the current study, where we have found reduced firing of nociceptors at chronic time
775 points in young animals and minimal changes in afferents from aged animals at acute or
776 chronic time points. It is difficult to discern exactly why the results differ between studies,
777 but several key differences may contribute. The current study utilizes substantially more
778 n's, which decreases the risk of a type I error. The current study also uses male mice
779 exclusively, while Garrison and Stucky largely utilized recordings from female mice.
780 Given the wide body of data showing that sex can affect afferent responses to pain, this
781 is a crucial difference (Mogil, 2012; Bartley and Fillingim, 2013). Finally, it should be
782 noted that the background strains of the mice utilized in each study were different; mice
783 used in Garrison and Stucky were C57Bl/6 mice, while the majority of mice utilized in
784 this study were on a mixed C57Bl/6 / Swiss Webster/CBA background.
785
786 ***Gene expression of key ion channels is largely unchanged during chronic***
787 ***inflammation***
788 Although we were unable to identify a specific gene responsible for the reduced
789 action potential firing during chronic inflammation, it is interesting to make note of the
790 overall trends observed amongst the different groups. Most of the examined genes were
791 elevated at the 2-day time point in young animals, suggesting that young animals are
792 able to quickly alter gene expression in sensory neurons in response to an injury. In
793 stark contrast, aged animals displayed minimal changes in gene expression at this same
794 time point. While a different set of genes may display altered expression in aged animals
795 than those examined in this study, it is intriguing to speculate that acute pain sensation
796 may occur via a different mechanism in aged animals than young animals. Interestingly,
797 the sole strongly-induced gene during acute inflammation in aged animals was TRPA1,
798 which has previously been shown to be important for both acute and chronic pain
799 behaviors in aged animals (Garrison and Stucky, 2014).

At chronic time points, young mice showed a general shift back toward baseline for gene expression levels; in fact, the only notable difference was a slight reduction in the expression of TRPC6 in chronically-inflamed animals compared to PBS controls. Gene expression was largely the same for aged animals between the CFA and PBS groups at chronic time points, with the exception of TRPA1 and TRPV1. It is interesting that TRPV1 was found to be expressed at higher levels only in aged animals based on a recent report that both TRPA1 and TRPV1 are important for the transition from acute to chronic pancreatic pain in young animals (Schwartz et al., 2013). Studies using a global TRPA1 knockout mouse line and specific TRPA1 antagonists have demonstrated that a removal/blockade of TRPA1 reduces nociceptive primary afferent firing (Brierley et al., 2009; Kerstein et al., 2009; Kwan et al., 2009). Therefore, it could be expected that elevations in gene expression of TRPA1 in aged animals at chronic time points would subsequently result in elevated C fiber firing rates. However, it is also known that TRPA1 plays an important role at the central synapse between nociceptive primary afferents and neurons in lamina I/II of the dorsal horn (Pertovaara and Koivisto, 2011; Sisignano et al., 2013). This raises the possibility that TRPA1's role in chronic pain in aged animals is not at the afferent terminals in the skin, but rather at the central terminal to promote greater fidelity at nociceptive synapses.

Correlation with clinical literature

The clinical literature paradoxically shows that while aged individuals have decreased tactile sensitivity (Thornbury and Mistretta, 1981), higher percentages of aged individuals have complaints of pain (Krueger and Stone, 2008; Maxwell et al., 2008). Furthermore, aged individuals have reduced mechanical pain thresholds experimentally (Lautenbacher et al., 2005). Our data in aged mice show the opposite with regards to tactile sensitivity - aged mice have increased sensitivity at baseline based on von Frey

826 thresholds. Additionally, while aged mice in this study exhibited significant pain
827 behaviors following CFA inflammation, they actually exhibited reduced allodynia
828 compared to young animals injected with CFA as judged by paw withdrawal thresholds.
829 However, hyperalgesia, as measured by responses to a suprathreshold stimulus, were
830 similar at chronic time points for both young and aged animals. This mirrors what is
831 observed clinically, with aged individuals and young individuals who complain of pain
832 reporting similar pain levels (Krueger and Stone, 2008).

833 In contrast to our findings that afferent drive is either unchanged or reduced
834 compared to controls at chronic time points, clinical studies seem to validate the idea
835 that peripheral afferent input must remain elevated during chronic pain. Evidence for this
836 stems from examples such as the elimination of chronic pain in patients suffering from
837 osteoarthritis who undergo total knee arthroplasties or patients with chronic pain that
838 experience relief following application of topical lidocaine (Richards and McMahon,
839 2013). However, these studies do not discriminate between reducing enhanced activity
840 of a sensitized nerve and reducing normal activity of a non-sensitized nerve.

841 For instance, topical lidocaine has been shown to reduce pain in patients
842 suffering from peripheral neuropathic pain syndromes (Meier et al., 2003). Yet lidocaine
843 may reduce chronic pain in some patients not because it returns elevated peripheral
844 drive to baseline, but rather because it blocks all input from a peripheral neuron from
845 ever reaching a central neuron. Indeed, applying a lidocaine patch to a healthy individual
846 will also be efficacious because it blocks transmission of sensory information. Likewise,
847 a joint replacement may result in decreased pain because nerve fibers are no longer
848 present in the joint to transmit any sort of sensory signal.

849 There has been some suggestion that age-related pain may be due to reduced
850 descending inhibition in aged adults (Edwards et al., 2003; Riley et al., 2010; Marouf et
851 al., 2014). While examining central mechanisms is outside the scope of the current

study, our results suggest that at least *some* of the elevated acute pain in aged individuals may be the result of peripheral mechanisms. Nociceptive primary afferents exhibited a strong trend towards increased firing in aged animals following acute inflammatory injury, and changes in TRPA1 gene levels were noted at this time point as well. However, given the overall blunting of the sensitization of primary afferents and the relative lack of changes in gene expression of nociceptive ion channels, it is possible that central mechanisms account for a large part of the acute pain response in this population.

Conclusion

Collectively, the results of this study question whether it is pertinent to examine mechanisms of pain sensation in the peripheral nervous system using *acute* inflammatory models, since nociceptive C and AM fibers seem to contribute minimally, if at all, to chronic inflammatory pain. Indeed, this point is buoyed by recent research examining the role of leukocyte elastase in a model of neuropathic injury (Vicuña et al., 2015). That study demonstrated that inhibiting leukocyte elastase is effective at blocking pain acutely, but has no effect on pain sensation at chronic time points. Finding the molecular cause of the reduced action potential firing at chronic time points may, however, lead to new therapies if this process can be taken advantage of during the acute pain phase prior to the installation of chronic pain.

Our findings also shed light on the processes that may contribute to differences in pain sensation between young and aged populations, and should serve as the impetus for future mechanistic research into this understudied area.

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Figure Legends

Figure 1: Acute and chronic inflammation sensitizes behavioral responses to mechanical stimuli to different extents in young and aged mice. A, Paw withdrawal thresholds to a mechanical stimulus are lower in aged animals (2.35 mN) as compared to young animals (3.23 mN) at baseline. **B and C,** Injection of CFA results in a dramatic reduction in paw withdrawal thresholds both acutely and chronically in young (B) and aged (C) mice compared to PBS injection. **D,** Young mice exhibit a greater reduction in paw withdrawal thresholds as compared to aged mice. **E,** As a percent of baseline, young mice exhibit a >90% reduction in paw withdrawal thresholds, while aged mice exhibit a 40-75% reduction in paw withdrawal thresholds. **F,** Baseline responses to a 3.61 mN suprathreshold stimulus are similar between young and aged mice. **G and H,** Injection of CFA results in a significant elevation in percent response to a suprathreshold 3.61 mN stimulus in both young (G) and aged (H) mice. **I,** In response to injection of CFA, aged mice respond with elevations in percent response to a suprathreshold stimulus on a different time course than young mice. **J,** Chronically inflamed mice continue to exhibit significant paw swelling at 8 weeks after inflammation induction. **K,** Top row: H&E-stained coronal sections through the entire paw at the metatarsophalangeal joint from young animals show significant inflammatory infiltrate present at both 2 days and 8 weeks after CFA injection. Bottom row: Increased magnification of the whole paw sections demonstrate significant infiltration of neutrophils and monocytes/macrophages at both 2 days and 8 weeks of CFA-mediated inflammation.

Figure 2: Acute inflammation sensitizes C fiber nociceptors to mechanical force only in young animals. A, Trace examples from young animals injected with either PBS (top left) or CFA (bottom left) and aged animals injected with either PBS (top right) or CFA (bottom right). **B,** C fiber nociceptors from acutely inflamed (2-day) young animals respond with significantly higher action potential firing rates in response to increasing mechanical forces. **C,** C fiber nociceptors from acutely inflamed aged animals trend toward responding with increased action potential firing in response to increasing mechanical forces, but this relationship is not significant. **D,** At baseline, C fibers from aged animals are more sensitive to mechanical stimuli than C fibers from young animals. **E,** Von Frey thresholds for individual C fibers were not different between the four cohorts. Each point on the graph represents the von Frey threshold of an individual C fiber and the black bars are indicative of the group mean. **F,** More C fibers from acutely inflamed animals tend to have ongoing, non-evoked activity (> 0.05 Hz), although this relationship is not significant.

Figure 3: Chronic inflammation results in a desensitization of C fibers to mechanical force in young, but not aged animals. A, Trace examples from young animals injected with either PBS (top left) or CFA (bottom left) and aged animals injected with either PBS (top right) or CFA (bottom right). **B,** After 8 weeks of inflammation, C fibers from young animals respond with significantly lower action potential firing rates in response to increasing mechanical forces. **C,** After 8 weeks of inflammation, C fibers from aged animals trend toward lower firing rates in response to increasing mechanical forces. **D,** The firing rates of C fibers from inflamed young animals are significantly lower after 8 weeks of chronic inflammation as compared to 2 days of acute inflammation. **E,** The firing rates of C fibers from inflamed aged animals are significantly lower after 8 weeks of chronic inflammation as compared to 2 days of acute inflammation. **F and G,**

Plots of the firing rates of individual C fibers at different forces for each cohort for young (F) and aged (G) animals. Note that after 2 days of acute inflammation the entire population of C fibers in both young and aged animals shifts toward elevated firing rates, rather than only a subpopulation of increased responders. **H,** Von Frey thresholds for individual C fibers are elevated in both young and aged animals after 8 weeks of chronic inflammation. Each point on the graph represents the von Frey threshold of an individual C fiber and the black bars are indicative of the group mean. **I,** Chronic inflammation results in an increased percentage of C fibers demonstrating ongoing, non-evoked activity in young animals, but not aged animals.

Figure 4: C fiber action potential firing patterns do not explain the significant behavioral sensitization, but reduction in action potential firing rates during chronic inflammation. A-D, Grouped instantaneous firing rates over the 12-second mechanical stimulus binned into 200-msec intervals for fibers from young acutely inflamed animals (A), aged acutely inflamed animals (B), young chronically inflamed animals (C), and aged chronically inflamed animals (D). **E,** C fibers from acutely inflamed young and aged animals fired with a significantly higher percentage of interspike intervals between 0-99 ms . **F,** Coefficients of Variation (CV_2 method) for a 140 mN stimulus were significantly different for C fibers from acutely inflamed young and aged animals and chronically inflamed young animals, but these relationships do not consistently demonstrate that variability may underlie the increased behavioral sensitization seen acutely and chronically. **G,** The time to first action potential after the onset of the mechanical stimulus is not different for any of the cohorts.

Figure 5: C fiber responses to capsaicin are reduced during chronic inflammation, while behavioral sensitization to capsaicin remains intact. A, C fiber responses to capsaicin are similar under naïve and acutely inflamed conditions in young animals, but

responses are strongly attenuated during chronic inflammation. **B,** The number of action potentials fired by capsaicin-sensitive C fibers is also reduced after 8 weeks of chronic inflammation in young animals (although this is not statistically significant). **C,** In aged animals, C fiber responses to capsaicin are similar across the naïve, acute inflamed, and chronic inflamed states. Note low n's due to lack of aged animal availability. **D,** Number of action potentials fired by aged C fibers in response to capsaicin. **E,** Young animals exhibit sensitized pain behaviors in response to capsaicin injection during both acute inflammatory and chronic inflammatory states, despite the reduced afferent responses to capsaicin at 8 weeks.

Figure 6: AM fibers from young animals exhibit reduced mechanical firing rates following inflammation. A, Following both 2-day acute and 8-week chronic inflammation, AM fibers from young animals exhibit reduced firing rates in response to mechanical stimuli. **B,** Plots of the firing rates of individual AM fibers at different forces for each cohort of young animals. Note the loss of a population of high-responding AM fibers at the 2-day and 8-week time points. **C**, von Frey thresholds of individual AM fibers from young animals are elevated after 8 weeks of chronic inflammation as compared to fibers from naïve animals.

Figure 7: Changes in gene expression of voltage-gated and mechanosensitive ion channels do not explain the reduced action potential firing after 8 weeks of chronic inflammation. A, Gene expression for voltage-gated sodium channels $Na_V1.7$ (SCN9A), $Na_V1.8$ (SCN10A), and $Na_V1.9$ (SCN11A). Bars indicate fold change of the CFA condition over the PBS condition for each cohort. The red dotted line indicates a fold change of 1, meaning no change in expression levels between CFA and PBS conditions. Stars (*) indicate significant fold changes for the CFA vs PBS condition, while hashtags (#) indicate significant differences in fold change between cohorts. **B,** Gene

1226 expression (shown as fold change compared to PBS controls) for Piezo2 and TRP
1227 channels. **C,** Gene expression (shown as fold change compared to PBS controls) for
1228 voltage-gated potassium channels $K_V1.1$ (KCNA1), $K_V7.2$ (KCNQ2), and $K_V7.3$
1229 (KCNQ3).

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Gene	Taqman Assay ID	Context Sequence
scn9a	Mm00450762_s1	ACGAAAGCAGGAAATAGAGCTTCGG
scn10a	Mm00501467_m1	TCCACTCCTGGTTCTCCATATTTAT
scn11a	Mm00449367_m1	TCTGTAATCTCAGGTCTGAAGGTCA
fam38b	Mm01265861_m1	ACAAGAGCCTCTTGTGCAAGAGGAG
trpa1	Mm01227437_m1	GAAGAAGGGAACACAGCACTCCACT
trpv1	Mm01246302_m1	TACTTTTCTTTGTACAGTCACTGTT
trpc3	Mm00444690_m1	CCTTGTAGCAGGCTGGGGAAGATTC
trpc6	Mm01176083_m1	TACCCCAGCTTCCGGGGTAATGAAA
kcna1	Mm00439977_s1	TGCGGCCGCACGCTCCCTGCCCCAC
kcnq2	Mm00440080_m1	CCACGCCTACGTGTTCCTTTTAGTC
kcnq3	Mm00548884_m1	TGTGCCCACAGCAAAGAACTCATCA
tbp	Mm00446971_m1	TCCCCACAGGGCGCCATGACTCCTG

Table 1: Context sequences for primers used for qPCR

Figure 1

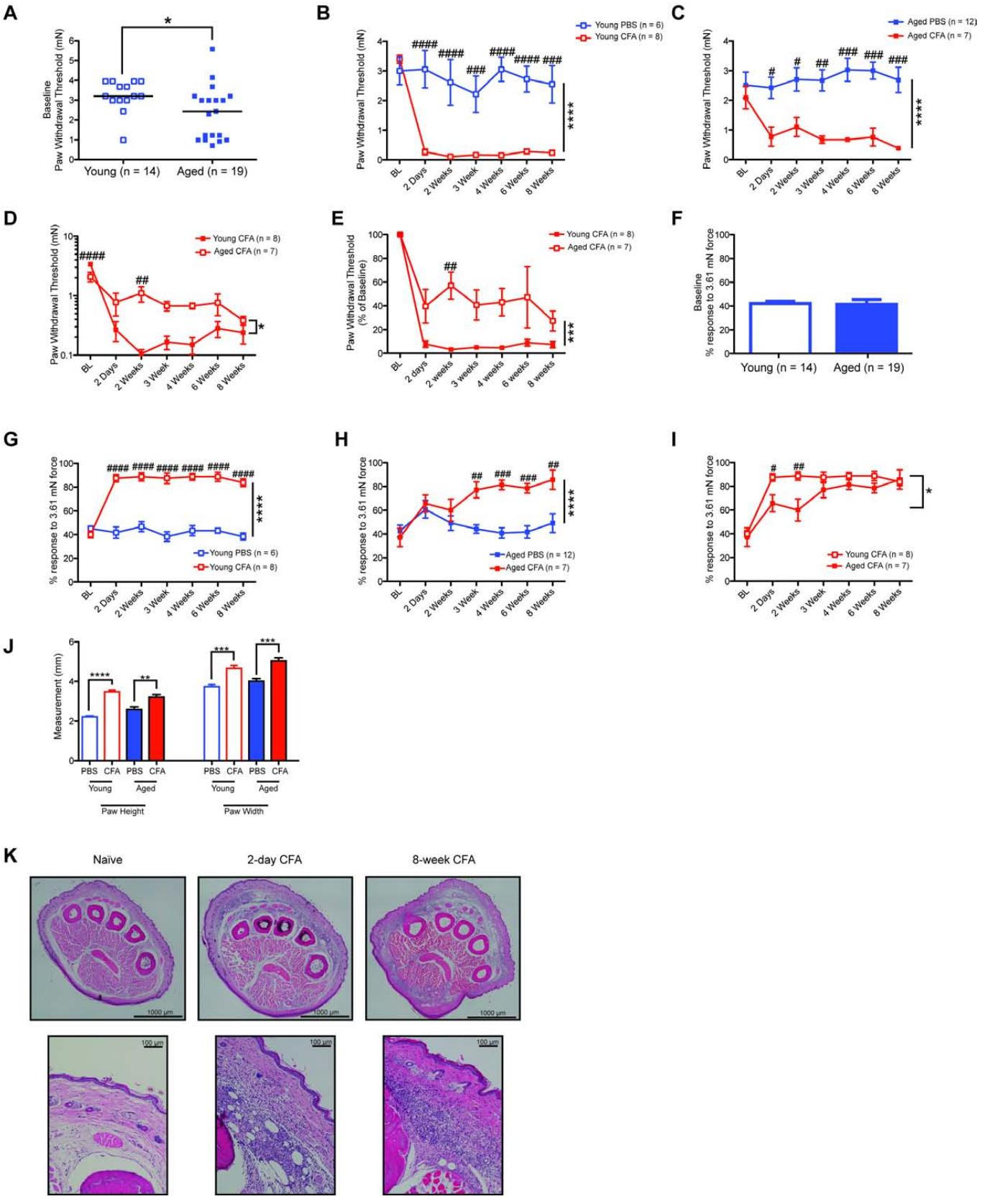

Figure 2

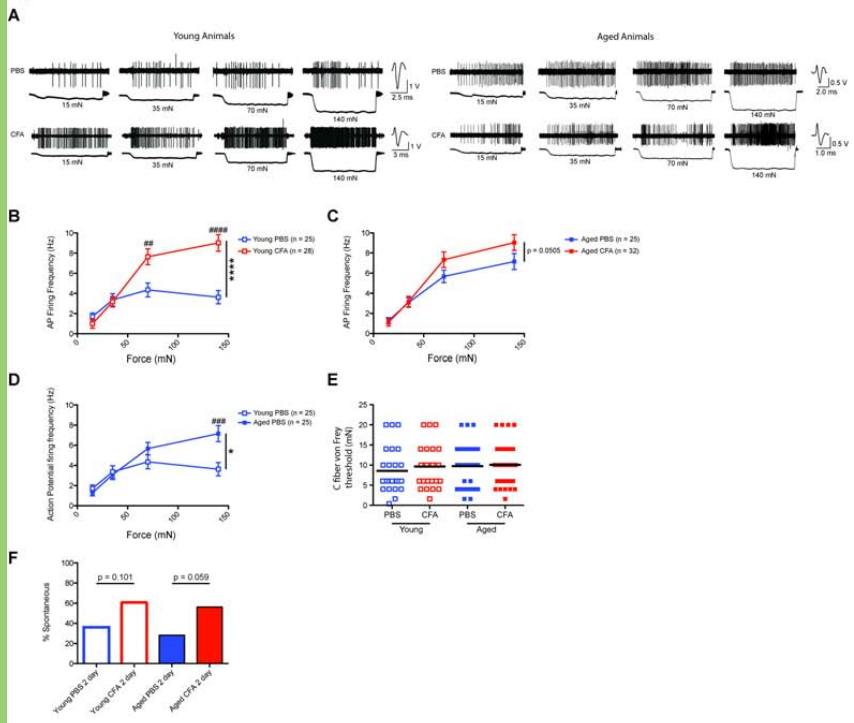

Figure 3

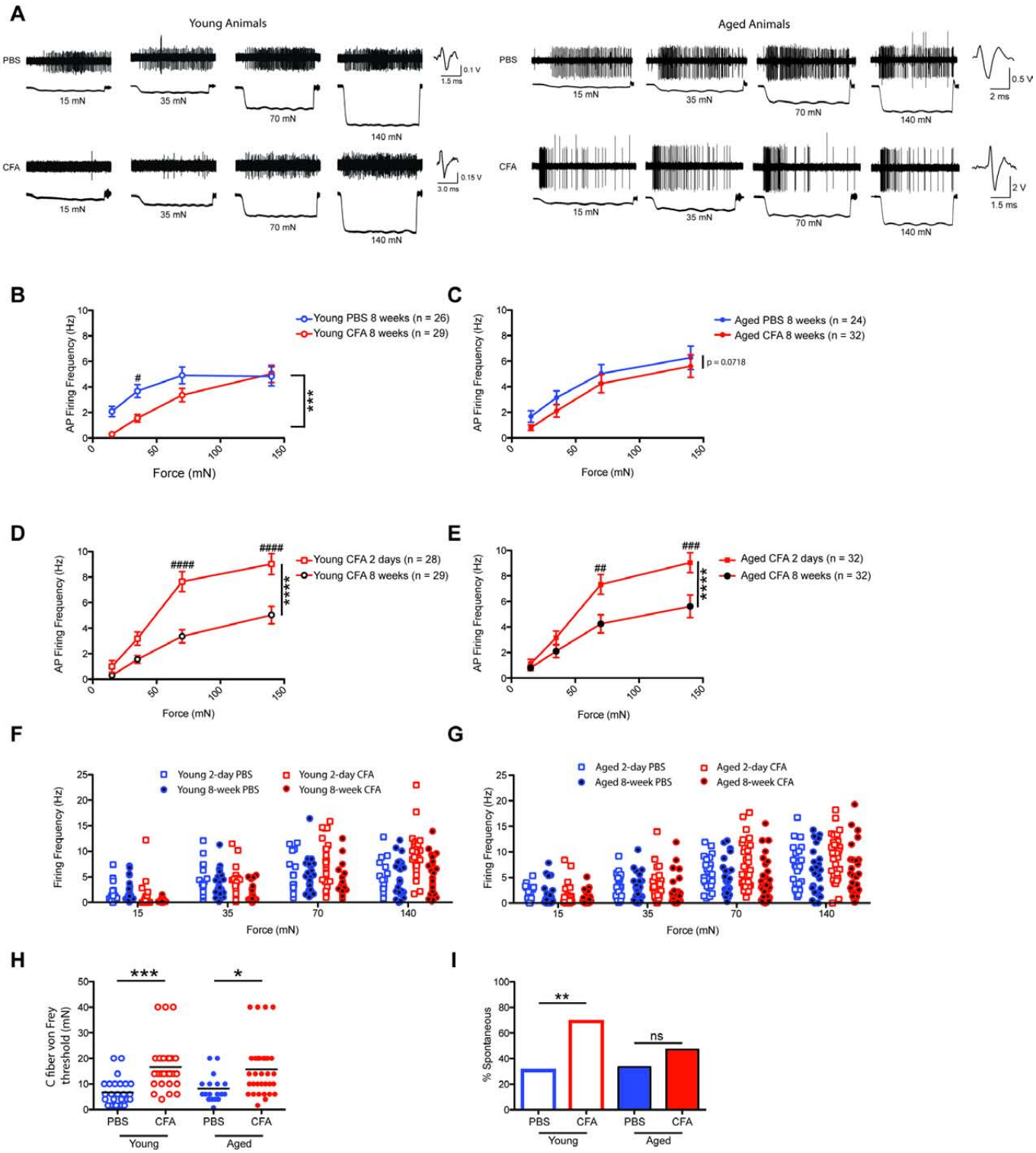

Figure 4

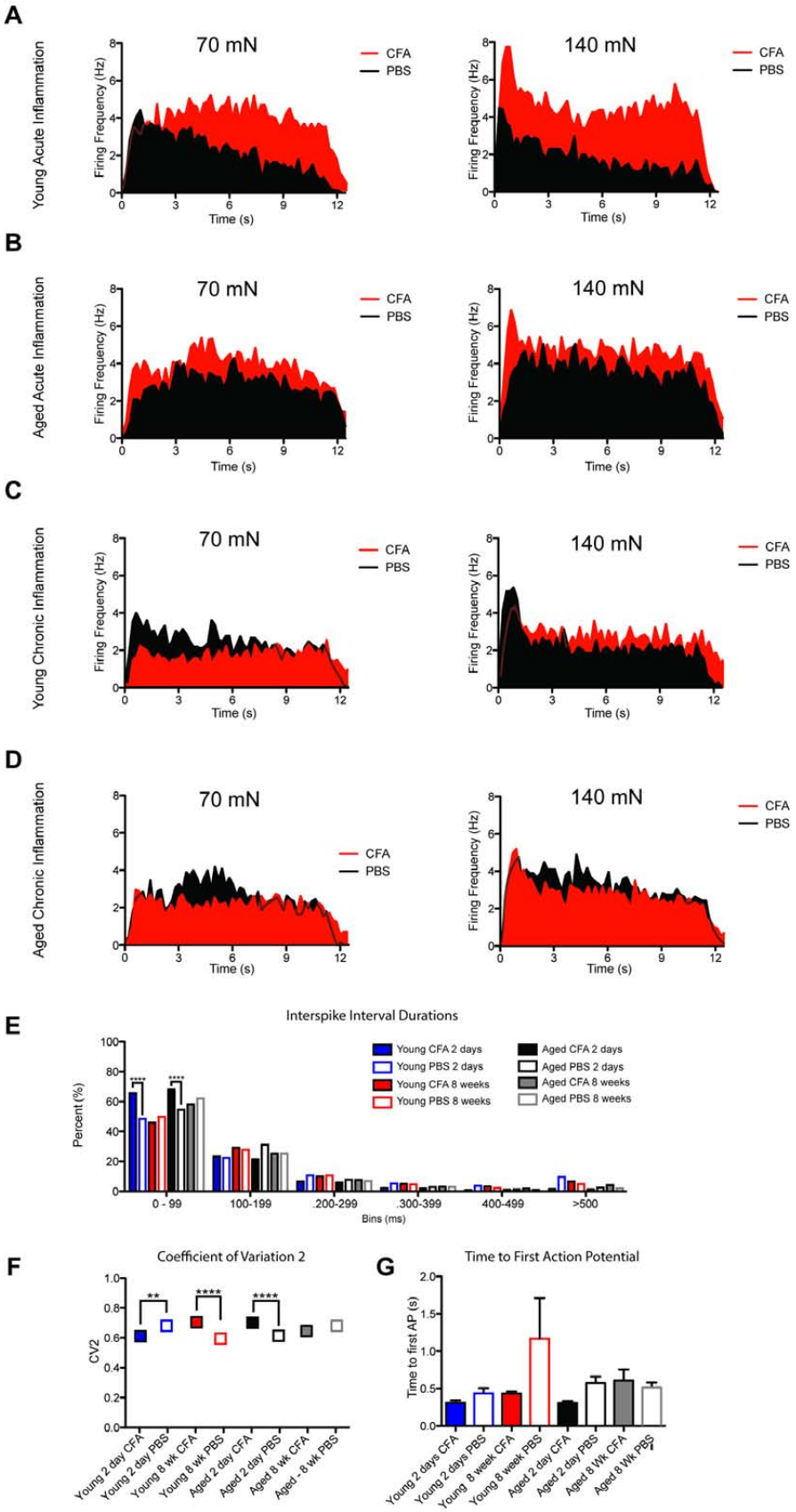

Figure 5

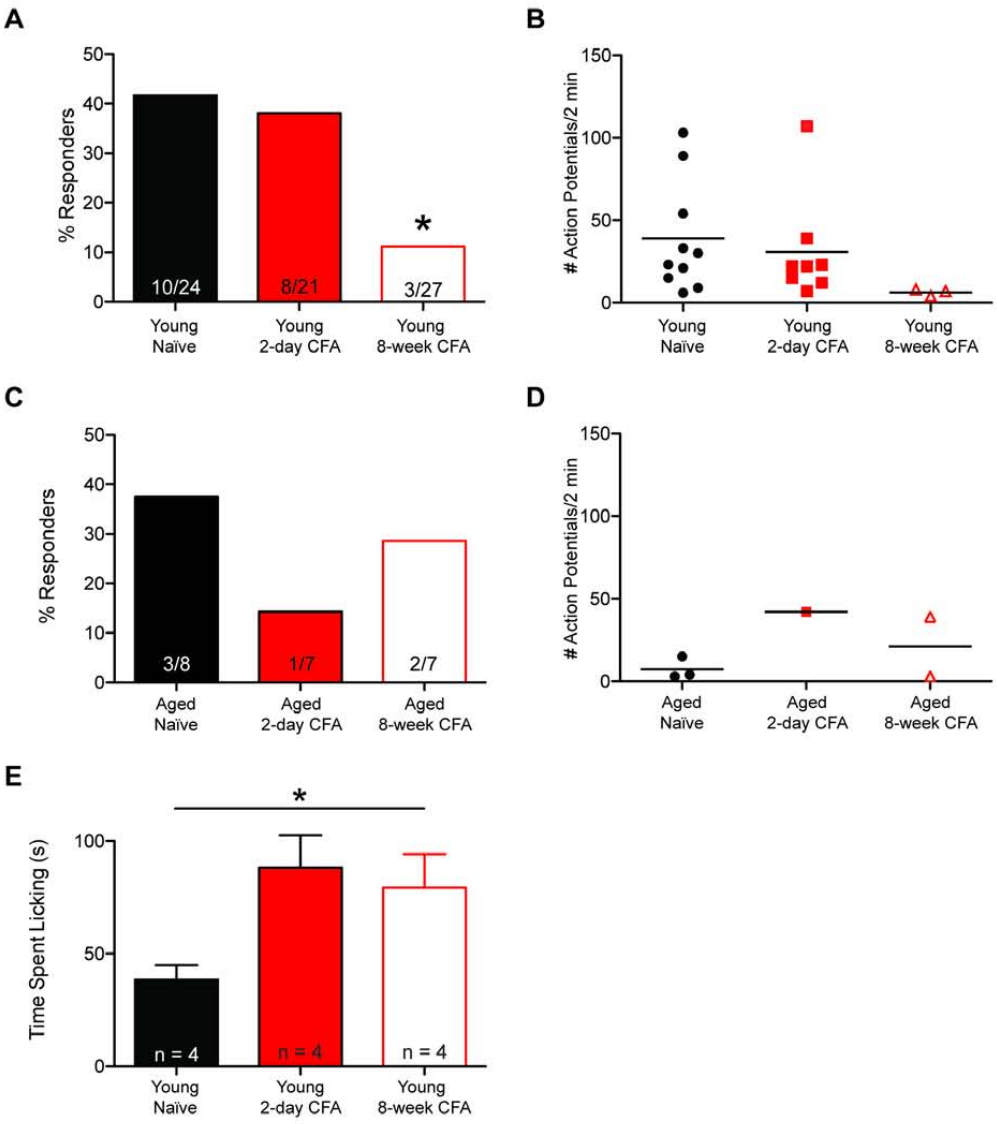

Figure 6

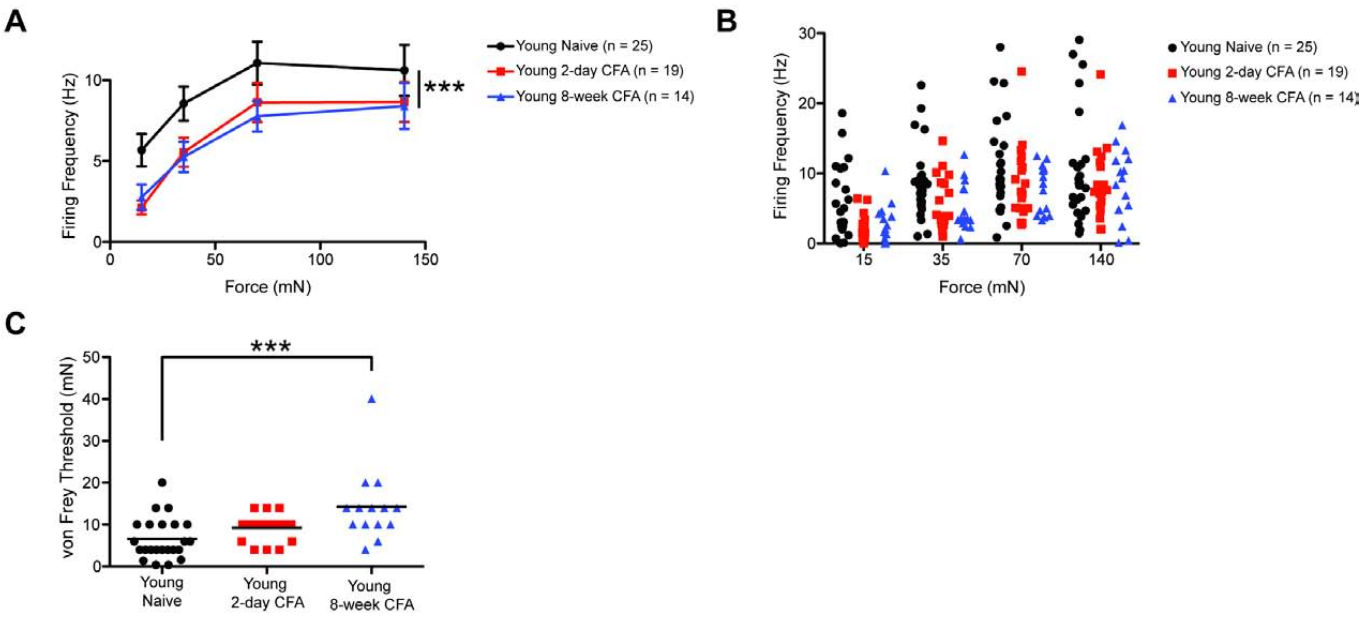

Figure 7