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Roles of glutamate, substance P and gastrin releasing peptide as spinal neurotransmitters of histaminergic and non-histaminergic itch

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Abstract

We investigated roles for substance P (SP), gastrin-releasing peptide (GRP), and glutamate in the spinal neurotransmission of histamine-dependent and –independent itch. In anesthetized mice, responses of single superficial dorsal horn neurons to intradermal (id) injection of chloroquine were partially reduced by spinal application of the AMPA/kainate antagonist CNQX. Co-application of CNQX plus a neurokinin-1 (NK-1) antagonist produced stronger inhibition, while co-application of CNQX, NK-1 and GRP receptor (GRPR) antagonists completely inhibited firing. Nociceptive-specific and wide dynamic range-type neurons exhibited differential suppression by CNQX plus either the GRPR or NK-1 antagonist, respectively. Neuronal responses elicited by id histamine were abolished by CNQX alone. In behavioral studies, individual intrathecal administration of a GRPR, NK-1 or AMPA antagonist each significantly attenuated chloroquine-evoked scratching behavior. Co-administration of the NK-1 and AMPA antagonists was more effective, and administration of all three antagonists abolished scratching. Intrathecal CNQX alone prevented histamine-evoked scratching behavior. We additionally employed a double-label strategy to investigate molecular markers of pruritogen-sensitive dorsal root ganglion (DRG) cells. DRG cells responsive to histamine and/or chloroquine, identified by calcium imaging, were then processed for co-expression of SP, GRP or vesicular glutamate transporter type 2 (VGLUT2) immunofluorescence. Subpopulations of chloroquine- and/or histamine-sensitive DRG cells were immunopositive for SP and/or GRP, with >80% immunopositive for VGLUT2. These results indicate that SP, GRP and glutamate each partially contributes to histamine-independent itch. Histamine-evoked itch is mediated primarily by glutamate with GRP playing a lesser role. Co-application of NK-1, GRP and AMPA receptor antagonists may prove beneficial in treating chronic itch.

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Introduction

Chronic itch is a burdensome clinical problem that decreases the quality of life [55], yet the neural mechanisms of itch are still not fully understood. Recent studies have implicated histamine-dependent and histamine-independent pathways in transmitting itch. The histamine-independent itch pathway involves members of the family of over 50 Mas-related G-protein coupled receptors (Mrgprs), in particular MrgprAs, MrgprB4-5, MrgprC11 and MrgprD, which are restricted to small diameter dorsal root ganglion (DRG) neurons in mice [21]. Chloroquine and the bovine adrenal medulla peptide 8-22 (BAM8-22) elicited itch-related scratching through MrgprA3 and MrgprC11, respectively, in mice [32], and both compounds elicit itch in humans [1; 46]. β-alanine elicited itch via MrgprD [31]. It was recently reported that MrgprA3-expressing primary sensory neurons play a predominant role in itch evoked by chloroquine and other pruritogens [27], implying that spinal neurons with input from such chloroquine-sensitive primary afferents selectively signal itch sensation.

Neurokinin-1 (NK-1) and gastrin releasing peptide (GRP) receptor (GRPR)-expressing spinal neurons are implicated in signaling itch [11; 50]. Their respective ligands, substance P (SP) and GRP, are partially involved in the spinal transmission of itch signals [9; 49]. The predominant excitatory neurotransmitter, glutamate, may additionally contribute to itch. A recent electrophysiological study suggested that glutamate acts as a neurotransmitter at GRP-sensitive spinal neurons [29]. In contrast, the genetic ablation of the vesicular glutamate transporter type 2 (VGLUT2), which is essential for glutamate release from the majority of A- and C-fiber nociceptors [42], resulted in reduced nocifensive behavior and enhanced spontaneous and pruritogen-evoked scratching [30; 36]. A very recent study reported that natriuretic polypeptide B (Nppb) is the primary transmitter released by pruritogen-sensitive primary afferents in mice [36]. Nppb excites GRPR-expressing spinal interneurons that are essential in transmitting itch, but not pain, signals to higher centers [36; 46; 47].

In the present study we used a multidisciplinary approach to investigate the roles of SP, GRP and glutamate in the spinal transmission of itch. In electrophysiological experiments we tested if chloroquine-evoked responses of superficial dorsal horn neurons are inhibited by spinal application of antagonists of NK-1, GRP and/or glutamate aminomethylphosphoric acid (AMPA)/kainate receptors. Complementary behavioral experiments investigated if these receptor antagonists alone or in combination attenuated chloroquine- and histamine-evoked scratching. Using a combination of calcium imaging followed by immunohistochemistry, we investigated the expression of SP, GRP and VGLUT2 in pruritogen-sensitive primary sensory neurons.

Materials and Methods

Electrophysiology

Experiments were performed using 118 adult male C57BL/6 mice (18–33 g) under a protocol approved by the UC Davis Animal Care and Use Committee. The single-unit recording from the lumbar spinal cord was conducted as previously detailed [3; 8]. Anesthesia was induced by sodium pentobarbital (60 mg/kg ip) and maintained by supplemental injections (10–20 mg/kg/hr). A gravity-driven perfusion system allowed artificial cerebrospinal fluid (Krebs: 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 11 mM glucose which was equilibrated with 95% O_2 and 5% CO_2 at 37°C) to be superfused continually over the exposed lumbosacral spinal cord [7]. A tungsten microelectrode recorded single-unit activity in the lumbar spinal cord. A chemical search strategy [3; 8] was used to identify and isolate chloroquine-responsive units. Our search strategy was intended to maximize the chance of

isolating a chloroquine-responsive neuron; we assume that such neurons either gave rise to ascending projections, or served as interneurons in segmental scratch-reflex circuitry, and no attempt was presently made to distinguish between these possibilities. Briefly, a small (~0.25 μl) intradermal (id) microinjection of chloroquine (100 μg/μl) was made in the ventral hindpaw and a spontaneously active unit in the superficial lumbar dorsal horn (depth < 300 um) was isolated. After the spontaneous activity had waned, chloroquine (1 μl, 100 μg/μl) was injected again at the same site through the same needle. Only units exhibiting an increase of >30% in firing to this second test microinjection of chloroquine were selected for further study. Responses were usually recorded for at least 30 min, although in many cases unit firing declined over a shorter period.

During the time that the unit exhibited a relatively stable level of chloroquine-evoked firing (usually 1 min post-injection), one of the following antagonists were successively delivered directly at spinal cord for 1 min; the GRP receptor antagonist RC-3095 (20 μM), the NK-1 receptor antagonist L-733060 (200 μM), the AMPA/kainate receptor antagonist CNQX (100 μM), a combination of CNQX (100 μM) and L-733060 (200 μM), or a combination of RC-3095 (20 μM), CNQX (100 μM) and L-733060 (200 μM).

When unit firing to the second chloroquine injection declined and reached a steady level, the unit's mechanosensitive receptive field was determined. The perimeter of the mechanical receptive field was mapped using a von Frey filament (55 mN bending force) by determining sites at which the unit either did (within receptive field) or did not (outside receptive field) respond to at least 3 of 5 repeated applications. The rationale for choosing this bending force is that it was sufficient to map the maximum extent of the mechanical receptive field as assessed by comparison of receptive field sizes mapped using a range of von Frey stimuli (0.7 mN: 1.2±0.6 mm^2, 6.9 mN: 3.7±0.9 mm^2, 55mN: 9.9±1.4 mm^2, 758 mN: 10.3±1.4 mm^2). Units were classified as wide dynamic range (WDR) –type if they responded in a graded manner to innocuous mechanical stimulation (brushing, cotton wisp) and noxious pinch, or nociceptive-specific (NS) if they responded to noxious pinch (and to the 55 mN von Frey stimulus) but not to the cotton wisp or brush stimuli. The properties are similar to those of WDR and NS units shown in previous studies to respond to histamine, serotonin, the PAR-2/ MrgprC11 agonist SLIGRL, or chloroquine [3; 8]. In some units, at least 5 min after the noxious pinch stimulus, either the NK-1 receptor antagonist, the AMPA/kainate receptor antagonist, or a mixture of both, was superfused directly over the spinal cord for 1 min. At the end of the antagonist superfusion, the noxious pinch stimulus was delivered again at the same site on the receptive field. Thirty min later, the noxious pinch stimulus was delivered in the same manner.

Following mechanical stimulation, histamine (50 μg) was injected id within the same receptive field at a different location via a separate injection cannula. Following the id histamine injection, we tested the effects of antagonists for the receptors of NK1, GRP, or AMPA/kainate in the same manner as described above for chloroquine. Units were then tested with topical hindpaw application of allyl isothiocyanate (AITC; mustard oil, Sigma; 75% in mineral oil, 2 μl). Following the AITC application, we tested the effects of the NK-1 receptor antagonist, the AMPA/kainate receptor antagonist, or a combination of both antagonists in the same manner as described above for chloroquine.

Action potentials were recorded to a computer and counted using Chart software (AD Instruments, Colorado Springs CO) and Spike2 software (CED Instruments). Ongoing responses elicited by chloroquine, histamine, or AITC were averaged at 20-sec intervals before, during and after the antagonist application, and compared by one way repeated-measures analysis of variance (ANOVA) followed by post-hoc Bonferroni test, with $p < 0.05$ set as significant. The mean firing rate was calculated over a 20-sec period 40 sec after the

antagonist application, and compared by one way ANOVA followed by post-hoc Bonferroni test, with $p< 0.05$ set as significant. The criterion for decrease in ongoing firing was >70% decrease below the ongoing activity elicited by pruritogen over a 20-sec period 40 sec after the antagonist application. Mean peak responses elicited by noxious pinch were compared by one way repeated-measures ANOVA followed by post-hoc Bonferroni test, with $p< 0.05$ set as significant. At the end of each experiment, an electrolytic lesion was made at the spinal cord recording site. The spinal cord was postfixed in 10% buffered formalin, cut in 50 μm frozen sections, and examined under the light microscope to identify lesions.

Behavior

Experiments were conducted using adult male C57BL/6 mice (Simonsen, Gilroy, CA; 19–25 g) under a protocol approved by the UC Davis Animal Care and Use Committee. The fur on the rostral back was shaved and mice were habituated to the Plexiglas recording arena one week prior to testing. For intrathecal injections, either vehicle (saline), the GRP receptor antagonist RC-3095 (0.3 nmol; Sigma-Aldrich, St. Louis MO), the NK-1 antagonist L-733060 (22.7 nmol; Tocris Bioscience, Minneapolis, MN), the AMPA/kainate antagonist CNQX (20 nmol; Tocris Bioscience), a combination of CNQX (20 nmol) and L-733060 (22.7 nmol), or a combination of RC-3095 (0.3 nmol), CNQX (20 nmol) and L-733060 (22.7 nmol), was administered by lumbar puncture, followed 5 min later by id injection (10 μl) of either chloroquine (193 nmol; Sigma-Aldrich) or histamine (271 nmol; Sigma-Aldrich). Microinjections were made id in the nape of the neck using a 30 G needle attached to a Hamilton microsyringe by PE-50 tubing. Immediately after the id injection the mouse was placed into the arena and videotaped from above for 30 min. Generally 3–4 mice were injected and videotaped simultaneously. Immediately after commencing videotaping all investigators left the room.

Videotapes were reviewed by investigators blinded to the treatment, and the number of scratch bouts was counted at 5-min intervals. A scratch bout was defined as one or more rapid back-and-forth hind paw motions directed toward and contacting the injection site, and ending with licking or biting of the toes and/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. One-way ANOVA followed by the Bonferroni post-test was used to compare the total number of scratch bouts across pretreatment groups. In all cases $p<0.05$ was considered to be significant. Data for effects of individual antagonist of either NK1 or GRP receptor on scratching evoked by either histamine or chloroquine were modified from our recent study [9].

Calcium Imaging

A total of 20 adult male C57BL/6 mice (Simonsen, Gilroy, CA; 7–9 weeks old, 18–21 g) was used under a protocol approved by the UC Davis Institutional Animal Care and Use Committee. The animal was euthanized under sodium pentobarbital anesthesia and lumbar DRGs were acutely dissected and enzymatically digested at 37°C for 10 min in Hanks's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) containing 20 units/ml papain (Worthington Biochemical, Lakewood, NJ) and 6.7 mg/ml L-cysteine (Sigma), followed by 10 min at 37°C in HBSS containing 3 mg/ml collagenase (Worthington Biochemical). The ganglia were then mechanically triturated using fire-polished glass pipettes. DRG cells were pelleted, suspended in MEM Eagle's with Earle's BSS (Gibco) containing 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 1×vitamin (Gibco) and 10% horse serum (Quad Five, Ryegate, MT), plated on poly-D-lysine-coated glass coverslips, and cultured for 16–24 hr.

DRG cells were incubated in Ringers solution (pH7.4; 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 4.54 mM NaOH) with 10 μM of Fura-2 AM and 0.05% of Pluronic F-127 (Invitrogen). Coverslips were mounted on a custom made aluminum perfusion block and viewed through an inverted microscope (Nikon TS100, Technical Instruments, San Francisco CA). Fluorescence was excited by UV light at 340 nm and 380 nm alternately and emitted light was collected via a CoolSnap camera attached to a Lambda LS lamp and a Lambda optical filter changer (Sutter Instrument Company, Novato, CA). Ratiometric measurements were made using Simple PCI software (Compix Inc, Cranberry Township, PA) every 3 sec.

Solutions were delivered by a solenoid-controlled 8-channel perfusion system (ValveLink, AutoM8). Chloroquine (300 μM) or histamine (100 μM) was delivered, followed by potassium at a concentration of 144 mM. Stimulus duration was 30 sec. Ratios were normalized to baseline. Cells were judged to be sensitive if the ratio value increased by more than 10% of the resting level following chemical application. Only cells responsive to high-K+ were included for analysis. After the experiment, coverslips were marked with a diamond pen to provide landmarks for alignment with subsequent immunohistofluoresence labeling of the same cells.

Immunocytochemistry

After calcium imaging, DRG cells in the culture dish were fixed in 4% paraformaldehyde followed by 30% sucrose and then incubated with 5% normal serum. They were immunostained with anti-rabbit GRP antibody (1:500; ImmunoStar Inc, Hudson, WI), anti-rat SP antibody (1:500; Millpore, Billerica, MA) and anti-guinea pig VGLUT2 antibody (1:300; Frontier Institute Co Ltd, Japan) at 4 °C over night, followed by incubation with the corresponding secondary antibody conjugated with Alexa Fluor 350 (1:300; Life Technologies Inc, Grand Island, NY), Alexa Fluor 488 (1:500; Life Technologies Inc) and Alexa Fluor 594 (1:500; Life Technologies Inc) for 2 hour. Images were captured using a fluorescence microscope (Nikon Eclipse Ti; Technical Instruments, San Francisco CA). Immunohistofluorescent images were aligned with images captured during calcium imaging to determine the percentages of pruritogen-responsive DRG cells that were triple-labeled for SP, GRP and VGLUT2.

Results

Electrophysiology

Chloroquine-evoked responses were inhibited more by antagonist co-application—A total of 210 chloroquine-responsive superficial dorsal horn neurons (78 NS, 73 WDR, 59 uncharacterized) was tested with spinal application of antagonists. The units were located in the superficial dorsal horn at a mean depth of 80.0 μm +/− 4.8 (SEM) below the surface (Fig 1K). All units had mechanosensitive receptive fields on the ipsilateral hind paw, and responded to id microinjection of chloroquine within the receptive field. Fig. 1A shows an example of the prolonged response of a superficial dorsal horn unit to id chloroquine. The graphs in Fig. 1C–H plot averaged neuronal responses, quantified as the mean firing rate averaged over the preceding 20-sec period. Neuronal activity increased immediately following id injection of chloroquine to a level that was significantly greater than the pre-injection baseline (Fig. 1A–H). The mean chloroquine-evoked response was also significantly greater than saline (vehicle)-evoked responses in all treatment groups (C: vehicle group, p=0.004 vs. pre; D: GRPR antagonist, p=0.0003; E: NK-1 antagonist, p=0.001; F: CNQX, p= 0.026; G: NK-1 +AMPA antagonist, p= 0.0007; H: all 3 antagonists, p= 0.007; unpaired t-test).

To investigate the role of SP, GRP and glutamate as neurotransmitters that excite chloroquine-sensitive spinal neurons, one or more antagonists of these neurotransmitters was superfused over the spinal cord during the chloroquine-evoked response. In Fig. 1C, mean responses are aligned with the onset of vehicle superfusion (black bar) at time 0. Chloroquine-evoked firing usually peaked within the first few seconds post-injection and continued over the ensuing 120 sec, allowing us to test the effect of antagonist superfusion during this period of activity. As a control, vehicle was superfused and shown to have no effect on chloroquine-evoked firing. Fig. 1A shows an individual example, and Fig. 1C shows that chloroquine-evoked firing rate remained significantly above baseline (Fig. 1C, *) during and after spinal superfusion of vehicle. Most units exhibited little or no adaptation in firing rate during the 60-sec period of vehicle superfusion, while one unit exhibited a decline of nearly 70%. Using this as a conservative criterion, 0/23 units tested exhibited a decline by more than 70% in the chloroquine-evoked firing rate relative to that observed prior to the spinal superfusion (Table 1).

Superfusion of the GRP receptor antagonist RC3095 resulted in an overall decline in mean firing rate (Fig. 1D). During spinal superfusion of RC3095, mean responses were still above pre-chloroquine baseline levels, but decreased significantly relative to the chloroquine-evoked response prior to spinal superfusion (Fig. 1D, #), indicating that the GRP receptor antagonist attenuated ongoing activity elicited by chloroquine. Twenty-four percent of units exhibited a decline of 70% or more during superfusion with the GRP antagonist (Table 1).

Chloroquine-evoked firing was also significantly attenuated after the cessation of superfusion with the NK-1 receptor antagonist L733060 (Fig. 1E, #), with 30% of units exhibiting a decline of 70% or more. Chloroquine-evoked firing was also significantly attenuated during and after spinal superfusion with the AMPA/kainate receptor antagonist CNQX (Fig. 1F), with 58% declining by 70% or more. Importantly, combinations of the NK-1 and AMPA/kainate receptor antagonist (Fig. 1G), or all three antagonists (Fig. 1B, H), significantly attenuated chloroquine-evoked firing during and after their spinal superfusion with 71% and 85% exhibiting reductions in firing rate of 70% or greater, respectively (Table 1). Fig. 1B shows an example in which spinal superfusion with all three antagonists completely suppressed chloroquine-evoked firing, followed by recovery of firing.

Fig. 1I summarizes the suppression of id chloroquine-evoked neuronal firing by antagonists. Chloroquine-evoked firing during the 40–60-sec period after onset of spinal superfusion was normalized to the firing rate 20 sec prior to the superfusion, and these normalized values were compared with the firing rate 40–60 sec after superfusion with vehicle. By this analysis, chloroquine-evoked firing was increasingly reduced by the GRPR, NK-1 and AMPA/kainate receptor antagonists applied individually, more strongly by the combination of NK-1 and AMPA/kainate antagonists, and most strongly by co-application all three antagonists.

We additionally determined if antagonists differentially affected dorsal horn neurons based on their subclassification as WDR or NS. Fig. 1J shows effects of the three antagonists on chloroquine-evoked responses of WDR (n=36) and NS units (n=37). The GRPR antagonist significantly reduced chloroquine-evoked firing in NS but not WDR cells. In contrast, the NK-1 antagonist significantly reduced chloroquine-evoked firing in WDR but not NS cells. CNQX significantly reduced chloroquine-evoked firing in both WDR and NS cells. It should be noted that the effects of antagonists were not related to whether the units additionally responded to histamine or not. Of the 75 chloroquine-responsive units that also responded to histamine, 42 were classified as NS cells and 33 as WDR. Of the units that responded to chloroquine but not histamine, 5 were NS and 5 were WDR.

CNQX alone inhibited histamine-evoked responses—The large majority of chloroquine-responsive units (75/ 85) also responded to id injection of histamine. Following id histamine, unit firing increased abruptly; an example is shown in Fig. 2A. The graphs in Fig. 2C–F plot averaged neuronal responses to histamine, quantified as the mean firing rate averaged over the preceding 20-sec period. In each instance, neuronal activity increased immediately following id injection of histamine to a level that was significantly greater than the vehicle (saline) -evoked response (C: vehicle, p=0.018 vs. pre; D: GRPR antagonist, p=0.011; E: NK-1 antagonist, p=0.015; F: CNQX, p= 0.009; unpaired t-test).

The individual example in Fig. 2B shows that spinal superfusion with CNQX completely suppressed histamine-evoked firing, as confirmed for the unit population (Fig. 2F) in which superfusion with CNQX significantly reduced the mean firing rate to the pre-histamine baseline. Overall, CNQX significantly attenuated histamine-evoked firing to a level not different from that elicited by id injection of vehicle (saline) (Fig. 2G, white bar), with 100% of units exhibiting a reduction in firing of more than 70% (Table 1). Both the GRPR and NK-1 antagonists numerically reduced histamine-evoked firing compared to vehicle (Figs. 2D, E, G), with 55% and 33% being reduced by more than 70%, respectively (Table 1). The overall effects, however, were not significantly different from vehicle (Fig. 2C).

Fig. 2H shows effects of the three antagonists on histamine-evoked responses of WDR (n=26) and NS units (n=21). Both NS and WDR unit responses to histamine were significantly reduced by CNQX (Fig. 2H). The GRP antagonist significantly reduced the mean histamine-evoked response of NS but not WDR units (Fig. 2H). The NK1 antagonist failed to reduce histamine-evoked firing in either WDR or NS units.

Lack of effect of antagonists on baseline activity—There was no significant effect of any of the antagonists on the baseline activity of superficial dorsal horn neurons that were subsequently show to respond to pruritogens. We compared the neuronal firing rate averaged over the 1-min period prior to antagonist application with that during a comparable 1-min period following application of the antagonist (before application of pruritic or noxious stimuli). Values prior to, and after administration of each antagonist individually or in combination, are as follows. NK-1 antagonist L-733060: pre-application 0.88 Hz ± 0.23 (SEM), post-antagonist 0.72 Hz ± 0.19 (n=10). GRPR antagonist RC-3095: pre-application 0.54 Hz ± 0.54, post-antagonist: 0.53 Hz ± 0.53 (n=4). CNQX: pre-application 0.53 Hz ± 0.14, post-antagonist 0.64 Hz ± 0.21 (n=22). CNQX + L-733060: pre-application 0.52 Hz ± 0.17, post-antagonists 0.43 Hz ±0.11 (n=26). CNQX + L-733060 + RC-3095: pre-application 0.14 Hz ± 0.06, post-antagonists 0.17 Hz ± 0.06 (n=9).

CNQX reduced firing elicited by AITC and noxious pinch—As a positive control, we wished to show that spinal superfusion with CNQX reduced responses of dorsal horn neurons to noxious stimuli as previously reported [22; 26; 28; 44]. Topical AITC elicited a significant increase in firing of superficial dorsal horn neurons that declined slightly over the ensuing 2 min (Fig. 3A, C). The NK-1 receptor antagonist numerically reduced AITC-evoked firing (Fig. 3D, G). CNQX significantly reduced AITC-evoked firing (Fig. 3E, G; $p<0.05$). Superfusion with both L733060 and CNQX further reduced AITC-evoked firing (Fig. 3B, F) to a level that was significantly different compared to either individual antagonist (Figs. 3F, G; $p<0.001$ vs. vehicle).

We additionally tested the effects these antagonists on spinal unit responses to noxious pinch. We tested chloroquine- and pinch-responsive units, as well as separate populations of NS and WDR units that were isolated by their response to pinch and that were unresponsive to id chloroquine. A total of 22 units was tested with the NK-1 antagonist (7 pinch + chloroquine responsive, 15 pinch but not chloroquine responsive), 39 with the AMPA/

kainite antagonist (24 pinch + chloroquine-responsive; 15 pinch but not chloroquine responsive) and 45 with both antagonists (23 pinch + chloroquine responsive, 22 pinch but not chloroquine responsive). Effects of antagonists on pinch-evoked responses were very similar for chloroquine-responsive and -unresponsive units, and for NS and WDR units, so data were pooled. Noxious pinch elicited a transient robust increase in firing that was not reduced by the NK-1 receptor antagonist (Fig. 4A, B), but was significantly reduced by CNQX, followed 30 min later by recovery (Fig. 4C, D; $p<0.001$). Co-application of the NK-1 antagonist and CNQX did not reduce the pinch-evoked response to any greater extent compared to CNQX alone (Fig. 4E, F; $p<0.001$ vs. pre). Those data are consistent with previous studies [18; 22; 23; 26; 28; 39; 44].

Behavior

Chloroquine-evoked scratching was inhibited more by antagonist co-application—Intrathecal administration of each antagonist significantly attenuated chloroquine-evoked scratching (Fig. 5; $p<0.005$). Co-administration of the NK-1 antagonist and CNQX attenuated chloroquine-evoked scratching to a greater extent than either antagonist individually (Fig. 5). Combined it coapplication of all three antagonists (GRPR, NK-1 and AMPA) reduced scratching to an even greater extent compared to co-application of the NK-1 antagonist and CNQX (Fig. 5).

CNQX reduced histamine-evoked scratching behavior—Intrathecal administration of neither the NK-1 nor the GRPR antagonist attenuated histamine-evoked scratching (Fig. 6). In contrast, the number of histamine-evoked scratch bouts was significantly decreased by CNQX (Fig. 6; $p<0.05$) to a level that did not differ from that elicited by id injection of vehicle (10.1 ± 4.0; $p=0.202$ unpaired t-test) [6].

Cell Imaging

Calcium imaging of DRG cells—Of a total of 898 DRG cells imaged, 8.4% responded to chloroquine and 12.9% responded to histamine, consistent with previous studies [4; 9; 32; 56]. Fig. 7A shows examples of two DRG cells that responded to chloroquine but not histamine.

Immunofluorescent labeling of pruritogen-responsive DRG cells—Following calcium imaging, the DRG cells were fixed and triple-immunostained for SP, GRP and VGLUT2. A total of 597 DRG cells was labeled. Fig. 7B–E show immunostained DRG cells from the calcium imaging experiment shown in Fig. 7A; the two chloroquine-responsive cells in A are indicated by yellow circles. Fig. 7B shows GRP-immunopositive DRG cells (green). Fig. 7C shows DRG cells immunopositive for SP (blue), including one of the chloroquine-responsive cells exhibiting weak immunoreactivity. Fig. 7D shows cells immunopositive for VGLUT2 (red), including one of the chloroquine-reponsive cells. The merged view in Fig. 7E shows cells that were triple-labeled (magenta; arrows) for VGLUT2, SP and GRP.

Overall, 27.1% of all DRG cells examined were immunopositive for SP, 23.8% for GRP, and 79.2% for VGLUT2, consistent with previous studies [9; 12; 15; 20; 42; 49; 52]. Of the DRG cells that responded to chloroquine, 16%, 18% and 80% were immunpositive for SP, GRP and VGLUT2, respectively (Fig. 7F). Of the histamine-responsive cells, 10%, 17.5% and 77% were immunpositive for SP, GRP and VGLUT2, respectively (Fig. 7G). All of the histamine-responsive cells that were immunopositive for either SP or GRP were also immunopositive for VGLUT2 (Fig. 7G). Fig. 7H shows the incidence of immunostaining of DRG cells that responded to both chloroquine and histamine; more cells co-expressed GRP (21%) than SP (7%). Fig. 7I similarly shows the incidence of immunostaining of DRG cells

that responded to chloroquine but not histamine, of which GRP and SP were approximately equally co-expressed (GRP 17%; SP 22%). Fig. 7J shows the incidence of immunostaning of DRG cells that responded to histamine but not chloroquine. Of these cells, SP was predominantly expressed (15%) compared to GRP (4%).

Discussion

The present findings imply that non-histaminergic itch is mediated by the combined intraspinal release of glutamate, SP and GRP from chloroquine-sensitive pruriceptors to activate itch-signaling spinal neurons. In contrast, histamine-mediated itch depends primarily on glutamate, with GRP playing a lesser role in NS neurons. These conclusions are supported by the following: (1) Chloroquine-evoked responses were partially suppressed by each individual antagonists and completely inhibited by co-application; CNQX alone abolished histamine-evoked responses. (2) Behavioral studies provided comparable results. (3) Ten-18% of chloroquine- or histamine-sensitive DRG neurons co-expressed substance P or GRP while ~80% co-expressed VGLUT2. These data support the concept of co-administration of AMPA/kainate, NK-1 and GRPR antagonists to treat antihistamine-resistant chronic itch. The NK-1 antagonist aprepitant shows promise for chronic itch [48]. An AMPA antagonist (Perampanel) is FDA-approved for epilepsy, and a GRPR antagonist shows promise in treating cancer [45]. Future clinical trials are needed to determine the efficacy and side-effects of combined antagonist treatment for chronic itch.

Chloroquine-responsive dorsal horn neurons in itch

MrgprA3-expressing afferents responded to chloroquine, histamine, capsaicin and other noxious stimuli [26], similar to the present and previously-recorded [2] dorsal horn neurons. Chloroquine acts at MrgprA3 expressed in primary afferent C-fibers [31], although we cannot exclude the possibility that chloroquine activates mast cells or keratinocytes to release substances that indirectly affect other afferent fibers. In mice lacking the capsaicin-sensitive ion channel TRPV1, TRPV1 was selectively re-expressed in MrgprA3-expressing DRG neurons [26]. In these animals, id injection of capsaicin, which normally elicits nocifensive wiping behavior, instead elicited hindlimb scratching indicative of itch [26]. This implies that MrgprA3-expressing primary afferent fibers are linked to a “labeled line” itch pathway, regardless of what type of stimulus activates them. Accordingly, we speculate that the chloroquine-responsive dorsal horn neurons recorded presently contribute to neural circuits selectively mediating itch and scratching behavior.

A subpopulation of histamine-sensitive, chloroquine-insensitive dorsal horn neurons, not presently studied, may also participate in histaminergic itch. Most chloroquine-responsive neurons also responded to histamine. Histamine-responsive dorsal horn neurons also responded to SLIGRL-NH2 [2] which acts at protease-activated receptor-2 (PAR-2) and MrgprC11 [33]. Knockout mice lacking MrgprA3 exhibited reduced scratching to chloroquine but not histamine [31], while a more recent study reported that ablation of MrgprA3-expressing sensory neurons attenuated scratching elicited by histamine, chloroquine and other itch mediators [26]. The latter results support the argument that histaminergic itch is mediated by the presently-reported population of chloroquine-sensitive spinal dorsal horn neurons. While this appears inconsistent with the differential effect of the GRPR antagonist to reduce chloroquine- but not histamine-evoked neuronal firing, we speculate that these two itch mediators elicit different patterns of spinal neurotransmitter release (see below). Alternatively, a small subpopulation of histamine-sensitive, chloroquine-insensitive neurons not presently recorded may be critical for glutamate-mediated histaminergic itch transmission. This is consistent with a recent report that histamine and chloroquine respectively activated separate populations of TRPV1- or TRPA1-expressing pruriceptors [40].

Spinal neurotransmitters mediating histaminergic and non-histaminergic itch

Chloroquine-evoked scratching and spinal neuronal firing was reduced by individually-applied GRPR, NK-1 (except for NS neurons) or AMPA antagonists, and was abolished by their co-application. One interpretation is that GRP, SP and glutamate are released from intraspinal terminals of chloroquine-sensitive pruriceptors to excite itch-signaling dorsal horn neurons (Fig. 8A). Consistent with this, chloroquine-sensitive DRG cells co-expressed GRP, SP and VGLUT2. In contrast, CNQX inhibited histamine-evoked neuronal responses, with the NK-1 and GRPR antagonists having lesser or no effect, implicating glutamate as the primary spinal neurotransmitter for histaminergic itch.

Most chloroquine-responsive neurons also responded to histamine. It is novel that responses of the same neuron to chloroquine and histamine were pharmacologically distinct. Chloroquine- and histamine-sensitive pruriceptors are functionally distinct subpopulations and might release different neurotransmitters [40]. Chloroquine excitation of NS neurons was reduced by NK-1 and AMPA antagonists (Fig. 1J), implying input primarily from pruriceptors that release SP and glutamate, i.e., chloroquine-sensitive histamine-insensitive (CQ+HIS−) and chloroquine-insensitive histamine-sensitive (CQ-HIS+) DRG cells (Fig. 6). Chloroquine excitation of WDR neurons was reduced by GRPR and AMPA antagonists, implying input from pruriceptors that release GRP (i.e., CQ+HIS+ and CQ+HIS−; Fig. 8A). Histamine excitation of both WDR and NS cells was inhibited by CNQX, implying input from histamine-sensitive pruriceptors (i.e., CQ-HIS+ and CQ+HIS+). That the GRPR antagonist partly inhibited responses of NS (but not WDR) neurons (Fig. 2) implies input from chloroquine- and histamine-sensitive (CQ+HIS+) DRG cells containing GRP (Fig. 8A). The lack of effect of the NK-1 antagonist on histamine-evoked responses implies that NS and WDR neurons either do not receive input from cells that co-express SP (CQ-HIS+), or that they do but SP participates exclusively in a peripheral role such as the axon reflex induced by histamine [43].

Alternatively, different firing patterns of primary afferents might account for the pharmacologically distinct effects of chloroquine and histamine. MrgprA3-expressing sensory neurons exhibited distinct firing patterns (bursting vs. steady state) to different itch mediators [27] that may determine if glutamate and/or neuropeptide is released from the presynaptic terminal.

Another possibility is that NS and WDR neurons receive inputs from different populations of interneurons using different neurotransmitters (Fig. 8B). Natriuretic polypeptide b (Nppb) released from central terminals of primary afferents excites GRPR-expressing spinal interneurons to account for all pruritogen-evoked scratching behavior in mice [38]. Our data are not inconsistent with this, since SP, GRP and glutamate are expressed in spinal interneurons [51; 54]. It is currently unknown if id chloroquine and histamine excite separate interneuronal populations that release different neurotransmitters.

We confirmed expression of SP in a subpopulation of chloroquine-sensitive DRG cells [9]. However, most MrgprA3-expressing sensory neurons coexpressed calcitonin gene-related peptide (CGRP) and IB-4, but not SP [27]. Chloroquine-evoked scratching was reduced by ~70% in mice lacking MrgprA3 [27]; residual scratching may be mediated by SP-expressing, MrgprA3-negative neurons. SP is presumably released by a small subpopulation of non-histaminergic pruriceptors [47]. In contrast, NK-1 antagonists failed to inhibit histamine-evoked scratching or neuronal firing, even though SP was expressed in some histamine-sensitive chloroquine-insensitive DRG neurons. That all neuropeptide-expressing histamine-sensitive DRG neurons co-expressed VGLUT2 suggests that glutamate is the main neurotransmitter released by histamine-sensitive pruriceptors. Consistent with this,

electrical stimulation of dorsal roots evoked responses in histamine-sensitive spinal neurons that were abolished by CNQX [29].

We confirm that GRP was coexpressed by some chloroquine-sensitive neurons [9]. Chloroquine-evoked scratching and neuronal firing were reduced by the GRPR antagonist, which did not affect histamine-evoked scratching (but reduced responses of NS neurons). Activity in the unaffected WDR neurons may have compensated for inhibition of NS neurons to account for the lack of GRPR antagonist effect on scratching.

The specificity of the GRP antibody and presence of GRP within DRG neurons has been questioned [36], although GRP mRNA and protein were reported to be expressed in substantial populations of primary sensory neurons [9; 10; 25; 30; 32; 34–36; 49; 52]. However, GRP staining of the rhizotomized spinal cord revealed that most GRP is synthesized locally [25]. Thus, GRP may be released primarily by spinal interneurons (Fig. 8B), rather than (or in addition to) pruriceptive afferents. In this scenario, spinal neurons sensitive to GRP may be synaptically excited by glutamate, rather than GRP, released from primary afferents [29].

Pruritogen-responsive DRG neurons expressed VGLUT2, implying that pruriceptors release glutamate. Knockout mice lacking VGLUT2 in primary afferents exhibited reduced nociception and enhanced scratching, explained by decreased release of glutamate from nociceptors and reduced excitation of inhibitory spinal interneurons, thereby disinhibiting itch-signaling neurons [30; 36; 41]. Fig. 8C shows a balance of excitatory pruriceptive and inhibitory interneuronal inputs onto itch-signaling spinal neurons. We speculate that knockout of VGLUT2 more strongly reduces nociceptive than pruriceptive afferent drive, shifting the balance toward enhanced itch transmission. Antagonism of spinal AMPA receptors more strongly blocks direct and indirect (via excitatory interneurons) glutamatergic pruriceptive input, shifting the balance toward reduced itch transmission.

Spinal nociceptive transmission

Noxious thermal, mechanical and chemical stimuli evoked spinal release of SP [24] which elicited prolonged excitatory postsynaptic potentials (EPSPs) [14; 53] that were inhibited by an NK-1 antagonist [18]. Spinal neurons exhibiting prolonged mechanically-evoked EPSPs received more synaptic contacts by SP-immunoreactive varicosities [19]. Spinal neuronal responses to formalin and capsaicin were inhibited by NK-1 antagonists [14; 23; 39]. The NK-1 antagonist presently inhibited dorsal neuronal responses to AITC, but not to noxious pinch, consistent with previous studies [18; 23; 37; 39]. Mice with genetic ablation of non-peptidergic MrgprD-expressing sensory neurons exhibited reduced behavioral responses to noxious mechanical but not thermal stimuli, suggesting a role for non-peptidergic sensory neurons in mechanical nociception [13].

Glutamate is generally associated with spinal nociceptive transmission. Spinal neuronal responses to pinch and AITC were presently reduced or blocked by CNQX, consistent with previous studies [22; 26; 28; 44]. Our present results indicate that spinal pathways signaling itch and pain likely share glutamate and SP as excitatory neurotransmitters. This is consistent with previous studies showing that most pruritogen-sensitive spinal neurons also respond to algogens, suggesting that the central nervous system discriminates between itch and pain based on input from partially overlapping subpopulations of itch- and pain-signaling neurons [3; 5; 8; 16; 17].

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References

1. Abila B, Ezeamuzie IC, Igbigbi PS, Ambakederemo AW, Asomugha L. Effects of two antihistamines on chloroquine and histamine induced weal and flare in healthy African volunteers. African journal of medicine and medical sciences. 1994; 23(2):139–142. [PubMed: 7625301]
2. Akiyama, T.; Tominaga, M.; Carstens, MI.; Carstens, E. Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience; 2012. Bombesin-responsive superficial dorsal horn neurons non-selectively respond to pruritic and noxious stimuli in the mouse. Program No 37511/PP82012online
3. Akiyama T, Carstens MI, Carstens E. Excitation of mouse superficial dorsal horn neurons by histamine and/or PAR-2 agonist: potential role in itch. Journal of neurophysiology. 2009; 102(4): 2176–2183. [PubMed: 19625538]
4. Akiyama T, Carstens MI, Carstens E. Enhanced scratching evoked by PAR-2 agonist and 5-HT but not histamine in a mouse model of chronic dry skin itch. Pain. 2010; 151(2):378–383. [PubMed: 20709455]
5. Akiyama T, Carstens MI, Carstens E. Facial injections of pruritogens and algogens excite partly overlapping populations of primary and second-order trigeminal neurons in mice. Journal of neurophysiology. 2010; 104(5):2442–2450. [PubMed: 20739601]
6. Akiyama T, Carstens MI, Ikoma A, Cevikbas F, Steinhoff M, Carstens E. Mouse model of touch-evoked itch (alloknesis). The Journal of investigative dermatology. 2012; 132(7):1886–1891. [PubMed: 22418875]
7. Akiyama T, Iodi Carstens M, Carstens E. Transmitters and pathways mediating inhibition of spinal itch-signaling neurons by scratching and other counterstimuli. PloS one. 2011; 6(7):e22665. [PubMed: 21818363]
8. Akiyama T, Merrill AW, Carstens MI, Carstens E. Activation of superficial dorsal horn neurons in the mouse by a PAR-2 agonist and 5-HT: potential role in itch. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009; 29(20):6691–6699. [PubMed: 19458238]
9. Akiyama T, Tominaga M, Davoodi A, Nagamine M, Blansit K, Horwitz A, Iodi Carstens M, Carstens E. Roles for substance P and gastrin releasing peptide as neurotransmitters released by primary afferent pruriceptors. Journal of neurophysiology. 2013; 109(3):742–748. [PubMed: 23155177]
10. Alemi F, Kwon E, Poole DP, Lieu T, Lyo V, Cattaruzza F, Cevikbas F, Steinhoff M, Nassini R, Materazzi S, Guerrero-Alba R, Valdez-Morales E, Cottrell GS, Schoonjans K, Geppetti P, Vanner SJ, Bunnett NW, Corvera CU. The TGR5 receptor mediates bile acid-induced itch and analgesia. The Journal of clinical investigation. 2013; 123(4):1513–1530. [PubMed: 23524965]
11. Carstens EE, Carstens MI, Simons CT, Jinks SL. Dorsal horn neurons expressing NK-1 receptors mediate scratching in rats. Neuroreport. 2010; 21(4):303–308. [PubMed: 20125052]
12. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science. 2000; 288(5464):306–313. [PubMed: 10764638]
13. Cavanaugh D, Lee H, Lo L, Shields S, Zylka M, Basbaum A, Anderson D. Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106(22):9075–9080. [PubMed: 19451647]
14. Chapman V, Dickenson A. The effect of intrathecal administration of RP67580, a potent neurokinin 1 antagonist on nociceptive transmission in the rat spinal cord. Neuroscience letters. 1993; 157(2):149–152. [PubMed: 8233046]

15. Chen CL, Broom DC, Liu Y, de Nooij JC, Li Z, Cen C, Samad OA, Jessell TM, Woolf CJ, Ma Q. Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron. 2006; 49(3):365–377. [PubMed: 16446141]

16. Davidson S, Zhang X, Khasabov SG, Moser HR, Honda CN, Simone DA, Giesler GJ Jr. Pruriceptive spinothalamic tract neurons: physiological properties and projection targets in the primate. Journal of neurophysiology. 2012; 108(6):1711–1723. [PubMed: 22723676]

17. Davidson S, Zhang X, Yoon CH, Khasabov SG, Simone DA, Giesler GJ Jr. The itch-producing agents histamine and cowhage activate separate populations of primate spinothalamic tract neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2007; 27(37):10007–10014. [PubMed: 17855615]

18. De Koninck Y, Henry JL. Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons in vivo by noxious stimulation. Proceedings of the National Academy of Sciences of the United States of America. 1991; 88(24):11344–11348. [PubMed: 1722327]

19. De Koninck Y, Ribeiro-da-Silva A, Henry J, Cuello A. Spinal neurons exhibiting a specific nociceptive response receive abundant substance P-containing synaptic contacts. Proceedings of the National Academy of Sciences of the United States of America. 1992; 89(11):5073–5077. [PubMed: 1375757]

20. Dirajlal S, Pauers LE, Stucky CL. Differential response properties of IB(4)-positive and -negative unmyelinated sensory neurons to protons and capsaicin. Journal of neurophysiology. 2003; 89(1): 513–524. [PubMed: 12522198]

21. Dong X, Han S, Zylka MJ, Simon MI, Anderson DJ. A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. Cell. 2001; 106(5):619–632. [PubMed: 11551509]

22. Dougherty P, Palecek J, Paleckova V, Sorkin L, Willis W. The role of NMDA and non-NMDA excitatory amino acid receptors in the excitation of primate spinothalamic tract neurons by mechanical, chemical, thermal, and electrical stimuli. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1992; 12(8):3025–3041. [PubMed: 1353793]

23. Dougherty P, Palecek J, Palecková V, Willis W. Neurokinin 1 and 2 antagonists attenuate the responses and NK1 antagonists prevent the sensitization of primate spinothalamic tract neurons after intradermal capsaicin. Journal of neurophysiology. 1994; 72(4):1464–1475. [PubMed: 7823080]

24. Duggan AW, Hendry IA, Morton CR, Hutchison WD, Zhao ZQ. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. Brain Res. 1988; 451(1–2):261–273. [PubMed: 2472190]

25. Fleming M, Ramos D, Han S, Zhao J, Son Y-J, Luo W. The majority of dorsal spinal cord gastrin releasing peptide is synthesized locally whereas neuromedin B is highly expressed in pain- and itch-sensing somatosensory neurons. Molecular pain. 2012; 8:52. [PubMed: 22776446]

26. Furue H, Narikawa K, Kumamoto E, Yoshimura M. Responsiveness of rat substantia gelatinosa neurones to mechanical but not thermal stimuli revealed by in vivo patch-clamp recording. The Journal of physiology. 1999; 521(2):529–535. [PubMed: 10581321]

27. Han L, Ma C, Liu Q, Weng HJ, Cui Y, Tang Z, Kim Y, Nie H, Qu L, Patel KN, Li Z, McNeil B, He S, Guan Y, Xiao B, Lamotte RH, Dong X. A subpopulation of nociceptors specifically linked to itch. Nature neuroscience. 2012; 16(2):174–182.

28. King A, Lopez-Garcia J. Excitatory amino acid receptor-mediated neurotransmission from cutaneous afferents in rat dorsal horn in vitro. The Journal of physiology. 1993; 472:443–457. [PubMed: 7908326]

29. Koga K, Chen T, Li XY, Descalzi G, Ling J, Gu J, Zhuo M. Glutamate acts as a neurotransmitter for gastrin releasing peptide-sensitive and insensitive itch-related synaptic transmission in mammalian spinal cord. Molecular pain. 2011; 7:47. [PubMed: 21699733]

30. Lagerstrom MC, Rogoz K, Abrahamsen B, Persson E, Reinius B, Nordenankar K, Olund C, Smith C, Mendez JA, Chen ZF, Wood JN, Wallen-Mackenzie A, Kullander K. VGLUT2-dependent sensory neurons in the TRPV1 population regulate pain and itch. Neuron. 2010; 68(3):529–542. [PubMed: 21040852]

31. Liu Q, Sikand P, Ma C, Tang Z, Han L, Li Z, Sun S, LaMotte R, Dong X. Mechanisms of itch evoked by β-alanine. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2012; 32(42):14532–14537. [PubMed: 23077038]
32. Liu Q, Tang Z, Surdenikova L, Kim S, Patel KN, Kim A, Ru F, Guan Y, Weng HJ, Geng Y, Undem BJ, Kollarik M, Chen ZF, Anderson DJ, Dong X. Sensory neuron-specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus. Cell. 2009; 139(7):1353–1365. [PubMed: 20004959]
33. Liu Q, Weng HJ, Patel KN, Tang Z, Bai H, Steinhoff M, Dong X. The distinct roles of two GPCRs, MrgprC11 and PAR2, in itch and hyperalgesia. Science signaling. 2011; 4(181):ra45. [PubMed: 21775281]
34. Liu T, Berta T, Xu ZZ, Park CK, Zhang L, Lu N, Liu Q, Liu Y, Gao YJ, Liu YC, Ma Q, Dong X, Ji RR. TLR3 deficiency impairs spinal cord synaptic transmission, central sensitization, and pruritus in mice. The Journal of clinical investigation. 2012; 122(6):2195–2207. [PubMed: 22565312]
35. Liu T, Xu ZZ, Park CK, Berta T, Ji RR. Toll-like receptor 7 mediates pruritus. Nature neuroscience. 2010; 13(12):1460–1462.
36. Liu Y, Abdel Samad O, Zhang L, Duan B, Tong Q, Lopes C, Ji RR, Lowell BB, Ma Q. VGLUT2-dependent glutamate release from nociceptors is required to sense pain and suppress itch. Neuron. 2010; 68(3):543–556. [PubMed: 21040853]
37. Mazario J, Basbaum AI. Contribution of substance P and neurokinin A to the differential injury-induced thermal and mechanical responsiveness of lamina I and V neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2007; 27(4):762–770. [PubMed: 17251415]
38. Mishra SK, Hoon MA. The cells and circuitry for itch responses in mice. Science. 2013; 340(6135):968–971. [PubMed: 23704570]
39. Rees H, Sluka KA, Urban L, Walpole CJ, Willis WD. The effects of SDZ NKT 343, a potent NK1 receptor antagonist, on cutaneous responses of primate spinothalamic tract neurones sensitized by intradermal capsaicin injection. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale. 1998; 121(3):355–358.
40. Roberson DP, Gudes S, Sprague JM, Patoski HA, Robson VK, Blasl F, Duan B, Oh SB, Bean BP, Ma Q, Binshtok AM, Woolf CJ. Activity-dependent silencing reveals functionally distinct itch-generating sensory neurons. Nature neuroscience. 2013; 16(7):910–918.
41. Ross SE, Mardinly AR, McCord AE, Zurawski J, Cohen S, Jung C, Hu L, Mok SI, Shah A, Savner EM, Tolias C, Corfas R, Chen S, Inquimbert P, Xu Y, McInnes RR, Rice FL, Corfas G, Ma Q, Woolf CJ, Greenberg ME. Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. Neuron. 2010; 65(6):886–898. [PubMed: 20346763]
42. Scherrer G, Low S, Wang X, Zhang J, Yamanaka H, Urban R, Solorzano C, Harper B, Hnasko T, Edwards R, Basbaum A. VGLUT2 expression in primary afferent neurons is essential for normal acute pain and injury-induced heat hypersensitivity. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107(51):22296–22301. [PubMed: 21135246]
43. Schmelz M, Michael K, Weidner C, Schmidt R, Torebjork HE, Handwerker HO. Which nerve fibers mediate the axon reflex flare in human skin? Neuroreport. 2000; 11(3):645–648. [PubMed: 10718329]
44. Schneider S, Perl E. Synaptic mediation from cutaneous mechanical nociceptors. Journal of neurophysiology. 1994; 72(2):612–621. [PubMed: 7983523]
45. Schwartsmann G, DiLeone LP, Horowitz M, Schunemann D, Cancella A, Pereira AS, Richter M, Souza F, da Rocha AB, Souza FH, Pohlmann P, De Nucci G. A phase I trial of the bombesin/gastrin-releasing peptide (BN/GRP) antagonist RC3095 in patients with advanced solid malignancies. Investigational new drugs. 2006; 24(5):403–412. [PubMed: 16505950]
46. Sikand P, Dong X, LaMotte RH. BAM8–22 peptide produces itch and nociceptive sensations in humans independent of histamine release. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011; 31(20):7563–7567. [PubMed: 21593341]

47. Song X, Zhao Z. Interaction between substance P and excitatory amino acid receptors in modulation of nociceptive responses of cat spinal dorsal horn neurons. Neuroscience letters. 1994; 168(1–2):49–52. [PubMed: 7518066]
48. Stander S, Siepmann D, Herrgott I, Sunderkotter C, Luger TA. Targeting the neurokinin receptor 1 with aprepitant: a novel antipruritic strategy. PloS one. 2010; 5(6):e10968. [PubMed: 20532044]
49. Sun YG, Chen ZF. A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature. 2007; 448(7154):700–703. [PubMed: 17653196]
50. Sun YG, Zhao ZQ, Meng XL, Yin J, Liu XY, Chen ZF. Cellular basis of itch sensation. Science. 2009; 325(5947):1531–1534. [PubMed: 19661382]
51. Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP, Maxwell DJ. The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. The European journal of neuroscience. 2003; 17(1):13–27. [PubMed: 12534965]
52. Tominaga M, Ogawa H, Takamori K. Histological characterization of cutaneous nerve fibers containing gastrin-releasing peptide in NC/Nga mice: an atopic dermatitis model. The Journal of investigative dermatology. 2009; 129(12):2901–2905. [PubMed: 19571818]
53. Urbán L, Randić M. Slow excitatory transmission in rat dorsal horn: possible mediation by peptides. Brain research. 1984; 290(2):336–341. [PubMed: 6198043]
54. Wang X, Zhang J, Eberhart D, Urban R, Meda K, Solorzano C, Yamanaka H, Rice D, Basbaum AI. Excitatory superficial dorsal horn interneurons are functionally heterogeneous and required for the full behavioral expression of pain and itch. Neuron. 2013; 78(2):312–324. [PubMed: 23622066]
55. Weisshaar E, Apfelbacher C, Jager G, Zimmermann E, Bruckner T, Diepgen TL, Gollnick H. Pruritus as a leading symptom: clinical characteristics and quality of life in German and Ugandan patients. The British journal of dermatology. 2006; 155(5):957–964. [PubMed: 17034525]
56. Wilson SR, Gerhold KA, Bifolck-Fisher A, Liu Q, Patel KN, Dong X, Bautista DM. TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. Nature neuroscience. 2011; 14(5):595–602.

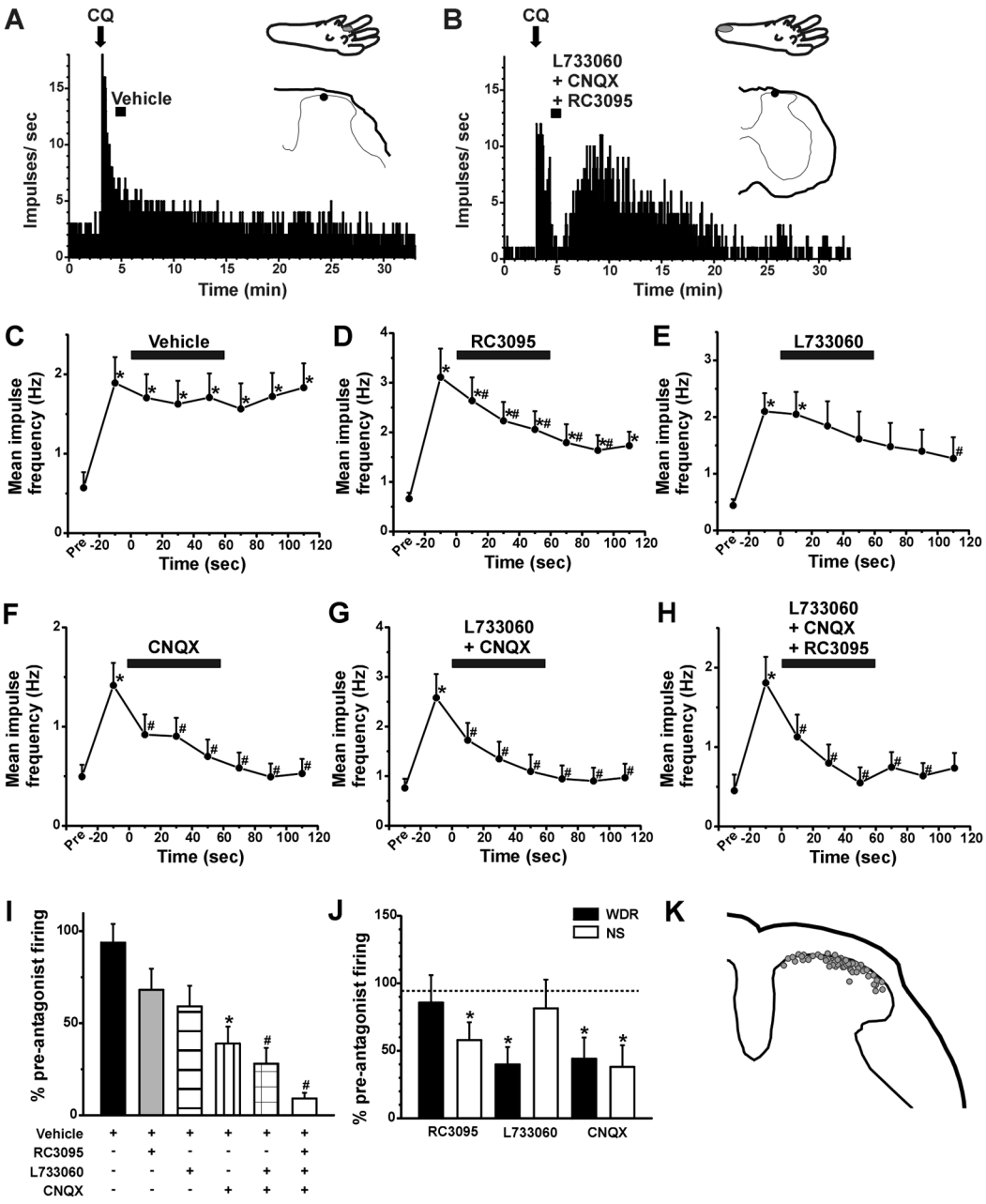

Fig. 1.

Combined effects of NK-1, GRP, and AMPA/kainate receptor antagonists on id chloroquine-evoked activity of superficial dorsal horn neurons. A: Individual example (vehicle control). Peristimulus-time histogram (PSTH; bins: 1s) shows response of superficial dorsal horn neuron to id chloroquine (at arrow). Chloroquine was injected id in the hindpaw receptive field area shown in the upper inset. Lower inset shows superficial recording site (dot). Vehicle (saline) was superfused over spinal cord (bar) during the initial part of the neuronal response to chloroquine. B: As in A for a combination of NK-1, AMPA/kainate and GRP receptor antagonists (PSTH for different superficial dorsal horn unit than in A). Chloroquine was injected id (at arrow) in hindpaw receptive field (upper inset). Shortly thereafter, a combination of NK-1, AMPA/kainate and GRP receptor antagonists

was superfused over the spinal cord (bar), resulting in marked suppression of chloroquine-evoked firing. C: Vehicle control. Graph plots mean responses (impulse frequency averaged over 20 sec) of superficial dorsal horn units before (Pre-) and after id chloroquine. Graphs are aligned at time 0 with the onset of spinal superfusion (horizontal bar above graph). Time point -20 represents mean response following id chloroquine, measured 20 sec prior to onset of spinal superfusion. Error bars: SEM. *: significantly different compared to Pre ($p < 0.05$; Bonferroni-test following one way repeated-measures ANOVA). D: GRP receptor antagonist RC-3095 (20 μM). Format and symbols as in C. #: significantly different compared to chloroquine-evoked response prior to spinal superfusion ($p < 0.05$; Bonferroni-test following one way repeated-measures ANOVA). E: NK-1 receptor antagonist L-733060 (200 μM). Format and symbols as in C. F: Glutamate receptor antagonist CNQX (100 μM). Format and symbols as in C. G: Combined NK-1 and AMPA/kainate receptor antagonists. Format and symbols as in C. H: Combined NK-1, AMPA/kainate and GRP receptor antagonists. Format and symbols as in C. I: Summary of antagonist effects. Bar graph plots mean responses during the 40–60 sec period of spinal superfusion of vehicle (black bar), GRP receptor antagonist RC-3095 (gray bar), NK-1 antagonist L-733060 (horizontal striped bar), AMPA/kainate receptor antagonist CNQX (vertical striped bar), L-733060 + CNQX (checkered bar) or RC-3095 + L-733060 + CNQX (white bar). All responses are normalized to the firing rate 20 sec prior to vehicle or antagonist application. Error bars: SEM. *, significantly different from vehicle group, $p < 0.05$, One-way ANOVA, Bonferroni post-test. #: significantly different from vehicle group, $p < 0.01$, One-way ANOVA, Bonferroni post-test, n=23–35/group. J: Summary of antagonist effects for NS and WDR cells. Bar graph plots mean responses of WDR (black bars) and NS units (white bars) during the 40–60 sec period of spinal superfusion of, from left to right, RC-3095 (GRP receptor antagonist), L-733060 (NK-1 antagonist) or CNQX (AMPA/kainate receptor antagonist). All responses are normalized to the firing rate 20 sec prior to vehicle or antagonist application. Dashed line: mean response during 40–60 sec period of spinal superfusion of vehicle. Error bars: SEM. *, significantly different from vehicle group, $p < 0.05$, unpaired t-test., n=9–14/group. K: Recording sites compiled on representative lumbar spinal cord section.

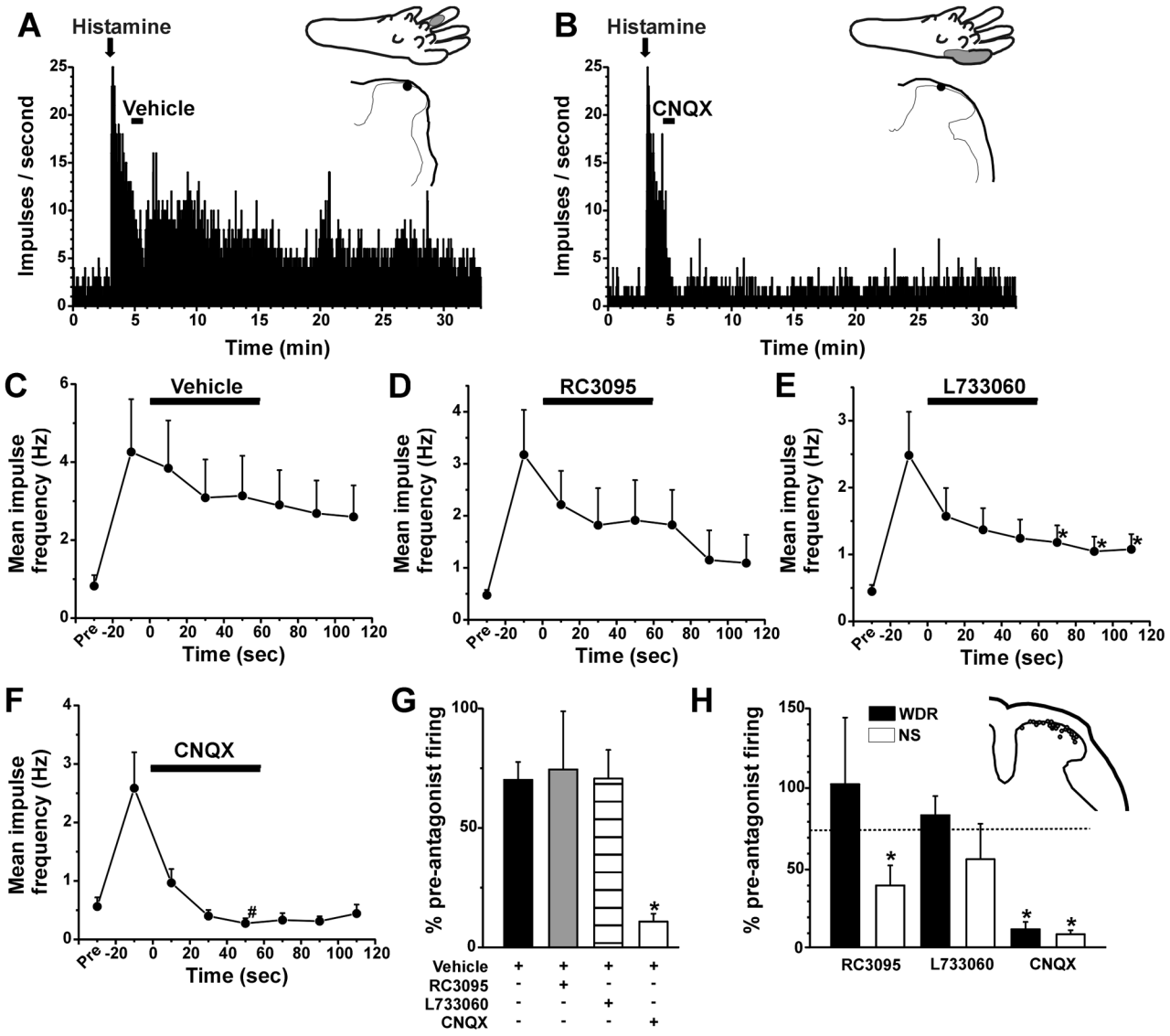

Fig. 2.

Effects of NK-1, GRP, or AMPA/kainate receptor antagonist on id histamine-evoked activity of superficial dorsal horn neurons. A: Individual example (vehicle control). PSTH (bins: 1s) shows response of superficial dorsal horn neuron to histamine (at arrow) injected id in the hindpaw receptive field (upper inset). Lower inset shows superficial recording site (dot). Vehicle (saline) was superfused over spinal cord (bar) during the initial part of the neuronal response to histamine. B: As in A for CNQX (PSTH for a different neuron than in A). Histamine was injected id (at arrow) in hindpaw receptive field (upper inset). Shortly thereafter, CNQX was superfused over the spinal cord (bar), resulting in marked suppression of histamine-evoked firing. C: Vehicle controls. Graph plots mean responses (impulse frequency averaged over 20 sec) of superficial dorsal horn units before (Pre-) and after id histamine (format as in Fig. 4C). D: as in C for spinal superfusion of GRP antagonist RC3095. E: As in C for L-733060. *: significantly different compared to the pre ($p < 0.05$; Bonferroni-test following one way repeated-measures ANOVA). F: As in C for CNQX. #: significantly different compared to histamine-evoked response prior to spinal superfusion ($p < 0.05$; Bonferroni-test following one way repeated-measures ANOVA). G: Summary of

antagonist effects. Bar graph plots, from left to right, the mean histamine-evoked responses during the 40–60 sec period of spinal superfusion of vehicle (black bar), GRPR antagonist, RC-3095 (gray bar), NK1R antagonist, L-733060 (horizontal striped bar) or AMPA/kainate receptor antagonist, CNQX (white bar). Responses are normalized to the firing rate before the antagonist application. Error bars: SEM. *, significantly different from vehicle group, $p < 0.05$, One-way ANOVA, Bonferroni post-test, n=14–18/group. H: Summary of antagonist effects for NS and WDR cells. Bar graph plots mean responses of WDR (black bars) and NS units (white bars) during the 40–60 sec period of spinal superfusion of, from left to right, RC-3095 (GRP receptor antagonist), L-733060 (NK-1 antagonist) or CNQX (AMPA/kainate receptor antagonist). Dashed line indicates mean responses during the 40–60 sec period of spinal superfusion of vehicle. All responses are normalized to the firing rate 20 sec prior to vehicle or antagonist application. Error bars: SEM. *, significantly different from vehicle group, $p < 0.05$, unpaired t-test., n=6–10/group. Inset: Recording sites.

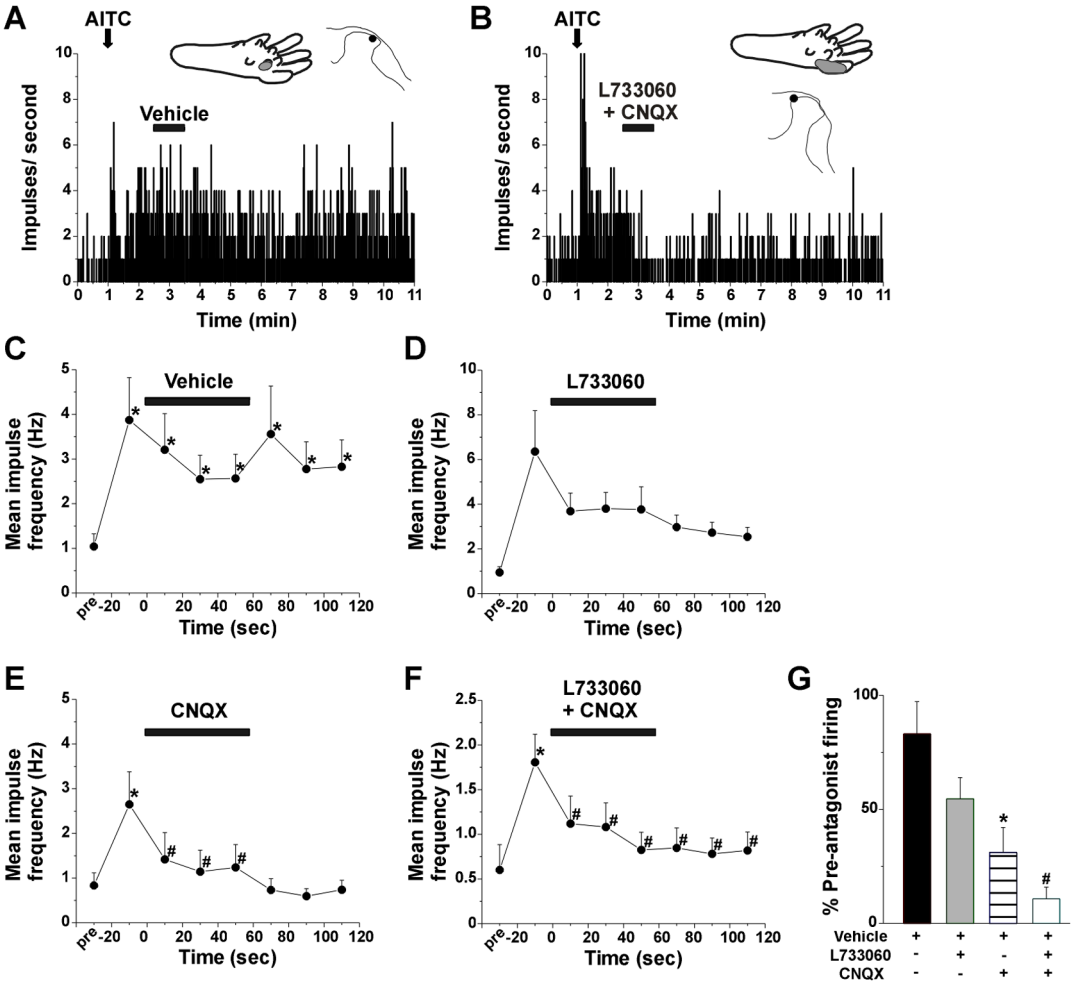

Fig. 3.

Combined effects of NK-1 and AMPA/kainate receptor antagonists on topical allyl isothiocyanate (AITC)-evoked activity of superficial dorsal horn neurons. A: Individual example shows PSTH of superficial dorsal horn unit's response to topical application of AITC to hindpaw receptive field (upper left inset). Vehicle (saline) was superfused during AITC-evoked response. B: As in A for different superficial dorsal horn neuron, with spinal superfusion of combined NK-1 (L733060) and AMPA/kainate (CNQX) receptor antagonists. C: Vehicle control. Graph plots mean responses (impulse frequency averaged over 20 sec) of superficial dorsal horn units before (Pre-) and after topical AITC (format as in Fig. 4C). Error bars: SEM. *: significantly different compared to Pre ($p < 0.05$; Bonferroni-test following one way repeated-measures ANOVA). D: as in C for spinal superfusion of NK-1 receptor antagonist. E: as in C for spinal superfusion of AMPA/kainate antagonist. #: significantly different compared to AITC-evoked response prior to spinal superfusion ($p < 0.05$; Bonferroni-test following one way repeated-measures ANOVA). F: as in C for spinal superfusion of combined NK-1 and AMPA/kainate antagonists. #: significantly different compared to AITC-evoked response prior to spinal superfusion ($p < 0.05$; Bonferroni-test following one way repeated-measures ANOVA). G: Summary of antagonist effects. Bar graph plots the mean AITC-evoked responses during the 40–60 sec period of spinal superfusion of vehicle (black bar), NK-1 receptor antagonist (gray bar), AMPA/kainate receptor antagonist (horizontal striped bar), or combined NK-1 and AMPA/kainate receptor antagonists (white bar). Responses are normalized to the firing rate before

the antagonist application. Error bars: SEM. *, significantly different from vehicle group, $p < 0.05$, One-way ANOVA, Bonferroni post-test #, significantly different from vehicle group, $p < 0.001$, One-way ANOVA, Bonferroni post-test, n=9–26/group.

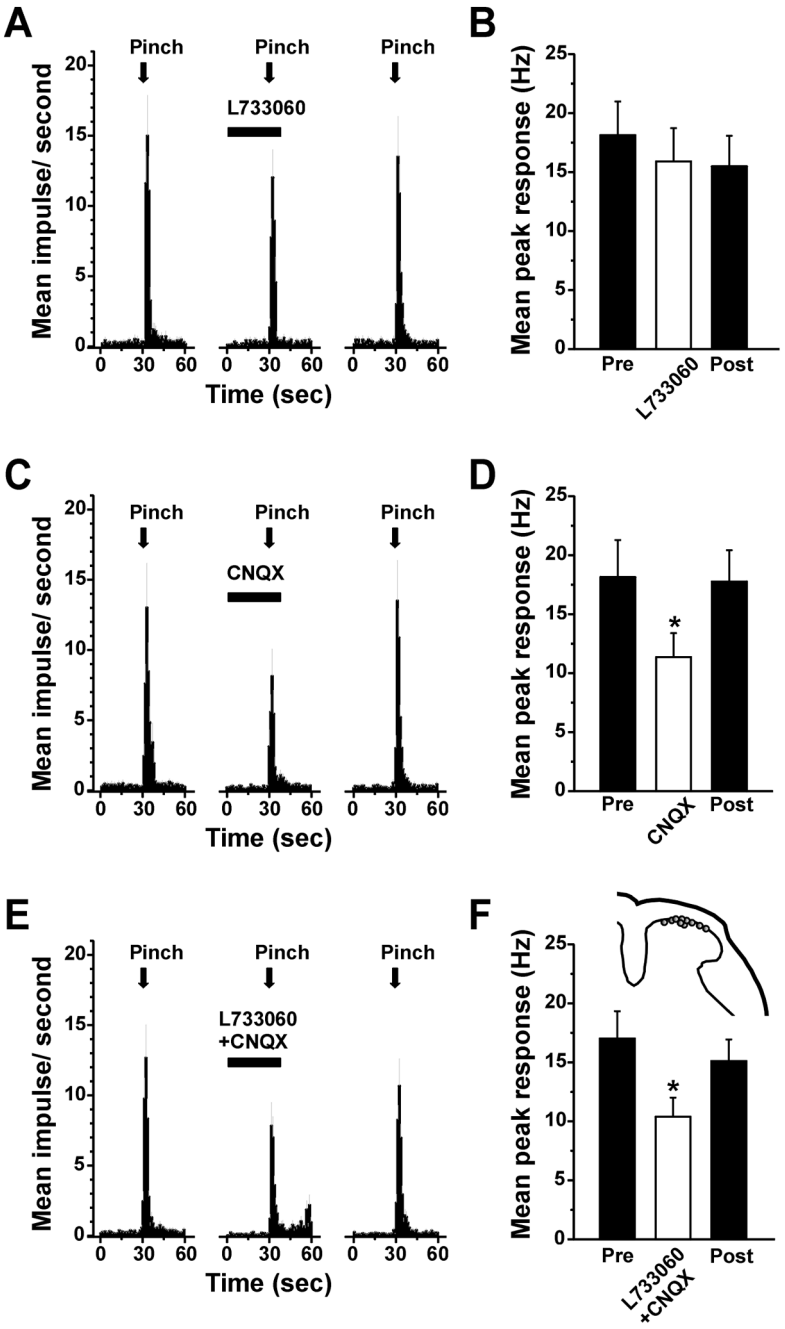

Fig. 4.

Effects of NK-1 antagonist, CNQX, and both, on noxious pinch-evoked activity of superficial dorsal horn neurons. A: Average responses to pinch. Averaged PSTHs (bins: 1s) show, from left to right, the mean pinch-evoked responses before, during, and after the spinal superfusion of NK-1 receptor antagonist L733060. Error bars: SEM. *n*=22/group. B: Summary of antagonist effects. Bar graph plots, from left to right, the mean pinch-evoked peak responses before (black bar), during (white bar), and after (black bar) the spinal superfusion of NK-1 receptor antagonist. *n*=22/group. C: As in A for CNQX. Error bars: SEM. *n*=39/group. D: As in B for CNQX. Error bars: SEM. *, significantly different from pre group, $p < 0.001$, One-way repeated measures ANOVA, Bonferroni post-test, *n*=39/

group. E: As in A for combined NK-1 antagonist and CNQX. Error bars: SEM. *n*=54/group. F: As in B for combined NK-1 antagonist and CNQX. Error bars: SEM. *, significantly different from pre group, $p < 0.001$, One-way repeated measures ANOVA, Bonferroni post-test, *n*=45/group. Inset above shows recording sites for units identified using pinch search stimulus.

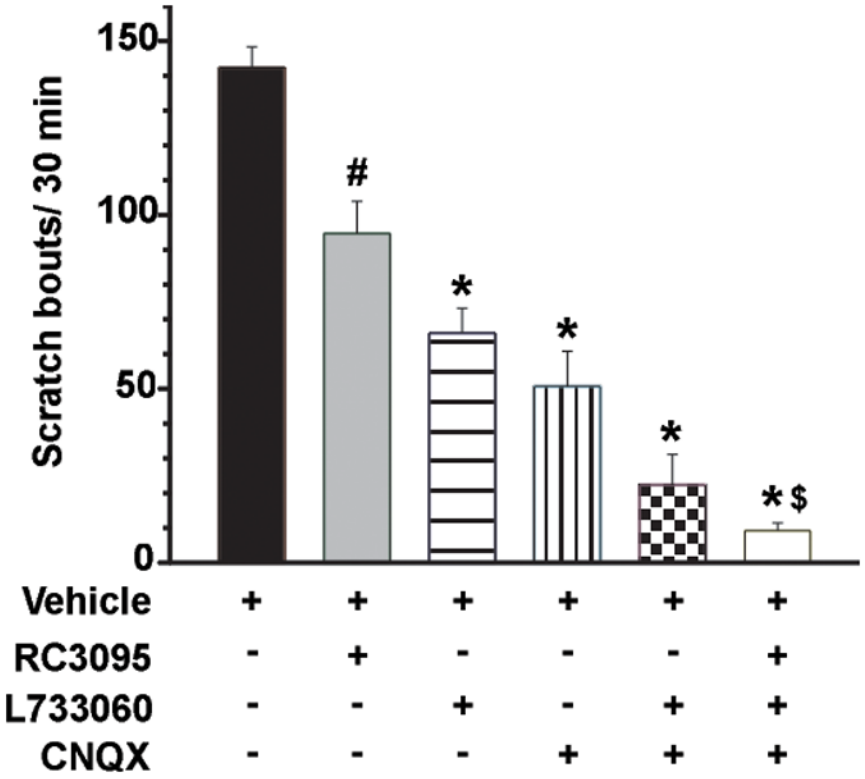

Fig. 5.

Combined effects of of intrathecally-administered NK-1, GRP, and AMPA/kainate receptor antagonists on chloroquine-elicited scratching. A. Bar graph plots, from left to right, the mean number of scratch bouts/30 min elicited by id chloroquine 5 min after prior intrathecal injection of vehicle (saline; black bar), the GRP receptor antagonist, RC-3095 (0.3 nmol; gray bar), the NK-1 antagonist L-733060 (22.7 nmol; horizontal striped bar), the AMPA/kainate receptor antagonist, CNQX (20 nmol; vertical striped bar), L-733060 + CNQX (checkered bar) or RC-3095 + L-733060 + CNQX (white bar). Error bars: SEM. *, significantly different from vehicle group, $p < 0.001$, One-way ANOVA, Bonferroni post-test, n=6/group. #, significantly different from vehicle group, $p < 0.005$, One-way ANOVA, Bonferroni post-test, n=6/group. $, significantly different from CNQX group, $p < 0.05$, One-way ANOVA, Bonferroni post-test, n=6/group. Data for NK-1 (striped bar) and GRPR (gray bar) antagonists adapted from [9].

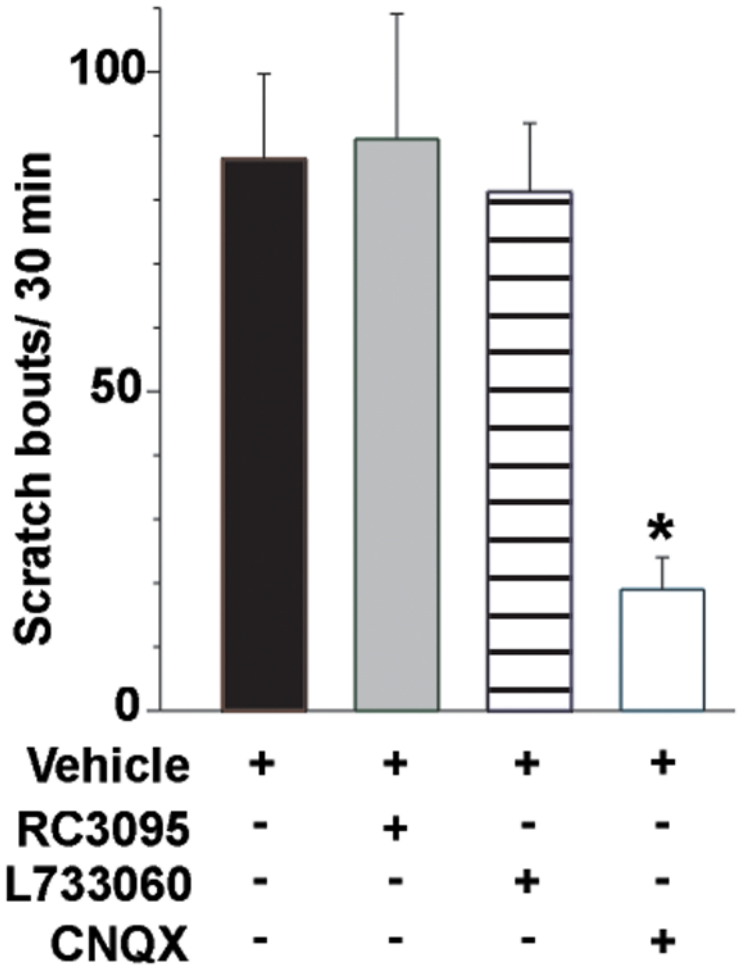

Fig. 6.

Effect of intrathecally-administered AMPA/kainate receptor antagonist on histamine-elicited scratching. A. Bar graph plots, from left to right, the mean number of scratch bouts/30 min elicited by id histamine 5 min after prior intrathecal injection of vehicle (saline; black bar), the GRP receptor antagonist RC-3095 (0.3 nmol; gray bar), the NK-1 receptor antagonist L-733060 (22.7 nmol; horizontal striped bar) or the AMPA/kainate receptor antagonist CNQX (20 nmol; white bar). Error bars: SEM. *, significantly different from vehicle group, $p < 0.05$, One-way ANOVA, Bonferroni post-test, n=6/group. Data for effects of NK-1 (striped bar) and GRPR antagonist (gray bar) adapted from [9].

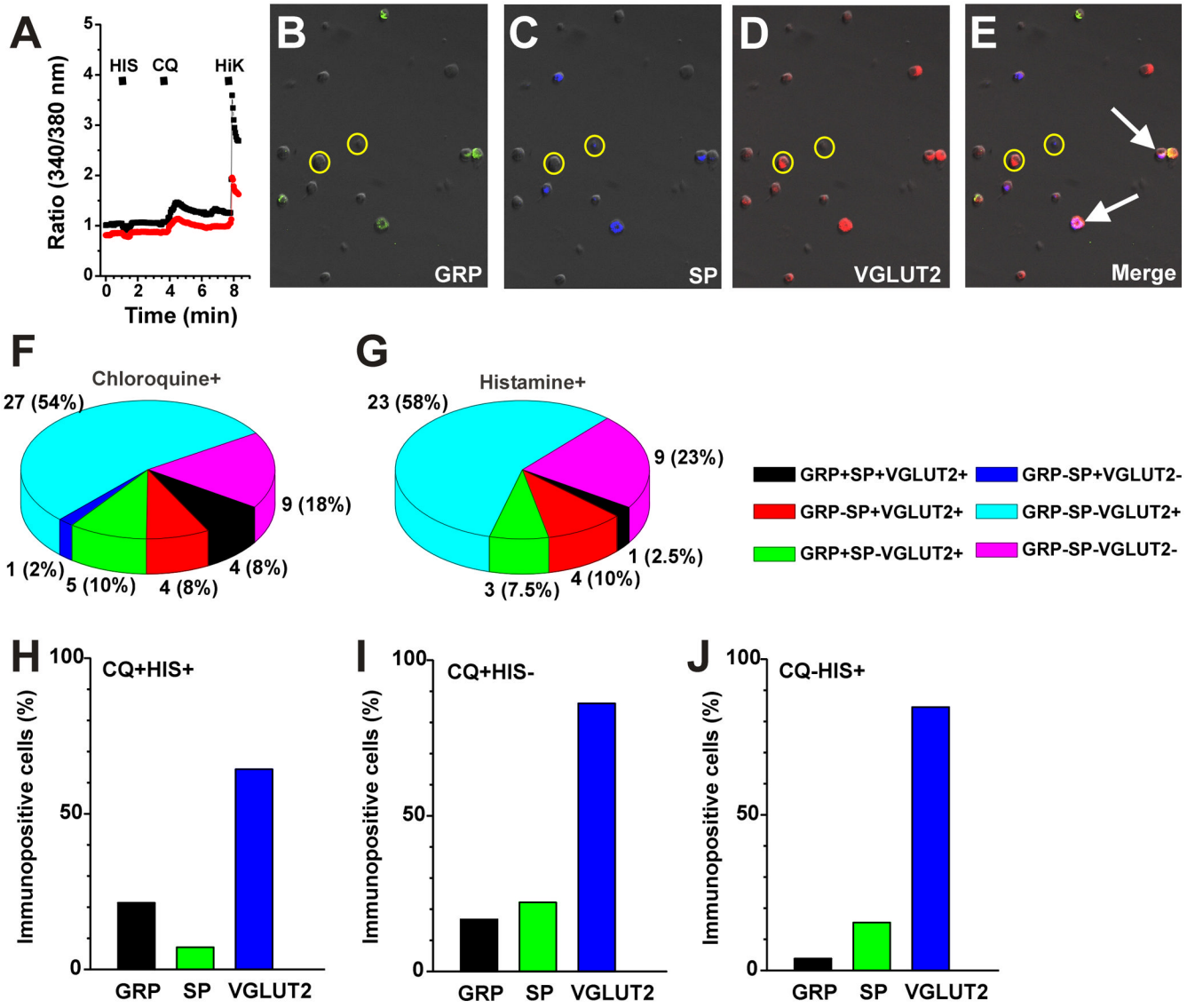

Fig. 7.

Pruritogen-responsive DRG cells double- and triple-labeled for SP-, GRP- and VGLUT2-immunoreactivity. A: Graph plots 340/380 nm ratio as a function of time for two cells (encircled in yellow in B-E) that responded to chloroquine but not histamine. Black bars indicate time of application of each indicated chemical. B: Fluorscence microscopic image of DRG cell labeled for GRP (green) following calcium imaging. Chloroquine-sensitive cells were not labeled with GRP. C: SP (blue). One chloroquine-sensitive cell (right) was lightly labeled. D: VGLUT2 (red). One-chloroquine-responsive cell (left) was labeled for VGLUT2. E: Triple-staining (Merge). Two cells indicated by arrows were triple-labeled for GRP, SP and VGLUT2. F: Pie chart summarizing the percentages of chloroquine-responsive DRG cells (assessed by calcium imaging) that were co-labeled for SP, GRP and/or VGLUT2 (*n*=50). G: As in F, summarizing percentages of histamine-responsive DRG cells (assessed by calcium imaging) that were co-labeled for SP, GRP and/or VGLUT2 (*n*=40). Legend to the right of pie chart applies to both F and G. H: Incidence of DRG cells that responded to both chloroquine and histamine (assessed by calcium imaging) that were co-labeled for SP, GRP and/or VGLUT2 (*n*=14). I: As in H, for DRG cells that responded to

chloroquine but not histamine (*n*=36). J: As in H, I for DRG cells that responded to histamine but not chloroquine (*n*=26).

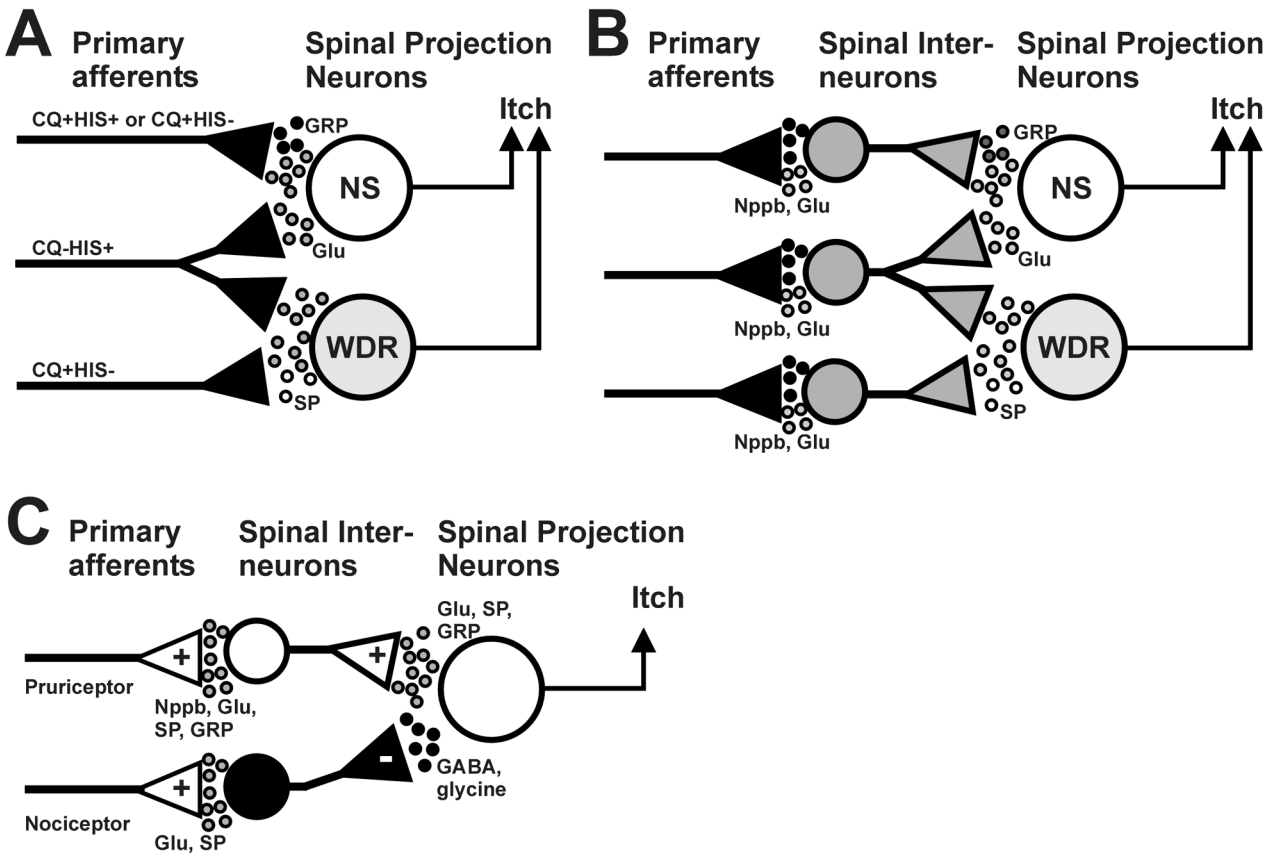

Fig. 8.

Schematic diagram showing primary afferents and spinal dorsal horn neurons that transmit itch. A. Different pruriceptors release differing proportions of glutamate (Glu) and neuropeptides GRP or SP to excite NS and/or WDR neurons that signal itch. ●: GRP; ○:SP; ◎:Glutamate. B: Pruriceptors release natriuretic polypeptide B (Nppb) and possibly glutamate to excite second-order spinal interneurons, which in turn release differing proportions of GRP, SP and glutamate to excite itch-signaling NS and/or WDR neurons. See text for further epxlanation. ●: GRP; ○:SP; ◎:Glutamate; ●:Nppb. C. Schematic of excitatory and inhibitory spinal interneurons. Itch mediators excite pruriceptors that may release glutamate and/or neuropeptides such as Nppb, GRP or SP. Intrathecal CNQX inhibits glutamatergic transmission from pruriceptors and/or excitatory spinal interneurons to itch-signaling neurons. Nociceptors release glutamate and SP to excite inhibitory spinal interneurons which inhibit itch-signaling spinal neurons. Loss of VGLUT2 in nociceptive afferents leads to reduced excitation of the inhibitory interneurons to disinhibit itch. This effect is proposed to outweigh any reduction in input from primary afferent pruriceptors.

Table 1

Percentages of dorsal horn units that exhibited a decrease of 70% or more in firing rate following spinal superfusion of antagonists.

	VH	RC3095	L733060	CNQX	L733060+CNQX	RC3095+L733060+CNQX
Chloroquine	0% (0/23)	24% (7/29)	30% (9/30)	58% (18/31)	71% (25/35)	85% (24/28)
Histamine	25% (4/16)	55% (10/18)	33% (6/18)	100% (14/14)		
AITC	27% (7/26)		22% (2/9)	62% (8/15)	89% (17/19)	