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Low-threshold mechanosensitive VGLUT3-lineage sensory neurons mediate spinal inhibition of itch by touch

Abbreviated title: Spinal Mechanism of Itch Relief by Tactile Stimuli

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Abstract

Innocuous mechanical stimuli, such as rubbing or stroking the skin, relieve itch through the activation of low-threshold mechanoreceptors (LTMRs). However, the mechanisms behind this inhibition remain unknown. We presently investigated whether stroking the skin reduces the responses of superficial dorsal horn neurons to pruritogens in male C57BL/6J mice. Single-unit recordings revealed that neuronal responses to chloroquine were enhanced during skin stroking, and this was followed by suppression of firing below baseline levels after the termination of stroking. Most of these neurons additionally responded to capsaicin. Stroking did not suppress neuronal responses to capsaicin, indicating state-dependent inhibition. Vesicular glutamate transporter 3 (VGLUT3)-lineage sensory nerves compose a subset of LTMRs. Stroking-related inhibition of neuronal responses to chloroquine was diminished by optogenetic inhibition of VGLUT3-lineage sensory nerves in male and female *Vglut3-cre*/*NpHR-EYFP* mice. Conversely, in male and female *Vglut3-cre/ChR2-EYFP* mice, optogenetic stimulation of VGLUT3-lineage sensory nerves inhibited firing responses of spinal neurons to pruritogens after the termination of stimulation. This inhibition was nearly abolished by spinal delivery of the κ-opioid receptor antagonist nor-binaltorphimine dihydrochloride, but not the neuropeptide Y receptor Y1 antagonist BMS193885. Optogenetic stimulation of VGLUT3-lineage sensory nerves inhibited pruritogen-evoked scratching without affecting mechanical and thermal pain behaviors. Therefore, VGLUT3-lineage sensory nerves appear to mediate inhibition of itch by tactile stimuli.

Significance Statement

Rubbing or stroking the skin is known to relieve itch. We investigated the mechanisms behind touch-evoked inhibition of itch in mice. Stroking the skin reduced the activity of itch-responsive spinal neurons. Optogenetic inhibition of VGLUT3-lineage sensory nerves diminished stroking-evoked inhibition, and optogenetic stimulation of VGLUT3-lineage nerves inhibited pruritogen-evoked firing. Together, our results provide a mechanistic understanding of touch-evoked inhibition of itch.

Introduction

Itch is often characterized by its relationship to scratching and other noxious counterstimuli, which are well known to inhibit itch. Less attention has been paid to the relationship between itch and innocuous stimuli, such as touch. Rubbing or stroking of the skin is commonly used to relieve itch in sensitive areas like the eyes or nose and is also frequently used by chronic itch patients (Stander et al., 2007). For example, one third of hemodialysis patients suffering from chronic itch reported that rubbing their skin combated itch (Hayani et al., 2016), and many patients with urticaria rub rather than scratch (Sabroe and Greaves, 2004). Rubbing or stroking the skin activates low-threshold mechanoreceptors (LTMRs), which are classified as Aβ-LTMR, Aδ-LTMR, and C-LTMR based on their conduction velocity (Abraira and Ginty, 2013). These fibers input to laminae II-IV of the spinal dorsal horn (Abraira et al., 2017). While it has been proposed that rubbing the skin activates itch inhibitory neuronal circuits within the spinal cord (Yosipovitch et al., 2003; Sabroe and Greaves, 2004), the mechanisms behind itch relief by innocuous mechanical stimuli are virtually unknown.

Many peripheral sensory neurons express vesicular glutamate transporters (VGLUT) 1-3, which are vesicular membrane-bound proteins that transport the excitatory neurotransmitter glutamate into presynaptic vesicles (Fremeau et al., 2004). VGLUT3-lineage sensory nerves consist of at least three subgroups, including (1) A-fibers innervating Merkel cells (a subset of Aβ-LTMR fibers), (2) C-fibers expressing tyrosine hydroxylase (TH; C-LTMR fibers), and (3) C-fibers not expressing TH (Seal et al., 2009; Lou et al., 2013; Draxler et al., 2014; Griffith et al., 2019), and are involved in sensory modulation in the spinal cord. Multiple lines of evidence suggest that VGLUT3-lineage neurons may mediate touch inhibition of itch. Firstly, A-fibers innervating Merkel cells have been shown to inhibit touch-evoked itch, presumably through activation of NPY^{+} inhibitory interneurons (Feng et al., 2018; Sakai and Akiyama, 2019). Secondly, C-LTMR fibers inhibit signaling from slowly conducting C-fibers (e.g., heat-sensitive C-fibers) in the rat spinal cord (Lu and Perl, 2003). Despite this evidence, the role of VGLUT3-lineage sensory nerves in the processing of itch in the spinal cord remains poorly understood.

We hypothesized that innocuous mechanical stimuli would inhibit itch signaling in the spinal cord. First, we performed *in vivo* single-unit electrophysiological recording from spinal neurons to investigate if the increased firing rates of pruritogen-responsive neurons are reduced by stroking the skin. We further hypothesized that VGLUT3-lineage primary afferents would mediate the relief of itch by innocuous mechanical stimuli. To directly activate or silence peripheral VGLUT3-lineage sensory nerves, we generated mice that

express the excitatory opsin channelrhodopsin-2 or the inhibitory opsin halorhodopsin in VGLUT3-lineage sensory nerves. Using these mice, we tested if innocuous mechanical stimuli-evoked inhibition could be attenuated or reversed by optogenetic inhibition of VGLUT3-lineage sensory nerves. Finally, we addressed the role of VGLUT3-lineage sensory nerves in spinal itch modulation by determining if optogenetic stimulation of VGLUT3-lineage sensory nerves reduced the firing rates of pruritogen-responsive neurons during pruritogen stimulation.

Materials and Methods

Mice

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Miami. All mice were group-housed (2-5 per cage), given standard food and water *ad libitum*, and maintained under a 12 hr light/dark cycle (6:00 lights on, 18:00 lights off). All mice were at least eight weeks old at the time of experiments. Adult male and female mice were randomly assigned to experimental conditions. Mice were typically used for a battery of behavioral tests, with a one-week break between each test. Before behavior testing, mice were habituated twice to each behavioral test apparatus for the equivalent of the recording time.

Vglut3-Cre mice (Tg(Slc17a8-icre)1Edw, The Jackson Laboratories (Grimes et al., 2011)) were crossed with the following strains: Ai32 mice expressing Cre-dependent channelrhodopsin-2 (ChR2)-EYFP (B6;129S-*Gt(ROSA)26Sor*$^{tm32(CAG-COP4*H134R/EYFP)Hze}$/J, The Jackson Laboratory (Madisen et al., 2012)); Ai39 mice expressing Cre-dependent halorhodopsin (NpHR)-EYFP (B6;129S-*Gt(ROSA)26Sor*$^{tm39(CAG-hop/EYFP)Hze}$/J, The Jackson Laboratory (Madisen et al., 2012)); and Ai14 mice expressing robust Cre-dependent tdTomato fluorescence (B6;129S6-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J, The Jackson Laboratory (Madisen et al., 2010)). *Vglut3-cre/ChR2-EYFP* and *Vglut3-cre*/*NpHR-EYFP* mice were used for electrophysiology recording, and immunohistochemistry. *Vglut3-cre*;*TdTomato* mice were used for immunohistochemistry.

C57BL/6J mice (male, 8-16 weeks, 22-38 g) were obtained from The Jackson Laboratories to perform electrophysiology recordings.

In vivo single unit recording from lumbar spinal cord

Single-unit recording from the lumbar spinal cord was conducted as previously detailed (Cuellar et al., 2004; Akiyama et al., 2009). Briefly, mice were anesthetized with sodium pentobarbital (80 g/kg, i.p.). The overlying muscles were dissected, and the L2-L4 lumbar spinal cord was exposed by laminectomy. A tungsten microelectrode (FHC, Bowdoin, ME) was driven into the superficial spinal cord by hydraulic microdrive (David Kopf, Tujunga, CA) to record extracellular single-unit activity, which was amplified and digitized using Powerlab (AD Instruments, Colorado Springs, CO) and Spike2 (CED, Cambridge, UK) software. The spinal cord was continually superfused with artificial cerebrospinal fluid (ACSF) consisting of (in mM) 117 NaCl, 3.6 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 25 NaHCO3, and 11 glucose, equilibrated with 95% O2-5% CO2 at 37°C.

A chemical search strategy (Jinks and Carstens, 2000) was used to isolate units in the superficial dorsal horn. In this study, chloroquine diphosphate salt (100 µg/1 µl in PBS; Sigma-Aldrich, St. Louis, MO) was used as the search stimulus, because chloroquine-responsive spinal neurons largely overlap with bombesin-responsive neurons postulated to signal itch (Sun et al., 2009; Mishra and Hoon, 2013; Akiyama et al., 2014a). Using a 30.5G needle connected to a Hamilton microsyringe, a small volume (~0.1 µl) was microinjected i.d. into the dorsal hindpaw skin, and the recording electrode was positioned to isolate an action potential in the superficial dorsal horn (300 µm from the surface) that had ongoing activity. After the ongoing activity subsided, ~1µl of chloroquine was injected through the same needle. Only units exhibiting a >3 SD increase in firing rate were selected for further study. In each experiment, some or all of the following additional stimuli were delivered to the dorsal hindpaw: innocuous stroking, pinch, i.d. PBS, serotonin (10 µg/1 µl in PBS; Alfa Aesar, Haverhill, MA), histamine (50 µg/1 µl in PBS; Sigma-Aldrich), 7% Tween 80 in PBS (vehicle for capsaicin), and capsaicin (30 µg/1 µl in PBS-Tween; Sigma-Aldrich).

During the time that the unit exhibited a relatively stable level of firing following i.d. injection of pruritogen or capsaicin (usually 3–5 min post-injection), stroking stimuli were successively delivered to the dorsal hindpaw. Stroking was accomplished by moving a cotton swab or different sized brushes (Table 1) in a back-and-forth motion across the dorsal hindpaw skin at a constant frequency of 2 Hz, excursion of 6 mm, and duration of 20-30 sec.

In A-fiber silencing experiments, flagellin (10 pmol (Xu et al., 2015); Enzo Life Sciences, Farmingdale, NY) and QX-314 (120 nmol (Xu et al., 2015); Tocris R&D Systems, Minneapolis, MN) were injected i.d. into the shaved dorsal hind paw. One hour after the i.d. injection, mice were used for electrophysiology experiments. Two-three hours after the i.d. injection, stroking stimuli were delivered during chloroquine responses.

In *Vglut3-cre/ChR2-EYFP* mouse experiments, blue or green light stimulation (20 Hz, 7 mW) was delivered to the dorsal hindpaw instead of stroking stimuli. In *Vglut3-cre/NpHR-EYFP* mouse experiments, blue or green light stimulation (constant, 7 mW) was delivered during stroking stimuli. In some experiments, either nor-binaltorphimine dihydrochloride (100 nM in ACSF; Santa Cruz Biotechnology, Dallas, TX) or BMS193885 (100 nM in ACSF; Tocris R&D) was delivered to the spinal cord for 30 sec, followed by switching back to ACSF alone.

At the conclusion of recording, an electrolytic lesion was made. The spinal cord was fixed in 4% paraformaldehyde, and 50-µm sections were cut and mounted on slides for microscopic verification of the lesion site.

In vivo single unit recording from dorsal root ganglia (DRG).

Mice were anesthetized with urethane (1.5 g/kg, i.p.). A 3-cm-long skin incision was made aseptically at the midline of the lower back. The overlying muscles were dissected and removed from the L4 -L5 spinous processes. To expose the L4 or L5 dorsal root ganglia (DRG), a small dorsolateral laminectomy was performed by removing the processus accessorius and part of the processus transversus. To loosen the neuronal somata from adjacent cells, collagenase P (3 mg/ml; Sigma-Aldrich) was applied to the DRG, incubated for 5 min and washed three times with ACSF. Collagenase application was repeated four times. The DRG was continually superfused with ACSF. A tungsten microelectrode was driven into the DRG by hydraulic microdrive to record extracellular single-unit activity, which was amplified and digitized using Powerlab and Spike2 software.

Units were categorized as LTMRs if they responded maximally to light touch. To estimate conduction velocity, transcutaneous electrical stimuli (0-8 mA, 2ms) were delivered from a pulse stimulator (Model 2100; A-M Systems) to receptive fields. Units were further classified by conduction velocity as follows: Aβ- (>9.0 m/s), Aδ- (1.0-9.0 m/s), and C-fibers (<1.0 m/s) (McIlwrath et al., 2007; Wetzel et al., 2007; Woodbury and Koerber, 2007). Each unit was retested with brush and pinch stimuli to establish a baseline response level.

To verify Aβ-fiber silencing, either QX-314 (24 or 120 nmol) or a mix of flagellin (10 pmol) and QX-314 (24 or 120 nmol) was microinjected id (10 µl volume) within the mechanosensitive receptive field. Units were then tested with brush and pinch stimuli at 30-min, 60-min, 120-min, and 240-min postinjection. In some experiments, *Vglut3-cre/ChR2-EYFP* mice were used to validate the optogenetic stimulation of VGLUT3-lineage nerves *in vivo*. In these experiments, blue light was delivered to the skin surface.

Immunohistochemistry

Mice were euthanized under sodium pentobarbital anesthesia, and the skin was immediately dissected. Skin was fixed in Zamboni fixative solution (Newcomer Supply, Middleton, WI) followed by 30% sucrose, frozen in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek, Torrance, CA), and cut in 40-µm sections on a cryostat.

Skin sections from *Vglut3-cre*/*ChR2-EYFP* and *Vglut3-cre*/*NpHR-EYFP* mice were blocked with 5% goat serum and 0.2% Triton X-100 in PBS for 2 hours at room temperature, then immunostained with chicken anti-green fluorescent protein (GFP) antibody (1:1000; Aves Labs, Tigard, OR) at 4°C overnight, followed by incubation with the corresponding secondary antibody conjugated with AlexaFluor 488 (1:300) at 37°C for 30 min. Then, the sections were immunostained with rabbit anti-VGLUT3 antibody (1:500) followed by the

corresponding secondary antibody conjugated with AlexaFluor 594 (1:300; Life Technologies Inc., Grand Island, NY) at 37°C for 30 min.

Skin sections from *Vglut3-cre*/*TdTomato* mice were blocked with 5% donkey serum and 0.2% Triton X-100 in PBS, then immunostained with either rabbit anti-PGP9.5 (1:200; EMD Millipore, Burlington, MA), rabbit anti-calcitonin gene-related peptide (CGRP) (1:300; Peninsula Laboratories International Inc, San Carlos, CA), rabbit anti-P2X3 (1:200; Neuromics Inc, Edina, MN), or rabbit anti-Neurofilament H (NFH) antibody (1:200, MilliporeSigma, Burlington, MA) at 4°C overnight, followed by incubation with the corresponding secondary antibody conjugated with AlexaFluor 488 (1:300) at 37°C for 30 min.

For immunostaining of VGLUT3, skin was dissected from *Vglut3-cre*/*TdTomato* mice and frozen in optimal cutting temperature compound (Tissue-Tek), and cut in 40-µm sections on a cryostat. Skin sections were fixed in Zamboni fixative solution and blocked with 5% donkey serum and 0.2% Triton X-100 in PBS for 2 hours at room temperature, then immunostained with rabbit anti-VGLUT3 antibody (1:500, Synaptic Systems, Goettingen, Germany) followed by the corresponding secondary antibody conjugated with AlexaFluor 488 (1:300; Life Technologies Inc., Grand Island, NY) at 37°C for 30 min. Then, the sections were immunostained with rabbit anti-red fluorescent protein (RFP) antibody conjugated with biotin (1:500; Rockland Immunochemicals, Pottstown, PA) at 4°C overnight, followed by incubation with the streptavidin conjugated with AlexaFluor 594 (1:100; Life Technologies Inc.) at 37°C for 30 min.

All sections were mounted on slides with Vectashield Hardset Antifade Mounting Medium with 4',6-diamino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were obtained with a Leica CTR6000 fluorescence microscope with 20X objective magnification (Leica Microsystems, Witzlar, Germany).

Optogenetics

For optic stimulation or inhibition, a fiber optic cable was connected to an LED light source (Prizmatix) that delivered blue light (460 nm wavelength) and green light (520 nm wavelength). For *Vglut3-cre*/*ChR2-EYFP* mice, blue light (20 Hz) was applied to the skin to stimulate ChR2-expressing VGLUT3-lineage nerve endings during behavioral tests and electrophysiology experiments. Green light (20 Hz) was used as a control. For *Vglut3-cre*/*NpHR-EYFP* mice, a constant green light was applied to the skin to inhibit NpHR-expressing VGLUT3-lineage nerve endings during electrophysiology experiments. A constant blue light was used as a control.

Scratching behavior

Fur on the rostral back was shaved with electric clippers one week before the scratching test. After 30 min habituation to a Plexiglas recording arena (15 x 15 x 15 cm^3), histamine (50 µg/10 µl), serotonin hydrochloride (10 µg/10 µl) or chloroquine diphosphate salt (100 µg/10 µl) was injected i.d. into the shaved rostral back skin. The light was delivered from the center of the ceiling of the recording arena. Behavior was recorded for 30 min with green light, blue light, or no light stimulation of the shaved skin (power: 2.5-7 mW, frequency: 20 Hz, distance between light and skin surface: 1-5 cm). The number of scratch bouts was analyzed in 5-min bins by a trained observer blinded to the treatment condition. One scratch bout was defined as one or more rapid back-and-forth hind paw motions directed toward and contacting the injection site, ending with licking or biting of the toes or placement of the hind paw on the floor. Hind paw movements directed away from the injection site (e.g., ear-scratching) and grooming movements were not counted.

Hargreaves test

After 120 min habituation to the Hargreaves arena, the plantar surface of the hind paws was exposed to 5 heat trials along with green light, blue light, or no light stimulation (4 mW, 20 Hz). Mice were assessed for paw withdrawal latencies (PWL). The beam active and idle intensities were 38 and 5, respectively. A cutoff of time of 10 sec was used to prevent excessive tissue damage.

Hind paw withdrawal test

After 120 min habituation to a perforated metal floor, the plantar surface of the hind paws was tested with a series of von Frey filaments (0.07 to 4 g) along with green light, blue light, or no light stimulation (4 mW, 20 Hz). The strength (g) of the von Frey filament which induced paw withdrawal was noted for each stimulus.

Statistical analysis

Results are presented as mean ± SEM. For comparison between 2 groups, a 2-tailed student's *t*-test was used. For comparison among more than 2 groups, a 1-way or 2-way repeated-measures ANOVA followed by Tukey multiple comparisons test was used. Statistical significance was set at $p<0.05$ for all tests. All statistical analyses and graphs were made using GraphPad Prism8.

Results

Stroking of the skin inhibits responses of spinal neurons to the itch stimulus chloroquine, but not the pain stimulus capsaicin

To test whether stroking the skin inhibits spinal firing evoked by the itch stimulus chloroquine, we performed *in vivo* single unit recording from C57BL/6J mice. We used a chemical search strategy to isolate chloroquine-responsive units (Akiyama et al., 2009; Akiyama et al., 2014b). Next, chloroquine was microinjected into the dorsal hind paw. During the chloroquine-evoked response, we stroked the dorsal hind paw skin with a cotton swab, a thin brush, or a thick brush (Table 1).

An example unit is shown in Fig. 1. This unit was located in the superficial dorsal horn and responded to i.d. injection of chloroquine (Fig. 1A). During the chloroquine-evoked response, stroking with the cotton swab, the thin brush, or the thick brush further excited the neuron (Fig. 1A-D). After stroking with the thick brush, the firing was decreased compared to pre-stroking. Neither PBS (vehicle for chloroquine) or PBS Tween (vehicle for capsaicin) elicited a response (Fig. 1E). Following vehicle injections, the two brushes, but not the cotton swab, excited the unit. An i.d. injection of capsaicin excited the unit, and capsaicin-evoked firing was not inhibited by stroking with the cotton swab, the thin brush, or the thick brush (Fig. 1F-I).

We identified ten chloroquine-responsive units in the dorsal horn. Unit recording sites were in the superficial dorsal horn based on micrometer depth (mean ± SEM, 95.3 ± 25.4 μm). For most units, the location was confirmed by histological identification of lesion sites (Fig. 2A inset). PBS vehicle did not increase mean unit firing (Fig. 2A). As expected, mean firing immediately post-chloroquine injection was significantly greater compared to the pre-injection level ($t_{(9)} = 3.06$, $p = 0.014$, Fig. 2B).

During the chloroquine-evoked response, mean firing was enhanced during stroking with the cotton swab, thin brush, or thick brush (cotton swab: from 2.35 ± 0.62 to 4.17 ± 0.9 impulses/sec; thin brush: from 2.69 ± 0.69 to 6.41 ± 1.51 impulses/sec; thick brush: from 2.59 ± 0.72 to 6.83 ± 1.65 impulses/sec, Fig. 2E-H). The mean firing rate post thick brush (1.03 ± 0.31 impulses/sec) was significantly lower than the mean firing rate pre thick brush (2.59 ± 0.72 impulses/sec; $F_{(1.21,\ 10.85)} = 11.24$, $p = 0.005$; Tukey's multiple comparison test: $p = 0.049$, Fig. 2E, 2G-H). Neither the cotton swab nor the thin brush affected the mean firing rate post stroking (cotton: from 2.35 ± 0.62 to 2.13 ± 1.72 impulses/sec, thin brush: from 2.69 ± 0.69 to 2.55 ± 1.02 impulses/sec, Fig. 2E-G).

Of the 10 recorded units, 9 (90%) also responded to capsaicin (Fig. 2C-D). Mean

unit firing immediately post capsaicin injection was significantly greater compared to the pre-injection level ($t_{(8)}$ = 4.77, p = 0.0014, Fig. 2D), while PBS Tween vehicle did not increase mean firing (Fig. 2C). During the capsaicin-evoked response, stroking did not have an effect on the mean firing rate post stroking (cotton: from 1.11 ± 0.26 to 1.27 ± 0.26 impulses/sec, thin brush: from 1.37 ± 0.36 to 1.59 ± 0.60 impulses/sec, thick brush: from 0.89 ± 0.23 to 1.34 ± 0.35 impulses/sec, Fig. 2I-K).

To test whether myelinated fibers mediate post-stroking inhibition of pruritogen-responsive neurons, we used a Toll-like receptor 5 (TLR5)-dependent A-fiber silencing approach: co-injection of a sodium channel blocker, QX-314, and a ligand of TLR5, flagellin (Xu et al., 2015; Pan et al., 2019; Sakai and Akiyama, 2019). Activation of TLR5 with flagellin leads to selective QX-314 entry into Aβ-LTMR fibers and subsequent blockade of sodium currents in these fibers. To verify the selectivity of the silencing approach, we performed *in vivo* single unit recording from the DRG. A representative Aβ-LTMR fiber was silenced for more than three hours by co-injection of flagellin (10 pmol) and QX-314 (120 nmol) (Fig. 3A). A lower dose of QX-314 (24 nmol) also silenced a representative Aβ-LTMR fiber, with a shorter duration (Fig. 3B). Co-injection of flagellin (10 pmol) and QX-314 (120 nmol) did not silence either an Aδ-LTMR fiber (Fig. 3C) or a C-LTMR fiber (Fig. 3D). Consistent with previous reports (Xu et al., 2015; Pan et al., 2019; Sakai and Akiyama, 2019), co-injection of QX-314 and flagellin into the hindpaw selectively silenced Aβ-LTMR fibers *in vivo*.

Next, following co-injection of QX-314 and flagellin, we performed *in vivo* single unit recording from the spinal cord. Ten units were isolated using an i.d. chloroquine search stimulus (Fig. 4). The mean recording depth was 61.8 ± 27.5 μm, and histologically identified recording sites were in the superficial dorsal horn (Fig. 4A inset). During the chloroquine-evoked response, the mean firing was not significantly changed during stroking with the thick brush (from 1.49 ± 0.37 to 1.81 ± 0.24 impulses/sec). The mean firing rate post thick brush was significantly lower (0.75 ± 0.19 impulses/sec) compared to the mean firing rate pre thick brush (1.49 ± 0.37 impulses/sec, $F_{(1.43, 7.16)}$ = 16.00, p = 0.033, Tukey's multiple comparison test: p=0.0034, Fig. 4C). Therefore, TLR5-dependent myelinated fiber silencing was not sufficient to block stroking-evoked inhibition of pruritogen-responsive neurons.

Optogenetic inhibition of VGLUT3-lineage sensory nerves reduces post-stroking inhibition of chloroquine-responsive neurons

VGLUT3-lineage sensory nerves are thought to mediate innocuous touch, such as stroking the skin (Seal et al., 2009; Abraira and Ginty, 2013; Lou et al., 2013). We next asked

whether VGLUT3-lineage sensory nerves mediate post-stroking inhibition of pruritogen-responsive spinal neurons. To visualize the innervation of VGLUT3-lineage sensory nerves in the skin, we bred *Vglut3-cre* mice with *tdTomato* reporter mice (Madisen et al., 2010). We then performed immunohistochemistry using skin sections from *Vglut3-cre*/*tdTomato* mice. The majority of tdTomato-expressing nerve fibers co-expressed VGLUT3 (Fig. 5A). The tdTomato-expressing nerve fibers (22.7%, 476/2101) represent a small proportion of PGP9.5-positive epidermal nerves (Fig. 5B). Fig. 5C shows that tdTomato-expressing nerve fibers also co-expressed NFH, previously reported as a marker for myelinated neurons in touch domes (Lou et al., 2013). A few tdTomato-expressing epidermal nerve fibers co-expressed CGRP, a peptidergic C-fiber marker (6.9%, 65/936; Fig. 5D), or P2X3, a nonpeptidergic C-fiber marker (10.0%, 67/665; Fig. 5E), which is consistent with a previous report (Draxler et al., 2014).

To silence VGLUT3-lineage sensory nerves, we prepared *Vglut3-cre/NpHR-EYFP* mice. In skin sections from these mice, peripheral EYFP-expressing nerve fibers stained positively for VGLUT3 (Fig. 6A). Using *in vivo* single unit recording, we identified 14 chloroquine-responsive dorsal horn units in *Vglut3-cre*/*NpHR-EYFP* mice (Fig. 6). Of these, 13 (93%) responded to capsaicin. None of the units responded to either blue or green light stimuli. Most unit recording sites were located in the superficial dorsal horn at a mean depth of 127.1 ± 27.0 μm below the surface of the lumbar spinal cord. For most units, the location was confirmed by histological identification of lesion sites (Fig. 6B inset).

PBS vehicle did not increase mean unit firing (Fig. 6B). As expected, mean firing immediately post-chloroquine injection was significantly greater compared to the pre-injection level (Fig. 6C). Likewise, mean unit firing immediately post-capsaicin injection was significantly greater compared to the pre-injection level, while PBS-Tween vehicle did not increase mean firing (Fig. 6D-E). Effects of stroking the hind paw with the thick brush on chloroquine-evoked firing were biphasic in *Vglut3-cre*/*NpHR-EYFP* mice. Chloroquine-evoked firing was significantly enhanced during stroking (from 0.62 ± 0.08 to 6.88 ± 2.33 impulses/sec, Fig. 6I), followed by a significant reduction in firing post-stroking (0.29 ± 0.07 impulses/sec, $F_{(1.00, 6.01)} = 7.44$, $p = 0.034$, Tukey's multiple comparison test: p=0.0020, Fig. 6I). Simultaneous green light stimulation during stroking canceled the post-stroking suppression of neuronal firing (from 0.75 ± 0.11 to 0.98 ± 0.20 impulses/sec, Fig. 6G-H) without affecting the enhancing effect of stroking with the thick brush. Simultaneous blue light (control) stimulation during stroking had no effect on post-stroking firing (from 0.68 ± 0.17 to 0.3 ± 0.07 impulses/sec, Fig. 6F, 6H), These data suggest that VGLUT3-lineage sensory nerves mediate stroking-evoked inhibition of pruritogen-responsive neurons.

Stroking the hind paw with the thick brush did not affect post-stroking activity elicited by capsaicin in *Vglut3-cre/NpHR-EYFP* mice (from 0.7 ± 0.12 to 0.9 ± 0.21 impulses/sec, Fig. 6L). Neither simultaneous blue nor green light stimulation during stroking had any significant effects on post-stroking firing (green: from 0.93 ± 0.17 to 1.23 ± 0.20 impulses/sec; blue: from 0.78 ± 0.13 to 0.81 ± 0.13 impulses/sec, Fig. 6J-K).

Optogenetic stimulation of VGLUT3-lineage sensory nerves inhibits responses of spinal neurons to itch stimuli

To investigate whether optogenetic stimulation of VGLUT3-lineage sensory nerves inhibits ongoing activity elicited by pruritogens in the spinal cord, we prepared *Vglut3-cre*/*ChR2-EYFP* mice. In skin sections from *Vglut3-cre/ChR2-EYFP* mice, peripheral EYFP-expressing nerve fibers stained positively for VGLUT3 (Fig. 7A). *In vivo* single unit recording from the DRG confirmed that blue light delivered to the hind paw increased the firing rate of a C-LTMR fiber in *Vglut3-cre/ChR2-EYFP* mouse (Fig. 7B left). The same unit did not respond to green light (Fig. 7B right). Using *in vivo* single unit recording from the lumbar spinal cord, we isolated 25 chloroquine-responsive units in the superficial dorsal horn. Of these units, 62.5% (10/16) responded to histamine injection to the hindpaw (Fig. 8A), 68.8% (11/16) responded to serotonin injection (Fig. 8D), and 100% (6/6) responded to capsaicin injection. While none of the chloroquine-responsive units responded to green light stimulus, 100% (25/25) responded to blue light stimulus. All unit recording sites were located in the superficial dorsal horn at a mean depth of 109.5 ± 19.3 μm below the surface of the lumbar spinal cord. For most units, the location was confirmed by histological identification of lesion sites (Fig. 7C inset).

As in C57BL/6 mice, mean firing immediately post-chloroquine or post-capsaicin injection was significantly greater compared to the pre-injection level, while PBS or PBS-Tween vehicle did not increase mean firing (Fig. 7C-D, 8G-H). During the chloroquine-evoked response, firing was enhanced during blue light stimulus (from 0.8 ± 0.12 to 4.11 ± 0.67 impulses/sec), followed by a significant reduction in firing post blue light stimulus (0.49 ± 0.09 impulses/sec, $F_{(1.00,\ 15.06)} = 25.88$, $p = 0.0001$, Tukey's multiple comparison test: p=0.00001, Fig. 7G, 7I). Similarly, during the histamine- or serotonin-evoked responses, firing post blue light stimulus was significantly reduced compared to firing pre blue light stimulus (histamine: before: 1.06 ± 0.24 impulses/sec, after: 0.57 ± 0.15 impulses/sec, $F_{(1.01,\ 10.06)} = 15.07$, $p = 0.0030$, Tukey's multiple comparison test: p=0.0011; serotonin; before: 1.23 ± 0.39 impulses/sec, after: 0.80 ± 0.30 impulses/sec, $F_{(1.01,\ 9.11)} = 10.7$, $p = 0.0094$, Tukey's multiple comparison test: p=0.0027, Fig. 8B, 8E).

In contrast, during the capsaicin-evoked response, the blue light stimulus did not significantly affect firing post blue light stimulus (before: 1.08 ± 0.26 impulses/sec; after 1.23 ± 0.27 impulses/sec, Fig. 8I). The green light (control) stimulus did not significantly affect ongoing activity elicited by any tested reagents (Fig. 7H-I, 8C, 8F, 8J). Therefore, optogenetic stimulation of VGLUT3-lineage sensory nerves was sufficient to inhibit firing elicited by pruritogens in the spinal cord in a state-dependent manner.

Recent studies revealed that there are two distinct subsets of spinal itch inhibitory interneurons, which regulate itch via neuropeptide Y receptor Y1 (NPY1R) or kappa opioid receptor (KOR) (Ross et al., 2010; Kardon et al., 2014; Bourane et al., 2015; Acton et al., 2019). To investigate which subsets of inhibitory interneurons are involved in inhibition of itch by VGLUT3-lineage sensory nerves, we applied either nor-binaltorphimine dihydrochloride, a KOR antagonist, or BMS193885, an NPY1R antagonist, to the spinal cord. To determine the duration of the inhibitory effects of antagonists, we identified mechano-sensitive spinal neurons and tested their responses to mechanostimuli pre- and post-treatment with the antagonists. The responses to pinch were increased at 2 min post-treatment with the antagonists, followed by returning to baseline (Fig. 9A-B). Therefore, the effects of antagonists were tested at 2 min post-application in the following experiments.

We tested nine chloroquine-responsive units with the two antagonists. None of the units exhibited an increased firing rate following the application of nor-binaltorphimine dihydrochloride or BMS193885 alone. During the chloroquine-evoked response, firing was significantly reduced post blue light stimulus (from 0.75± 0.12 to 0.40 ± 0.09 impulses/sec, $F(1.01, 8.09) = 14.53$, $p = 0.005$, Tukey's multiple comparison test: $p=0.0005$, Fig. 9C). Application of nor-binaltorphimine dihydrochloride during the blue light stimulus canceled the post-stimulus suppression of neuronal firing (from 1.27 ± 0.25 to 1.26 ± 0.22 impulses/sec, Fig. 9D). Application of BMS193885 during the blue light stimulus had no effect on post-stimulus firing (from 1.29 ± 0.28 to 0.49 ± 0.11 impulses/sec, Fig. 9E). Neither antagonist had an effect on blue light-evoked firing post PBS injection (Fig. 9F-H). These data suggest that KOR-expressing spinal interneurons mediate the itch-inhibitory effects of VGLUT3-lineage nerves.

Optogenetic stimulation of VGLUT3-lineage sensory nerves inhibits itch-related behavior, but not pain-related behavior

To investigate if optogenetic stimulation of VGLUT3-lineage sensory nerves affects itch-related behavior, we used *Vglut3-cre*/*ChR2-EYFP* mice and videotaped their behaviors following i.d. injection of the pruritogens histamine, serotonin, and chloroquine with and

without simultaneous blue or green light stimulation. Blue light stimulation of peripheral VGLUT3-lineage sensory nerves significantly reduced the total scratching response to each pruritogen (histamine: $F_{(1.62,\ 12.92)} = 9.719$, $p = 0.0039$, Tukey's multiple comparison test: $p=0.011$; serotonin: $F_{(1.22,\ 10.96)} = 6.62$, $p = 0.022$, Tukey's multiple comparison test: $p=0.0069$; chloroquine: $F_{(1.67,\ 15.05)} = 10.7$, $p = 0.0017$, Tukey's multiple comparison test: $p=0.012$, Fig. 10A-C). In contrast, green light (control) stimulation did not have any significant effects on the total scratching response. Neither blue nor green light affected spontaneous scratching in *Vglut3-cre*/*ChR2-EYFP* mice (Fig. 10D).

Finally, to assess if optogenetic stimulation of peripheral VGLUT3-lineage sensory nerves affects pain sensation, we used the Hargreaves test and von Frey filament test in *Vglut3-cre*/*ChR2-EYFP* mice. Neither blue light nor green light stimulation had any effect on thermal or mechanical pain thresholds (Fig. 10E-F).

Collectively, these data demonstrate that optogenetic stimulation of VGLUT3-lineage sensory nerves is capable of inhibiting itch-related behavior without affecting pain-related behaviors.

Discussion

Innocuous mechanical stimuli (e.g., rubbing or stroking the skin) are thought to relieve itch through the activation of LTMRs. Here we show that stroking the skin inhibits the responses of spinal neurons to pruritogens via VGLUT3-lineage sensory nerves. Moreover, optogenetic stimulation of VGLUT3-lineage sensory nerves inhibits itch-related behavior without affecting pain-related behaviors.

A cotton swab and two different sizes of brushes were used to stroke the skin. Interestingly, only the thick brush was sufficient to inhibit post-stroking pruritogen-evoked firing of spinal neurons. The strength of stroking with the thick brush was 24-35 mN, which is higher than that with cotton swab (< 1mN), implying that the strength of stroking is an important factor for itch inhibition. In line with this, weak stroking (1-2 mN) of the hind paw skin did not show any effects on spontaneous firing in a mouse model of dry skin. In addition to strength, the width of the device is likely a key factor. While the stroking forces of the two brushes were comparable, the thick brush was 6 mm wide (stroked area 36 mm^2) and the thin brush was 2.6 mm wide (stroked area 15 mm^2). The receptive fields of rapidly adapting type 1 LTMRs, slowly adapting type 1 LTMRs, and C-LTMRs are 22, 9, and 0.2-0.4 mm^2, respectively (Li et al., 2011; Roudaut et al., 2012). The number of activated LTMRs during stroking may be important for post-stroking inhibition of itch.

Stroking the skin activates various types of LTMRs (Abraira and Ginty, 2013; Bai et al., 2015). The velocity of stroking is one of the factors that determines which LTMRs respond. While C-LTMRs respond optimally to skin stroking in the range of 1–10 cm/s, A-LTMRs show stronger responses in proportion to higher velocities (Loken et al., 2009). In the present study, the stroking velocity was approximately 1.2 cm/s. This could likely activate both C- and A-LTMRs. Stroking-evoked inhibition of itch was prevented by optogenetic silencing of VGLUT3-lineage nerves, suggesting that VGLUT3-lineage C-LTMRs or Aβ-LTMRs are involved in itch inhibition. Given that selective inhibition of $TLR5^+$ A-fibers did not affect stroking-evoked inhibition of itch, VGLUT3-lineage C-LTMRs might play a major role in inhibition of itch by stroking.

There are at least two distinct subsets of itch inhibitory spinal interneurons, including NPY^+ and $Bhlhb5^+$ interneurons (Ross et al., 2010; Kardon et al., 2014; Bourane et al., 2015; Pan et al., 2019). NPY^+ inhibitory interneurons receive mono-synaptic or polysynaptic input from Aβ-, Aδ- and C-LTMRs (Bourane et al., 2015), suggesting that

stroking the skin activates NPY^+ inhibitory interneurons. A recent study showed that NPY^+ inhibitory interneurons regulate mechanical itch through NPY1R (Acton et al., 2019). In this study, the NPY1R antagonist BMS193885 failed to prevent inhibition of itch by VGLUT3-lineage nerve stimulation. Post-stroking itch inhibition may be mediated by interneurons other than NPY^+ inhibitory interneurons. Another subset of itch inhibitory interneurons has been shown to suppress chemical itch via KOR (Kardon et al., 2014). In the present study, the KOR antagonist nor-binaltorphimine dihydrochloride reversed the inhibitory effects of VGLUT3-lineage nerve stimulation. This result suggests that $Bhlhb5^+$ inhibitory spinal interneurons may mediate post-stroking itch inhibition. In line with this, C-LTMR fibers establish direct synaptic contact with GABAergic interneurons to suppress C-fibers in the superficial spinal cord (Lu and Perl, 2003; Kambrun et al., 2018). Whether C-LTMR fibers directly send signals to $Bhlhb5^+$ GABAergic interneurons is still unknown.

Previous studies reported that scratching inhibits itch-signaling neurons in the spinal cord in a state-dependent manner (Davidson et al., 2009; Akiyama et al., 2012; Nishida et al., 2013). In primate and rodent pruritogen-responsive spinal neurons, scratching reduces pruritogen-induced firing, but not capsaicin-induced firing. Likewise, in the present study, stroking or stimulation of VGLUT3-lineage nerves reduced the responses of spinal neurons to pruritogens, but not capsaicin. These findings suggest that scratching, stroking, and stimulation of VGLUT3-lineage nerves may engage similar inhibitory mechanisms during itch, but not pain.

Stroking the skin at the speeds of 0.1-10 cm/s (the optimal speed for C-LTMRs) is known to reduce pain (Shaikh et al., 2015; Habig et al., 2017; Liljencrantz et al., 2017; Gursul et al., 2018). A previous study showed that stroking the skin with sandpaper, an unpleasant stimulus, enhanced underlying muscle pain, while stroking with velvet, a pleasurable stimulus, relieved pain (Shaikh et al., 2015). This finding suggests that stroking-evoked analgesia is a type of pleasure-related analgesia, which is elicited by any pleasant stimuli (e.g., pleasant odors, positive images, pleasurable music, and sweet food) (Leknes and Tracey, 2008). Pleasure-related analgesia is expected to be mediated via cortical mechanisms. In contrast to pain, itch relief and pleasant sensation are likely mediated by different mechanisms. Scratch-evoked relief of itch is independent of pleasant sensation and mediated by spinal mechanisms (Davidson et al., 2009; Mochizuki et al., 2017). Likewise, our data suggest that the spinal cord plays an important role in stroking-evoked inhibition of itch.

The gate control theory proposes that touch relieves pain in the spinal cord (Melzack and Wall, 1965). Electrophysiological studies imply that multi-receptive neurons in the deep dorsal horn are responsible for pain relief by touch (Hillman and Wall, 1969; Salter and Henry, 1990; Le Bars, 2002). These neurons have segmental receptive fields, which are characterized by an excitatory receptive field surrounded by an inhibitory receptive field. The excitatory receptive field is activated by both innocuous and noxious stimuli, while the inhibitory receptive field is activated by innocuous stimuli. The activation of inhibitory receptive field can reduce the firing of multi-receptive neurons to nociceptive stimuli. The pruriceptive neurons recorded in the present study are not likely to be multi-receptive neurons, because they were recorded from the superficial dorsal horn and not the deep dorsal horn. In line with this, stroking the skin did not inhibit the firing of spinal neurons during responses to capsaicin in the current study.

In our recordings of dorsal horn neurons, stroking with the thick brush markedly increased the firing rate of chloroquine-responsive neurons during chloroquine exposure. A similar phenomenon has been observed for scratching. Most pruritogen-sensitive spinal neurons exhibit an increase in firing rate during scratching within the receptive field (Davidson et al., 2009; Akiyama et al., 2012; Nishida et al., 2013). Previous studies showed that most pruritogen-sensitive spinal neurons are a subpopulation of algogen-sensitive spinal neurons (Akiyama et al., 2009; Davidson et al., 2012; Akiyama et al., 2014a; Cevikbas et al., 2014). Based on these findings, it has been postulated that pruritogens activate pruritogen- and algogen-sensitive spinal neurons to elicit itch, while algogens activate a wider population of algogen-sensitive spinal neurons to elicit pain (Akiyama et al., 2009; Ma, 2010; Akiyama et al., 2014a). Therefore, while scratching does excite pruritogen-sensitive neurons, the overall sensation elicited by scratching is pain and not itch. A similar idea can be used to interpret the present result. Stroking activates low-threshold mechano-sensitive spinal neurons, wide dynamic range spinal neurons including pruritogen-sensitive spinal neurons, and dorsal column nuclei to elicit tactile sensation (Abraira and Ginty, 2013; Abraira et al., 2017). Therefore, while stroking enhanced the response to pruritogens during stimulation, the overall sensation elicited by stroking (and presumably by VGLUT3-lineage nerve stimulation) is touch and not itch. In line with this idea, optogenetic VGLUT3-lineage nerve stimulation alone did not elicit scratching.

Additionally, the pharmacological silencing of $TLR5^{+}$ A-fibers diminished the enhancing effect of stroking with the thick brush during the chloroquine-evoked response. On

585 the other hand, the optical silencing of VGLUT3-lineage nerves did not have such an effect.
586 These data indicate that the enhancement of chloroquine-evoked response by stroking with
587 the thick brush may be mediated by $TLR5^{+}$ A-fibers rather than VGLUT3-lineage nerves.
588
589 This study has shown that gentle skin stroking can reduce itch signaling in the
590 spinal cord through the activation of VGLUT3-lineage sensory nerves. The findings reported
591 here shed new light on the role of VGLUT3-lineage LTMRs in itch processing.
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Figure Legends

Figure 1.

Example *in vivo* single-unit electrophysiological recording from a chloroquine-responsive unit in the spinal cord in C57BL/6J mice. (A) This unit displayed a positive response over baseline to intradermal chloroquine. During chloroquine-evoked firing, the unit showed an enhanced response to (B) stroking with a cotton swab, (C) stroking with a thin brush, and (D) stroking with a thick brush. The unit's firing rate was decreased post stroking with the thick brush. (E) The same unit did not display a positive response over baseline to intradermal PBS (vehicle for chloroquine), or PBS-Tween (vehicle for capsaicin). Following vehicle injections, the unit displayed a positive response to the thin brush and thick brush, but not to the cotton swab. (F) The unit displayed a positive response over baseline to intradermal capsaicin. During capsaicin-evoked firing, the unit had no response to (G) the cotton swab but showed a positive response to (H) the thin brush and (I) the thick brush. The recording site is shown in the inset of (A) and injection/stroking site in the inset of (D).

Figure 2.

Stroking the skin with a thick brush reduced chloroquine-evoked firing in the spinal cord in

775 C57BL/6J mice. (A) Average firing of 10 units recorded from the superficial dorsal horn of
776 spinal cord before (pre) and after (post) intradermal PBS. Recording sites shown in inset. (B)
777 As in A, for chloroquine injection. (C) As in A, for PBS-Tween injection (vehicle control for
778 capsaicin injection). (D) As in A, for capsaicin injection. (E) Averaged peristimulus-time
779 histogram (PSTH, bins: 1 sec) of activity following i.d. chloroquine in 10 superficial dorsal
780 horn units, before, during, and after stroking with the thin or thick brushes. Horizontal lines
781 indicate duration of stroking. (F) Individual (grey lines) and mean (black line) responses of
782 superficial dorsal horn units following i.d. chloroquine, before (pre), during, and after (post)
783 stroking with a cotton swab. (G) As in F, for thin brush. (H) As in F, for thick brush. (I)
784 Individual (grey lines) and mean (black line) responses of superficial dorsal horn units
785 following i.d. capsaicin, before, during, and after stroking with the cotton swab. (J) As in I,
786 for thin brush. (K) As in I, for thick brush. Data are shown as mean ± SEM. *$p < 0.05$, **$p <$
787 0.01, for paired t-test (A-D) or 1-way repeated measure ANOVA followed by Tukey multiple
788 comparisons test (F-K).
789
790 Figure 3.
791 Pharmacological silencing of $TLR5^{+}$ myelinated fibers. Cutaneous low-threshold

792 mechanoreceptors (LTMRs) were recorded using *in vivo* single-unit electrophysiological
793 recording from the dorsal root ganglia in C57BL/6J mice. The recorded units were classified
794 as Aβ-, Aδ- and C-LTMR fibers based on their conduction velocities and
795 mechanosensitivities. $TLR5^{+}$ myelinated fibers were silenced by co-injection of QX-314 and
796 flagellin to the hind paw. (A) Co-injection of QX (QX-314; 120 nmol) and FL (flagellin; 10
797 pmol) abolished the responses of Aβ-LTMR to cotton and thick brush for up to 3 hours. (B)
798 Co-injection of low-dose QX (24 nmol) and FL (10 pmol) abolished the responses of Aβ-
799 LTMR to cotton and thick brush for a shorter duration. (C-D) Co-injection of QX (120 nmol)
800 and FL (10 pmol) had no effects on the mechanosensitivities of Aδ- (C) and C- (D) LTMR
801 fibers.
802
803 Figure 4.
804 Myelinated fiber silencing was not sufficient to block stroking-evoked inhibition of
805 chloroquine-evoked firing in the spinal cord in C57BL/6J mice. $TLR5^{+}$ myelinated fibers
806 were silenced by co-injection of QX-314 (120 nmol) and flagellin (10 pmol) to the hind paw.
807 (A) Average firing of units recorded from the superficial dorsal horn of spinal cord before
808 (pre) and after (post) i.d. PBS. Recording sites shown in inset. (B) As in A, for i.d.

809 chloroquine. (C) Individual (grey lines) and mean (black line) responses of superficial dorsal
810 horn units following i.d. chloroquine, before (pre), during, and after (post) stroking with the
811 thick brush. Data are shown as mean ± SEM. *$p < 0.05$, for paired t-test (A, B) or 1-way
812 repeated measures ANOVA followed by Tukey multiple comparisons test (C).
813
814 Figure 5.
815 Innervation of VGLUT3-lineage sensory nerves in the skin. Skin sections from *Vglut3-*
816 *cre*/*TdTomato* mice were immunostained with antibodies for VGLUT3 (green; A), PGP9.5
817 (B), NFH (C), CGRP (D), or P2X3 (E). Arrows indicate double-positive nerves. Arrowheads
818 indicate tdTomato single-positive nerves. Scale bars indicate 10 μm.
819
820 Figure 6.
821 Optogenetic inhibition of VGLUT3-lineage sensory nerves blocked post-stroking inhibition
822 of chloroquine-evoked firing in the spinal cord of *Vglut3-cre*/*NpHR-EYFP* mice. (A) Skin
823 sections from *Vglut3-cre*/*NpHR-EYFP* mice were immunostained for VGLUT3 (red) to show
824 expression of halorhodopsin in VGLUT3+ sensory nerves of the skin. Arrows indicate
825 double-positive nerves. Scale bars indicate 10 μm. (B) Average firing of units recorded from

826 the superficial dorsal horn of spinal cord before (pre) and after (post) i.d. PBS. Spinal
827 recording sites shown in inset. (C) As in B, for i.d. chloroquine injection. (D) As in B, for i.d.
828 PBS-Tween injection (vehicle control for capsaicin injection). (E) As in B, for i.d. capsaicin
829 injection. (F) Individual (grey lines) and mean (black line) responses of superficial dorsal
830 horn units following i.d. chloroquine, before (pre), during, and after (post) stroking with the
831 thick brush and simultaneous blue light exposure. (G) As in F, for green light exposure. (H)
832 Averaged peristimulus-time histograms (PSTH, bins: 1 sec) of activity following i.d.
833 chloroquine before, during, and after stroking with the thick brush and simultaneous blue or
834 green light exposure. Horizontal lines indicate duration of stroking and light exposure. (I) As
835 in F, for no light exposure. (J) Individual (grey lines) and mean (black line) responses of
836 superficial dorsal horn units following i.d. capsaicin, before (pre), during, and after (post)
837 stroking with the thick brush and simultaneous blue light exposure. (K) As in J, for green
838 light. (L) As in J, for no light. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, for
839 paired t-test (B-E) or 1-way repeated measures ANOVA followed by Tukey multiple
840 comparisons test (F, G, I-L).
841
842 Figure 7.

Optogenetic stimulation of VGLUT3-lineage sensory nerves inhibited chloroquine-evoked firing in the spinal cord in *Vglut3-cre*/*ChR2-EYFP* mice. (A) Skin sections from *Vglut3-cre*/*ChR2-EYFP* mice were immunostained for VGLUT3 (red). Arrows indicate double-positive nerves. Scale bars indicate 10 μm. (B) Firing responses of a C-LTMR fiber to blue or green light pulses (20 Hz). A C-LTMR unit was recorded from the dorsal root ganglia in *Vglut3-cre/ChR2-EYFP* mice. (C) Average firing of units recorded from the superficial dorsal horn of spinal cord before (pre) and after (post) i.d. PBS. Recording sites shown in inset. (D) As in C, for chloroquine injection. (E) Individual (grey lines) and mean (black line) responses of superficial dorsal horn units before (pre), during, and after (post) blue light stimulation. (F) As in E, for green light stimulation. (G) Individual (grey lines) and mean (black line) responses of superficial dorsal horn units following i.d. chloroquine, before (pre), during, and after (post) blue light stimulation. (H) As in G, for green light stimulation. (I) Averaged peristimulus-time histogram (PSTH, bins: 1 sec) of activity following i.d. chloroquine before, during, and after blue or green light stimulation. Horizontal lines indicate duration of light stimulation. Data are shown as mean ± SEM. ***$p < 0.001$, ****$p < 0.0001$, for paired t-test (C, D) or 1-way repeated measure ANOVA followed by Tukey multiple comparisons test (E-H).

860

861 Figure 8.

862 Optogenetic stimulation of VGLUT3-lineage sensory nerves inhibited histamine- or
863 serotonin-evoked firing in the spinal cord in *Vglut3-cre*/*ChR2-EYFP* mice. (A) Average firing
864 of units recorded from the superficial dorsal horn of spinal cord before (pre) and after (post)
865 i.d. histamine. (B) Individual (grey lines) and mean (black line) responses of superficial
866 dorsal horn units following i.d. histamine, before (pre), during, and after (post) blue light
867 stimulation. (C) As in B, for green light stimulation. (D) As in A, for serotonin. (E) As in B,
868 for serotonin. (F) As in C, for serotonin. (G) As in A, for PBS-Tween injection (vehicle
869 control for capsaicin injection). (H) As in A, for capsaicin. (I) As in B, for capsaicin. (J) As in
870 C, for capsaicin. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, for paired t-test (A,
871 D, G, H) or 1-way repeated measure ANOVA followed by Tukey multiple comparisons test
872 (B, C, E, F, I, J).

873

874 Figure 9.

875 A kappa opioid receptor antagonist canceled the itch inhibitory effects of optogenetic
876 stimulation of VGLUT3-lineage sensory nerves in *Vglut3-cre*/*ChR2-EYFP* mice. (A)

877 Responses of a superficial dorsal unit to cotton and pinch before and after superfusion of the
878 KOR (kappa opioid receptor) antagonist nor-binaltorphimine dihydrochloride. (B) As in A,
879 for the neuropeptide Y receptor Y1 (NPY1R) antagonist BMS193885. (C) Individual (grey
880 lines) and mean (black line) responses of superficial dorsal horn units following i.d.
881 chloroquine and superfusion of artificial cerebrospinal fluid over the spinal cord, before (pre),
882 during, and after (post) blue light stimulation. (D) As in C, for KOR antagonist. (E) As in C,
883 for NPY1R antagonist. (F) As in C, for PBS. (G) As in D, for PBS. (H) As in E, for PBS.
884 Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for 1-way repeated
885 measure ANOVA followed by Tukey multiple comparisons test (C-H).
886
887 Figure 10.
888 Optogenetic stimulation of VGLUT3-lineage sensory nerves inhibited itch-related behavior
889 without affecting pain-related behaviors in *Vglut3-cre*/*ChR2-EYFP* mice. (A) Number of
890 scratch bouts after i.d. injection of histamine (n=9/group). (B) As in A, for i.d. chloroquine (n
891 = 10/group). (C) As in A, for i.d. serotonin (n=10/group). (D) Number of spontaneously-
892 occurring scratch bouts (n=4/group). (E) Paw withdrawal threshold to von Frey filament (n =
893 11/group). (F) Paw withdrawal latency in the Hargreaves test (n = 6/group). All experiments

were performed under no light, green light, and blue light conditions. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, for 1-way repeated measure ANOVA followed by Tukey multiple comparisons test.

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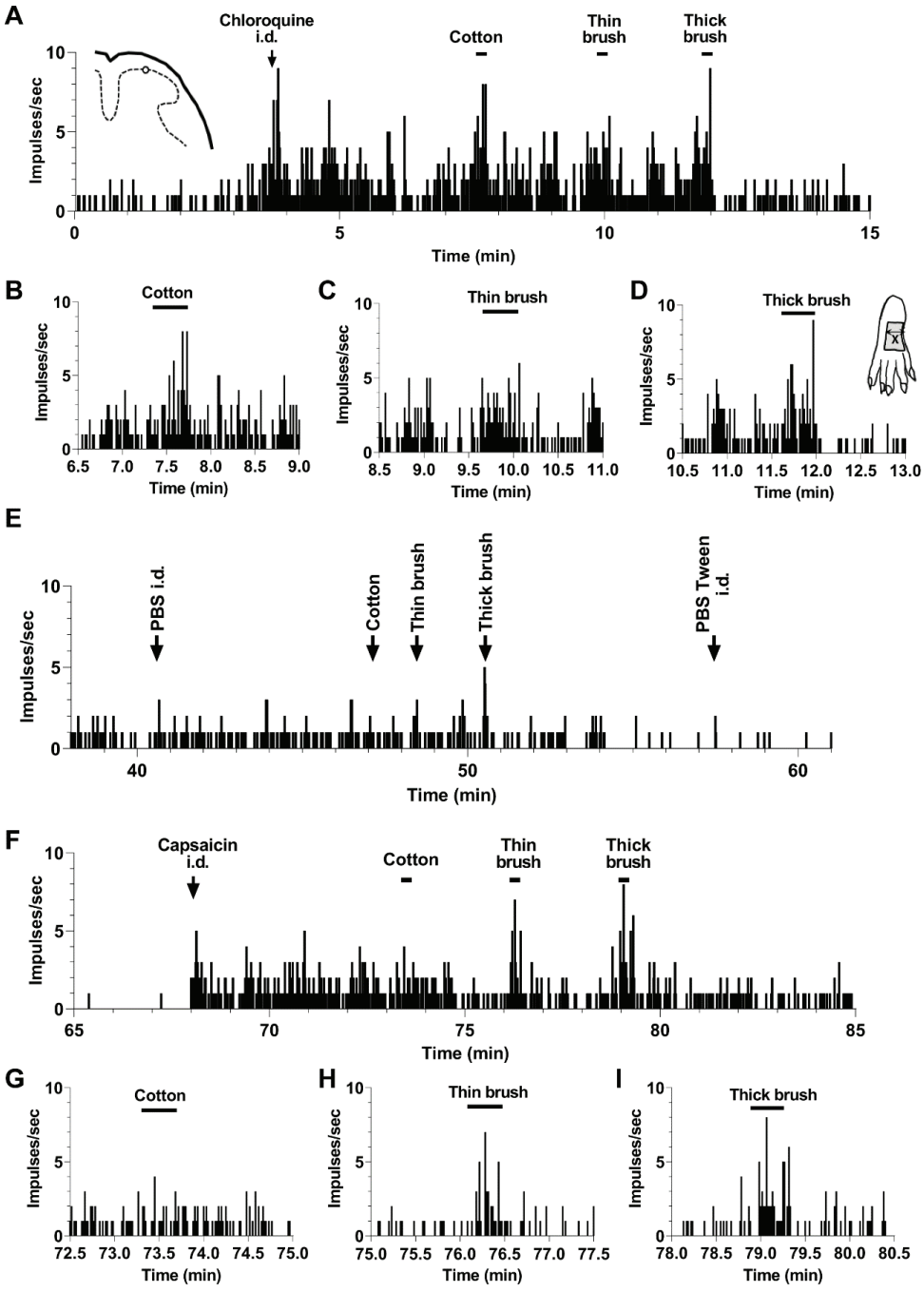

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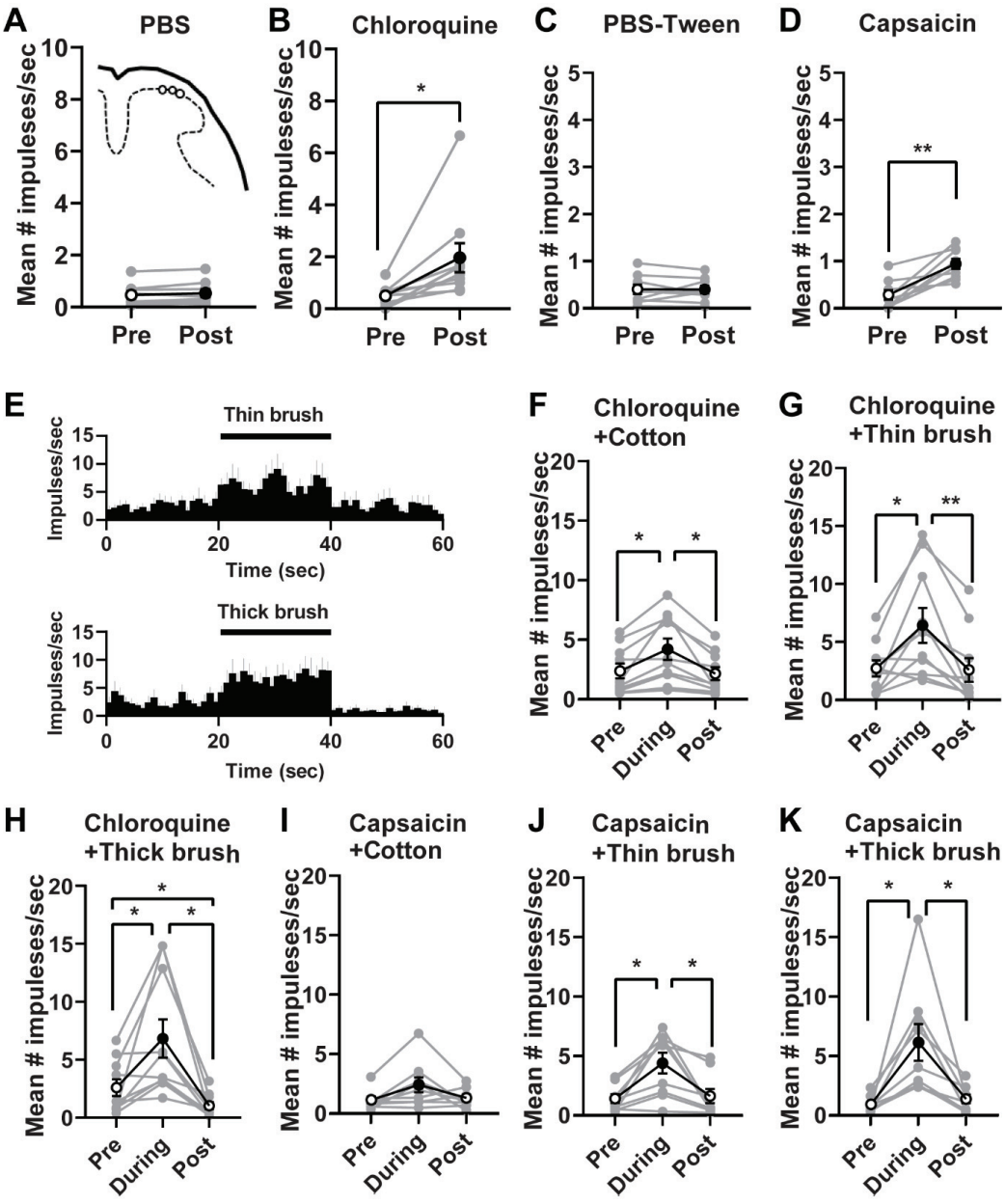

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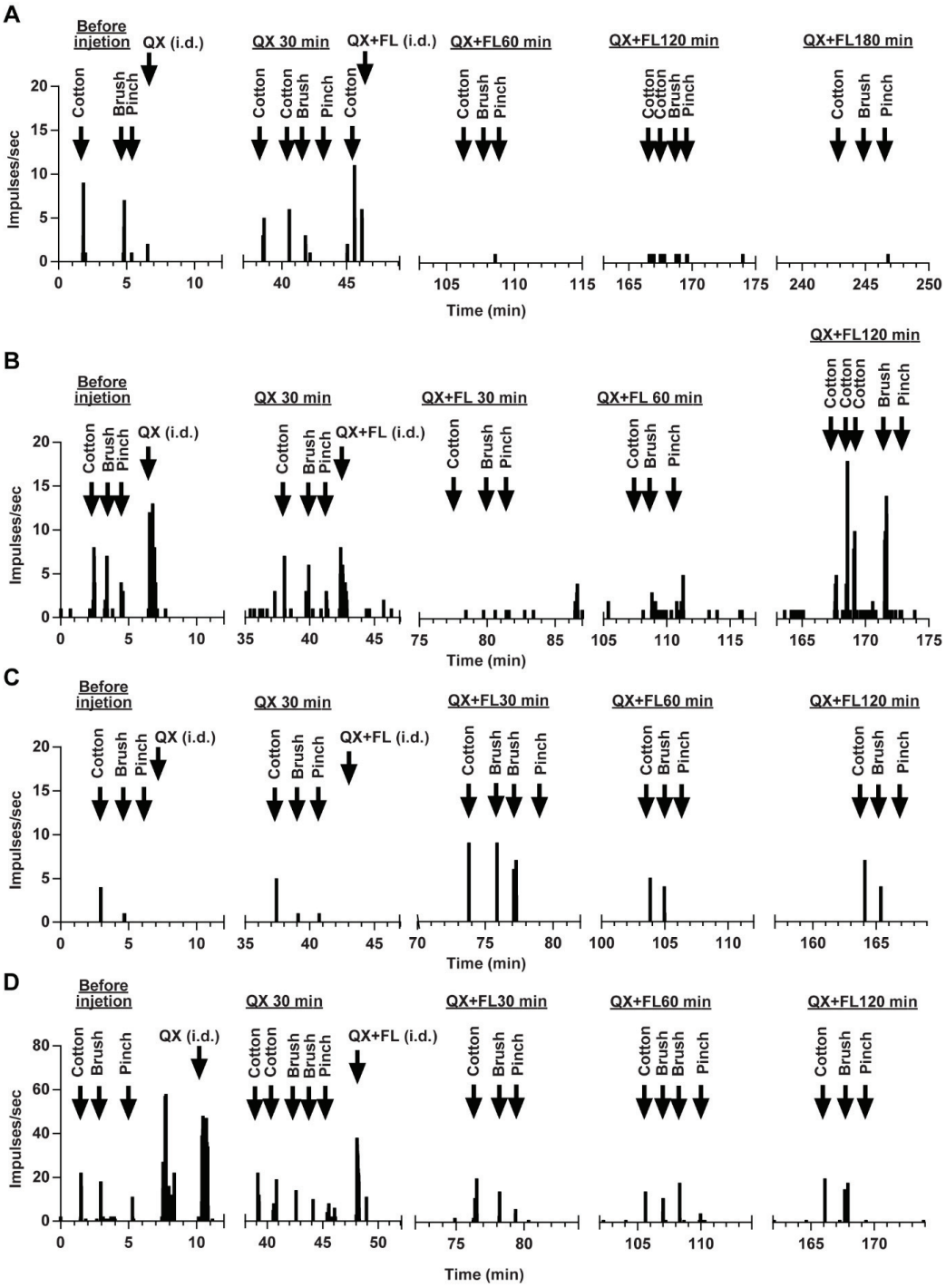

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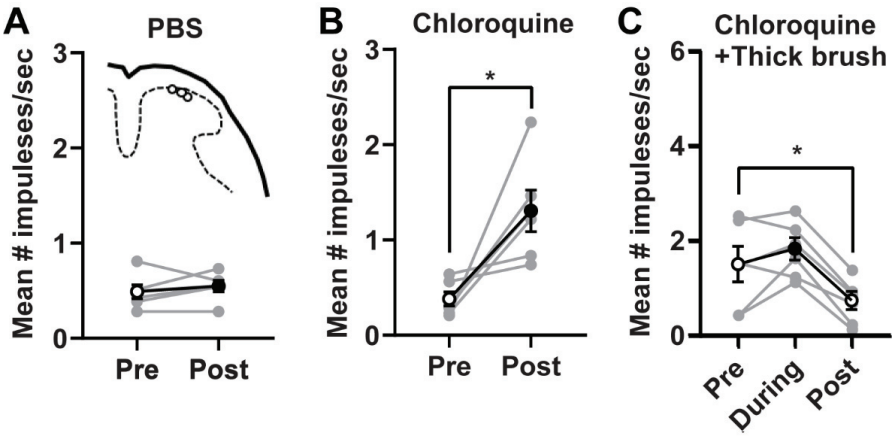

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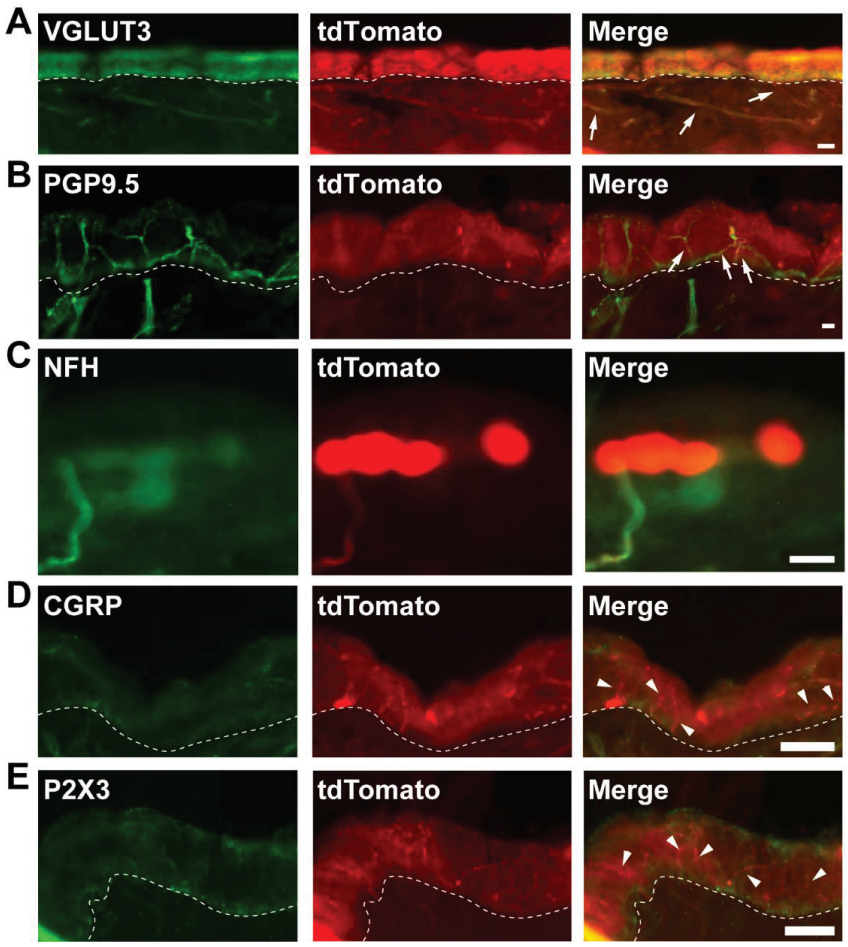

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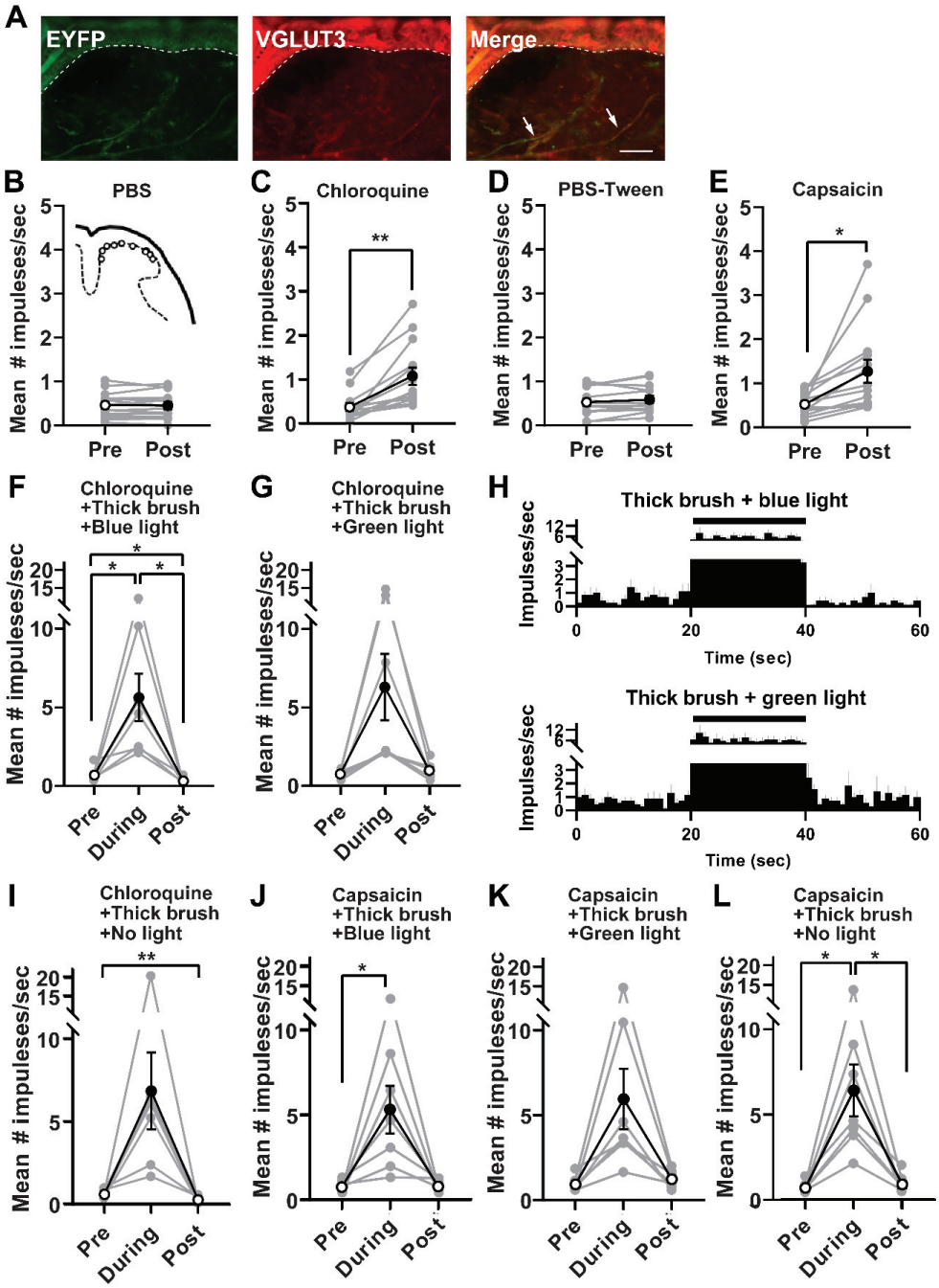

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934 Figure 7.

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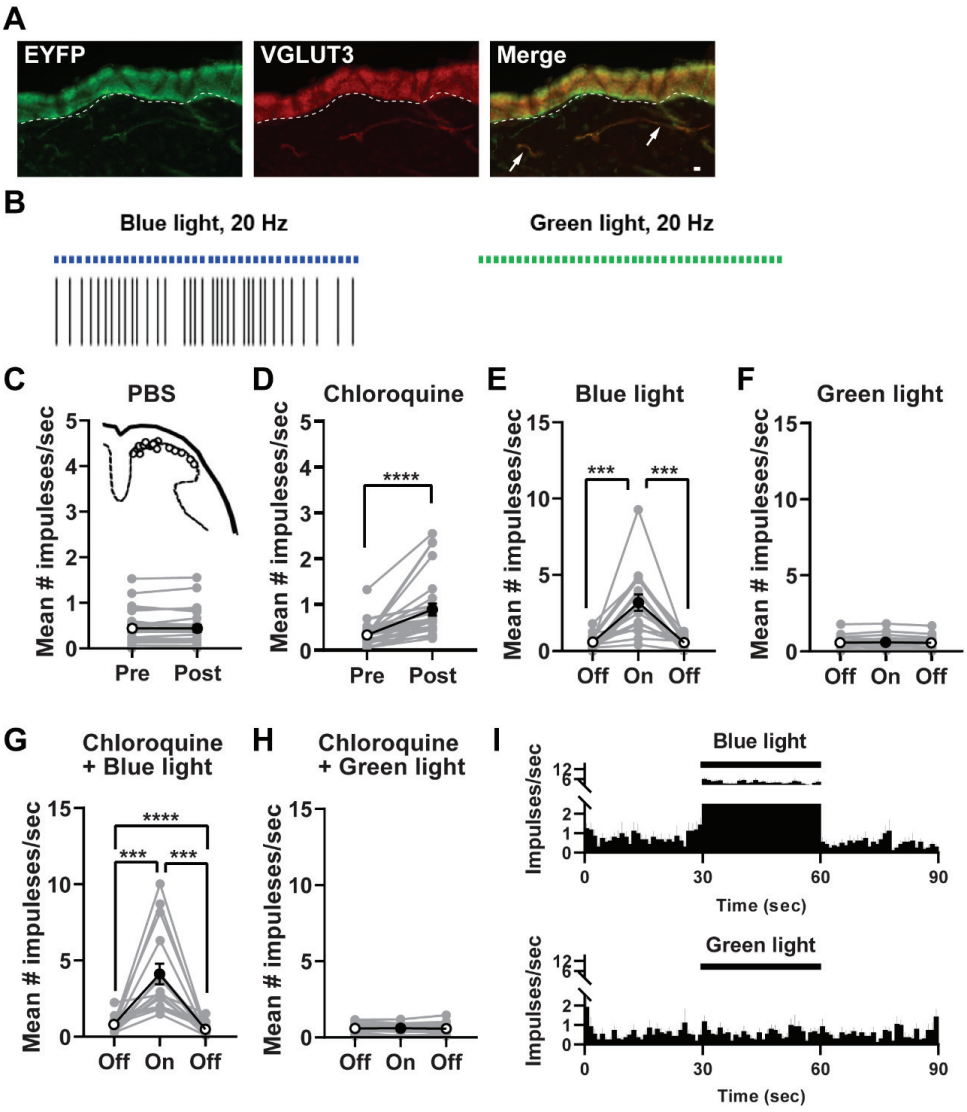

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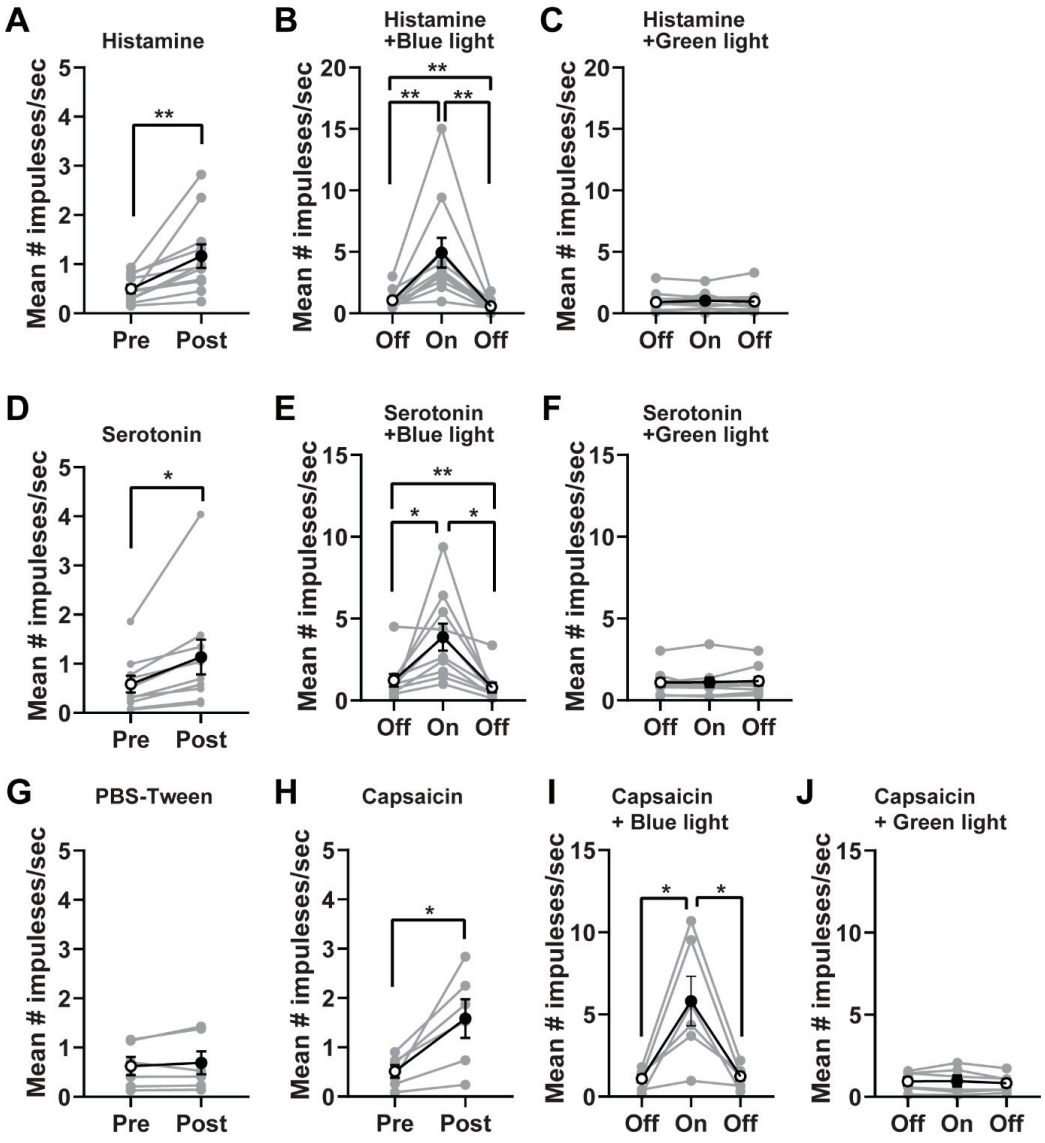
939 Figure 8.



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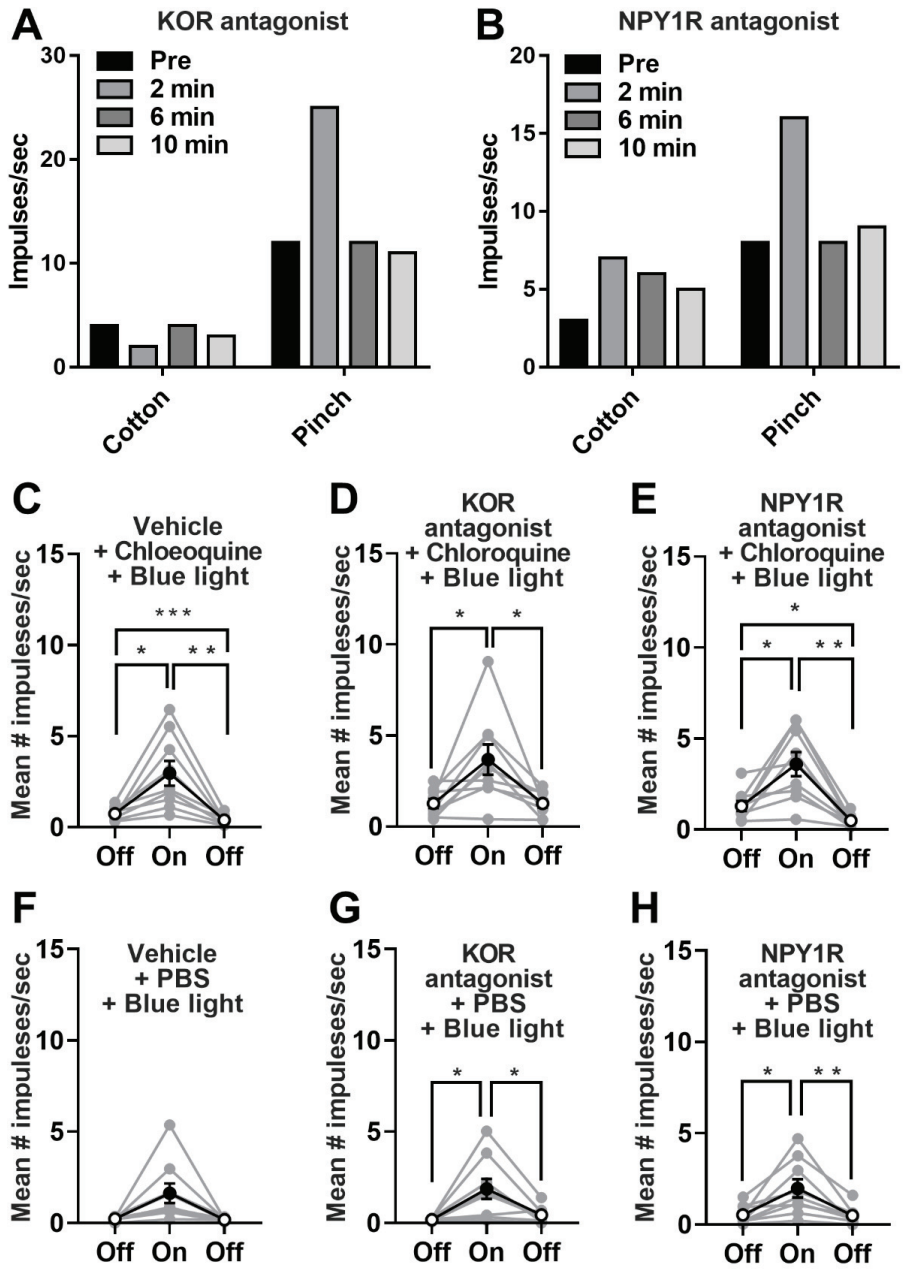

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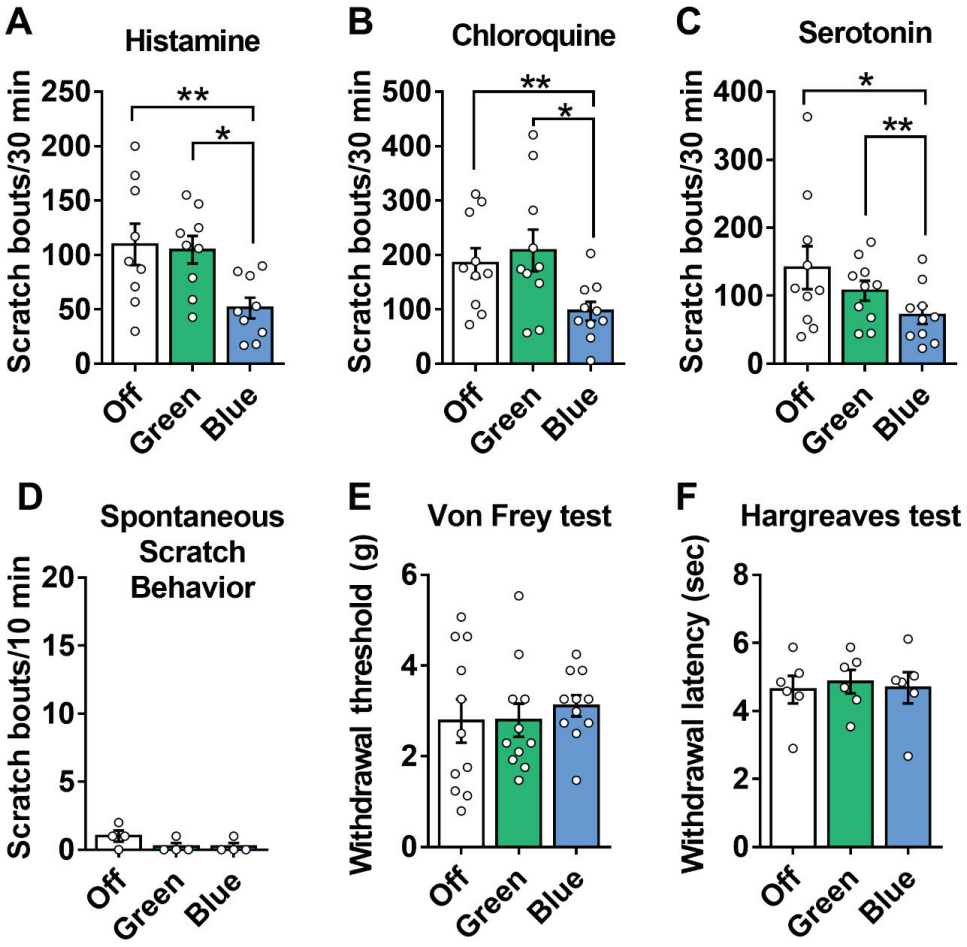

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951 Table 1. Stroking and scratching devices.

	Strength (mN)	Width (mm)	Speed (cm/sec)
Cotton swab	0.21	4	2
Thin brush	49-64	2.5	2
Thick brush	24-35	6	2

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