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Gate control of mechanical itch by a subpopulation of spinal cord interneurons

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Abstract

Light mechanical stimulation of the hairy skin can induce a form of itch known as mechanical itch. This itch sensation is normally suppressed by inputs from mechanoreceptors, however, in many forms of chronic itch, including alloknesis, this gating mechanism is lost. Here we demonstrate that a population of spinal inhibitory interneurons (INs) that are defined by the expression of neuropeptide Y::Cre (NPY::Cre) act to gate mechanical itch. Mice in which dorsal NPY::Cre-derived neurons are selectively ablated or silenced develop mechanical itch without an increase in sensitivity to chemical itch or pain. This chronic itch state is histamine-independent and is transmitted independently of the GRP-GRPR signaling pathway. Our studies thereby reveal a dedicated spinal cord inhibitory pathway that gates the transmission of mechanical itch

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The sensation of itch elicits stereotypical scratching behaviors that are an important protective response to cutaneous irritants and parasites. Animals appear to have evolved two forms of itch: chemical itch which is activated by chemical mediators such as histamine and proteases (1–6) and can be effectively gated by noxious painful stimuli (7), and mechanical itch that is evoked by light tactile stimuli, such as when insects or parasites come in contact with the skin. In humans, this latter pathway can be activated by vibrating the fine vellous hair (8). Itching is also frequently evoked by light mechanical stimuli in patients suffering from chronic itch, (9, 10).

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SUPPLEMENTARY MATERIAL
Material and Methods
Figs. S1–S8

While progress has been made on identifying the spinal inhibitory neurons that gate chemical itch (11, 12), little is known about the spinal pathways that gate mechanical itch. The dorsal horn of the spinal cord contains multiple inhibitory IN populations including cells that express the neuropeptide NPY (13, 14). These cells are distinct from those that express dynorphin, galanin, nNOS and parvalbumin (15, 16). When *NPY::Cre* transgenic mice (Gensat, RH26) were crossed with *R26*$^{\text{LSL-tdTomato;}}$ (*ai14*)) reporter mice to trace the provenance of the INs in the dorsal horn that express NPY, NPY::Cre-derived INs were localized in laminae III/IV (70.4 ± 0.3%) and to a lesser extent in laminae I/II (29.6 ± 0.3%) (Fig. 1A,B). The number of NPY^{+}/tdTomato^{+} INs decreases postnatally, with only 35% of the tdTomato cells expressing NPY at P30 (Fig. 1C). NPY::Cre thus captures two populations of NPY-expressing neurons: one that transiently expresses NPY during late embryonic/early neonatal development and another that shows persistent expression into the adult.

Over 98% of the NPY::Cre-tdTomato^{+} cells expressed glutamic acid decarboxylase 1 (Gad1) and/or the glycine transporter 2 (GlyT2^{+}) (Fig. 1D,E), with the majority displaying a tonic firing pattern following current injection (Fig. 1F; 34/42 cells) that is characteristic of many dorsal horn inhibitory INs (17). These NPY::Cre INs make up 31% and 45% of the inhibitory Gad1^{+}/GlyT2^{+} INs in laminae I/II and III/IV, respectively. Very few of the NPY::Cre INs cells expressed nNOS (4.9 ± 0.6%), galanin (5.0 ± 1.1%), dynorphin (8.2 ± 1.6%) or parvalbumin (5.9 ± 1.7%), indicating they constitute a distinct population of dorsal horn inhibitory INs.

An intersectional genetic strategy that restricts diphtheria toxin receptor (DTR) expression to NPY::Cre-derived INs in the dorsal spinal cord and medulla (18) was then used to determine the contribution the NPY::Cre INs make to gating cutaneous sensory stimuli. Injecting *NPY::Cre; Lbx1*$^{\text{FlpO}}$; *Tau*$^{\text{ds-DTR}}$ mice with diphtheria toxin (DTX) markedly reduced the number of NPY::Cre-tdTomato INs in the dorsal spinal cord (fig. S1A–C). This cell loss was restricted to inhibitory INs that express NPY, Gad1/GlyT2 (Fig. 1G–L, fig. S1G–I) and Pax2 (fig. S1D–F). Neighboring dorsal inhibitory IN subtypes expressing nNOS, dynorphin, and parvalbumin (fig. S1J–R), and dorsal excitatory IN subtypes (fig. S2A–L) were spared, and there was no noticeable change in the central projections of sensory afferents or in the distribution of NPY::Cre derived neurons in other regions of the CNS (fig. S3A–B).

Two weeks after injecting DTX, the NPY::Cre IN-ablated mice began to display spontaneous scratching, followed by the appearance of skin lesions (Fig. 2A,B). This scratching was not related to chemical itch, as injection of the chemical pruritogens 48/80 and chloroquine into the nape region of NPY::Cre IN-ablated mice before the onset of spontaneous scratching revealed no difference in the level or intensity of scratching (Fig. 2C–D). Using a modified protocol for analyzing alloknesis in mice in which von Frey hairs were used to deliver graded mechanical forces to the nape of the neck (19) (Fig. 2E), we observed a significant increase in evoked hindlimb scratching in NPY::Cre IN-ablated mice with low force (0.02–0.4 g) von Frey hairs as compared to control mice (Fig. 2F). By contrast, high-threshold mechanical stimuli (0.6–1 g) did not induce pronounced scratching. Additional behavioral tests revealed no marked differences between control and NPY::Cre IN-ablated mice with regard to their responsiveness to noxious mechanical and thermal

stimuli (fig. S4A). Acute chemical pain sensitivity was also normal, with capsaicin injection into the cheek of control and NPY::Cre IN-ablated mice producing similar levels of pain-indicating wiping, with little or no itch-indicating scratching (20) (fig. S4B).

To exclude the possibility that the increased scratching in NPY::Cre IN-ablated mice arises from secondary changes to the spinal circuitry following neuronal ablation, an intersectional genetic strategy employing mice carrying a conditional double-stop allele encoding the inhibitory hM4D DREADD receptor (21) (*R26*$^{ds\text{-}hM4D\text{-}tdTomato}$; fig. S5A–B) was used to acutely silence the NPY::Cre-derived INs (Fig. 2G). Activation of hM4D with clozapine-N-oxide (CNO) precipitated a mechanical itch phenotype closely resembling the itching behavior seen after NPY::Cre IN ablation (Fig. 2H). 40 minutes after CNO injection, low-threshold mechanical stimuli (0.04 and 0.07 g) produced robust scratching. High-threshold mechanical stimuli (0.6 and 1 g) that typically produce pain did not. Silencing the NPY::Cre INs did not increase scratching after exposure to 48/80 and chloroquine (Fig. 2I,J). Responses to von Frey, brush and Hargreaves test were also unchanged (fig. S5C) and there was no increase in mechanical allodynia following CFA injection (fig. S5D–E), indicating NPY::Cre-derived INs primarily inhibit mechanical itch.

Dorsal horn neurons that express GRPR are required for itch transduction by a variety of chemical pruritogens (22, 23). To address whether the mechanical itch pathway gated by the NPY::Cre INs differs from this chemical itch pathway, chemical itch was blocked either pharmacologically or by ablating the GRPR neurons in the dorsal horn. NPY::Cre IN-ablated mice treated with a H1/H4 histamine receptor antagonist displayed no reduction in the number of scratch events after mechanical stimulation on the nape of the neck (Fig. 3A) as compared to saline controls, despite the antagonist being highly effective in reducing 48/80-induced scratching (Fig. 3B). Intrathecal injection of a GRPR antagonist or ablation of GRPR-expressing neurons with conjugated bombesin-saporin (fig. S6), while effective in blocking chloroquine-evoked itch (22) (Fig. 3D,F), failed to blunt scratching in response to mechanical stimulation in NPY::Cre IN-ablated mice (Fig. 3C,E). Mechanical itch gated by NPY::Cre INs is therefore independent of the histaminergic and GRP-GRPR itch pathways described to date (1–7).

To assess whether the NPY::Cre INs contribute to the tactile inhibition of itch, we asked if the NPY::Cre INs are innervated by cutaneous low-threshold mechanoreceptors (LTMs). In *Pitx2-EGFP* mice (Gensat), myelinated hair follicle afferents that selectively express GFP (fig. S7A–C) form multiple contacts on the cell bodies and dendrites of NPY::Cre INs (Fig. 4A–B). When Cholera Toxin B (CTB) was injected into the hairy skin (fig. S7D–G, (24)), presumptive CTB^{+}/vGluT1^{+} Aβ- and Aδ-LTM synaptic boutons and putative CTB^{+}/vGluT1^{-} C-LTM synaptic contacts were detected on NPY::Cre-tdTomato^{+} INs in laminae III/IV (Fig. 4C, fig. S7Ga, arrows) and lamina II (Fig. 4C, fig. S7Gb), respectively. The synaptic nature of these contacts was confirmed by single synapse transsynaptic rabies tracing (Fig. 4D–G, fig. S7H–J) and whole-cell recordings from NPY::Cre-tdTomato neurons (Fig. 4H). Our demonstration that the NPY::Cre INs receive LTM inputs, coupled with evidence from humans that mechanical itch is gated by LTMs (8), suggest the NPY::Cre INs mediate the tactile inhibition of mechanical itch.

We then examined how neurons in laminae I–III respond to innocuous touch (brush, Fig. 4I,J) and painful stimuli (pinch, fig. S8B,C) following NPY::Cre IN ablation. Neurons with hairy skin receptive fields displayed a significant increase in afterdischarge spike number in NPY::Cre IN-ablated mice as compared to control mice, (Fig. 4I). This occurred in the absence of any concomitant increase in spontaneous activity (Fig. S8A). By contrast, afterdischarge activity following brush in neurons with glabrous skin receptive fields (Fig. 4J), or after noxious stimulation (pinch) of both hairy and glabrous skin (fig. S8B–C), was unchanged. The NPY::Cre INs therefore have a specific role in gating innocuous mechanosensory inputs from hairy skin. This is consistent with our observation that scratching and skin lesions in the NPY::Cre IN-ablated mice are restricted to hairy sites (Fig. 2A), and sensitivity to noxious or mechanical stimulation on the glabrous skin is unchanged (fig. S4).

Our findings reveal that inhibitory spinal INs marked by the expression of NPY::Cre selectively gate low-threshold mechanical itch. By contrast, *Bhlhb5* conditional knockout mice show increased chemical itch sensitivity (11). NPY expression is not affected in the *Bhlhb5* mutant cord (17), and several Bhlhb5-dependent inhibitory IN subtypes are spared following NPY::Cre IN ablation (fig. S1). This suggests that NPY::Cre-derived and Bhlhb5–dependent inhibitory INs are required to gate mechanical and chemical itch pathways, respectively (summarized in Fig. 4K). The loss of NPY::Cre INs (fig. S4) or Bhlhb5-dependent inhibitory INs (11) does not affect mechanical pain, which is gated by dynorphin-expressing inhibitory INs (18). It therefore appears that inhibitory INs in the dorsal horn are organized into discrete functional modules that gate different streams of somatosensory information.

In identifying a previously uncharacterized gate for low-threshold mechanical itch, this study highlights a largely overlooked driver of chronic itch, namely the light touch pathway that is insensitive to anti-histamine or anti-GRPR drugs (8). Human patients with chronic itch (trichoknesis) (25) display a phenotype similar to that seen in the NPY::Cre IN-ablated mice. In both instances, itch sensitivity is restricted to the hairy skin. Our finding that the NPY::Cre-derived INs are innervated by tactile inputs from hairy skin LTMs (Fig. 4), suggests the NPY::Cre INs are key components of a spinal inhibitory circuit by which hairy skin LTMs gate mechanical itch. We propose that the NPY::Cre INs function as rheostat for low-threshold tactile stimuli to suppress itch and prevent excessive scratching.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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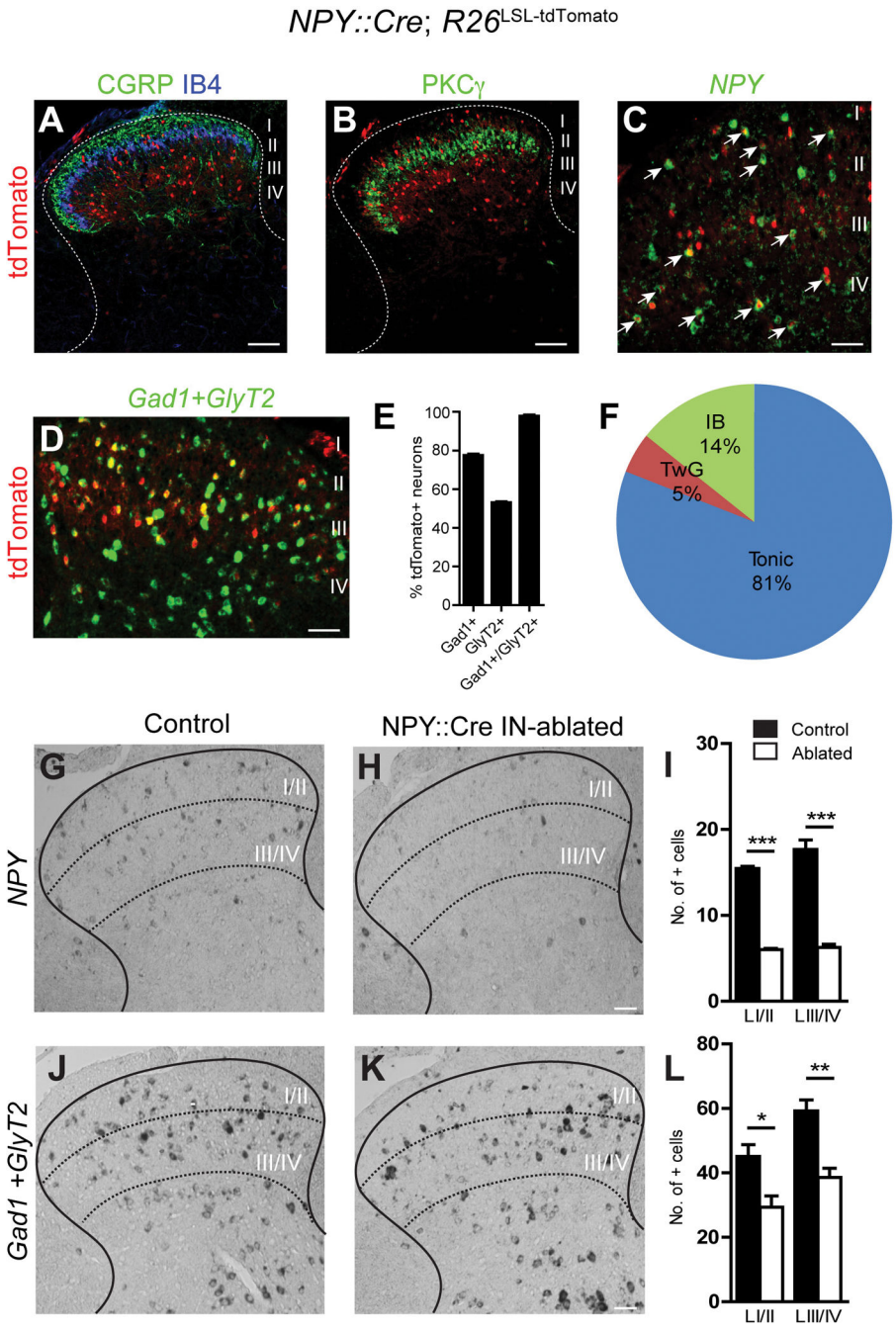

Fig. 1. NPY::Cre delineates a population of inhibitory neurons in the dorsal spinal cord

(A–B) Sections through the lumbar dorsal spinal cord of a P30 *NPY::Cre; R26*$^{\text{LSL-tdTomato}}$ mouse stained with CGRP and IB4 **(A)** and PKCγ **(B)**. tdTomato^{+} fluorescence was visualized without staining. **(C)** Section through the lumbar dorsal spinal cord of a P30 *NPY::Cre; R26*$^{\text{LSL-tdTomato}}$ mouse comparing tdTomato reporter (red) and NPY (green, in situ) expression. **(D)** Section through P30 *NPY::Cre; R26*$^{\text{LSL-tdTomato}}$ lumbar dorsal horn showing co-expression of tdTomato with the inhibitory markers *Gad1* and *GlyT2*. **(E)** Quantification of co-expression of NPY-tdTomato^{+} with Gad1-GFP, GlyT2-GFP and

Gad1+GlyT2 in situ hybridization. **(F)** Firing properties of NPY::Cre INs. The majority of NPY-tdTomato$^+$ INs (34/42 cells) show a tonic firing pattern upon current injection. **(G–L)** In situ analysis and quantification of NPY (G–I) and Gad1/GlyT2 (J–L) expression in lumbar dorsal spinal cord of P60 control and NPY::Cre IN-ablated mice. NPY$^+$ cell numbers were reduced by 59.0% and 69.5% in laminae I/II and III/IV, respectively (I, *** $P<0.001$). Gad1$^+$ and GlyT2$^+$ cell numbers were reduced by 34.7% in laminae I/II (L, *$P<0.05$) and 34.9% in laminae III/IV (L, **$P<0.01$). *P* values were calculated using the Student's unpaired *t*-test.). Scale bars: 50 μm except in A and B (200 μm).

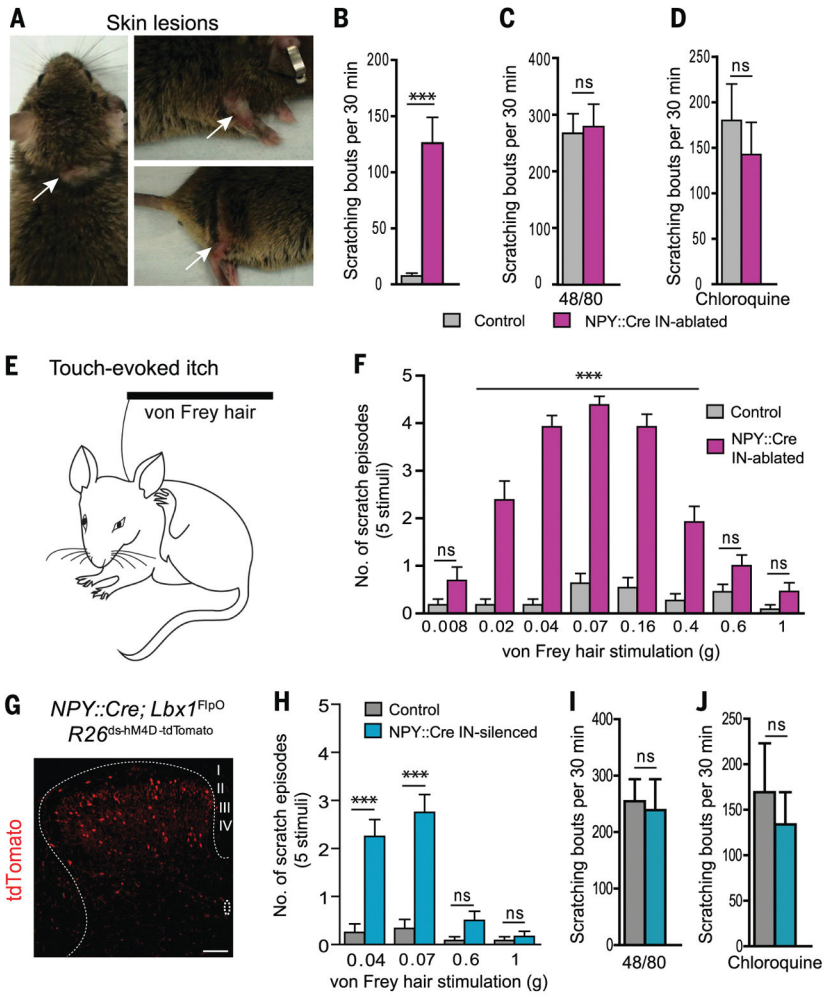

Fig. 2. Ablation and silencing of NPY::Cre INs induces touch-evoked itch

(A) Skin lesions on the neck and body of NPY::Cre IN-ablated mice 2 weeks post DTX-injection. **(B)** The number of spontaneous scratch events over a 30 min period. (Control: 7.5 ± 2.6; NPY::Cre IN-ablated: 125.8 ± 23.2; n = 10, ***$P<0.001$). **(C–D)** Equivalent responses to chemical-evoked itch are seen in control and NPY::Cre IN-ablated mice 7 days post DTX-injection and before the spontaneous scratch phenotype develops. 48/80 (Control: 266.8 ± 34.9; NPY::Cre IN-ablated: 279 ± 39.6; n=6 mice; P=0.82); chloroquine (Control: 180.1 ± 39.8; n=7 mice; NPY::Cre IN-ablated: 142.5 ± 35.3; n=6 mice; P=0.50). **(E)** Schematic showing the touch-evoked itch (alloknesis) test. **(F)** Increase in the number of scratch events after applying von Frey hairs to the shaved nape of the neck (Control: n=11 mice; NPY::Cre IN-ablated: n=13 mice; ***P<0.001). **(G)** tdTomato reporter expression in a section through the lumbar dorsal spinal cord of *NPY::Cre; Lbx1*$^{\text{Flpo}}$; *R26*$^{\text{ds-hM4Di-tdTomato}}$ mice. **(H)** An increase in the number of scratches to low intensity (0.04 and 0.07 g) but not to high intensity force (0.6 and 1 g) is seen in NPY::Cre IN-silenced compared to control mice. (Control: n=12 mice; NPY::Cre IN-silenced: n=12 mice; ***P<0.001). **(I–J)** Equivalent responses to chemical-evoked itch are seen in control and NPY::Cre IN-silenced mice. 48/80 (Control: 254.8 ± 39.03; NPY::Cre IN-silenced: 239 ± 54.78; n=6 mice;

P=0.82); chloroquine (Control: 169.3 ± 53.6; n=6 mice; NPY::Cre IN-silenced: 133.9 ± 35.49; n=7 mice; *P*=0.58). ns, no significant difference, *P* values were calculated using the Student's unpaired *t* test. Scale bar: 200μm (G).

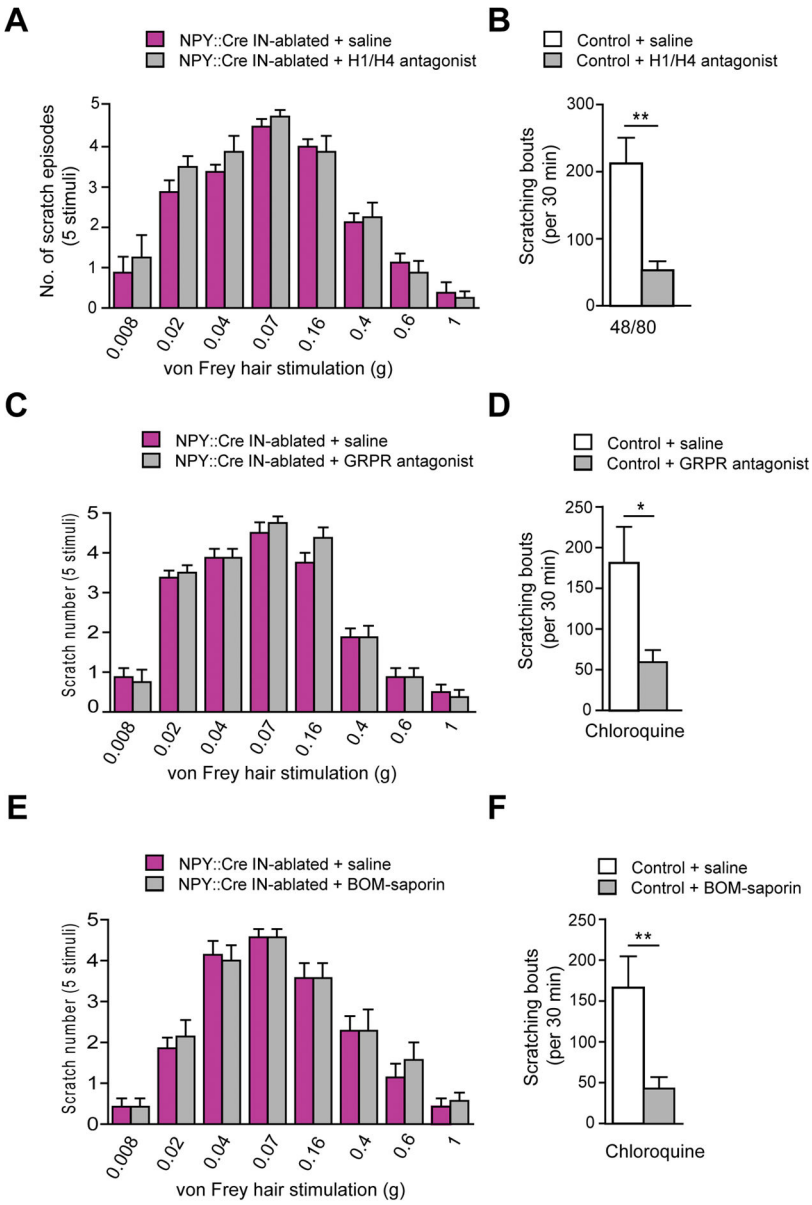

Fig. 3. The touch-evoked itch pathway gated by NPY::Cre INs is histamine and GRPR-independent

(A) Mechanical alloknesis response in NPY::Cre IN-ablated mice was not affected after oral administration of histamine H1/H4 receptor antagonists. **(B)** A significant reduction of scratch events was observed in response to 48/80 when H1/H4 receptor antagonists were administered to control (naïve) mice (control: 212 ± 38.5; n=4; control-H1/H4 antagonist: 52.8 ± 13.7; n=5; ***P*<0.01). **(C)** The mechanical alloknesis response in NPY::Cre IN-ablated mice was not altered after intrathecal injection of a GRPR antagonist. **(D)** Scratch events in response to chloroquine injection were reduced in control (naïve) mice (Control-saline: 180.8 ± 44.6; n=5; control-GRPR antagonist: 59.4 ± 14.7; n=5; **P*<0.05). **(E)** Mechanical alloknesis in NPY::Cre IN-ablated mice was not affected 2 weeks after ablating $GRPR^+$ INs in the spinal cord. **(F)** Ablation of the $GRPR^+$ INs in control (naïve) mice

causes a significant reduction of scratch events induced by injecting chloroquine (Control-saline treated mice: 166.1 ± 38.5; n=7; control-bombesin-saporin (BOM-saporin) treated mice: 42.9 ± 13.9; n=7; $^{**}P<0.01$). *P* values were calculated using the Student's unpaired *t* test.

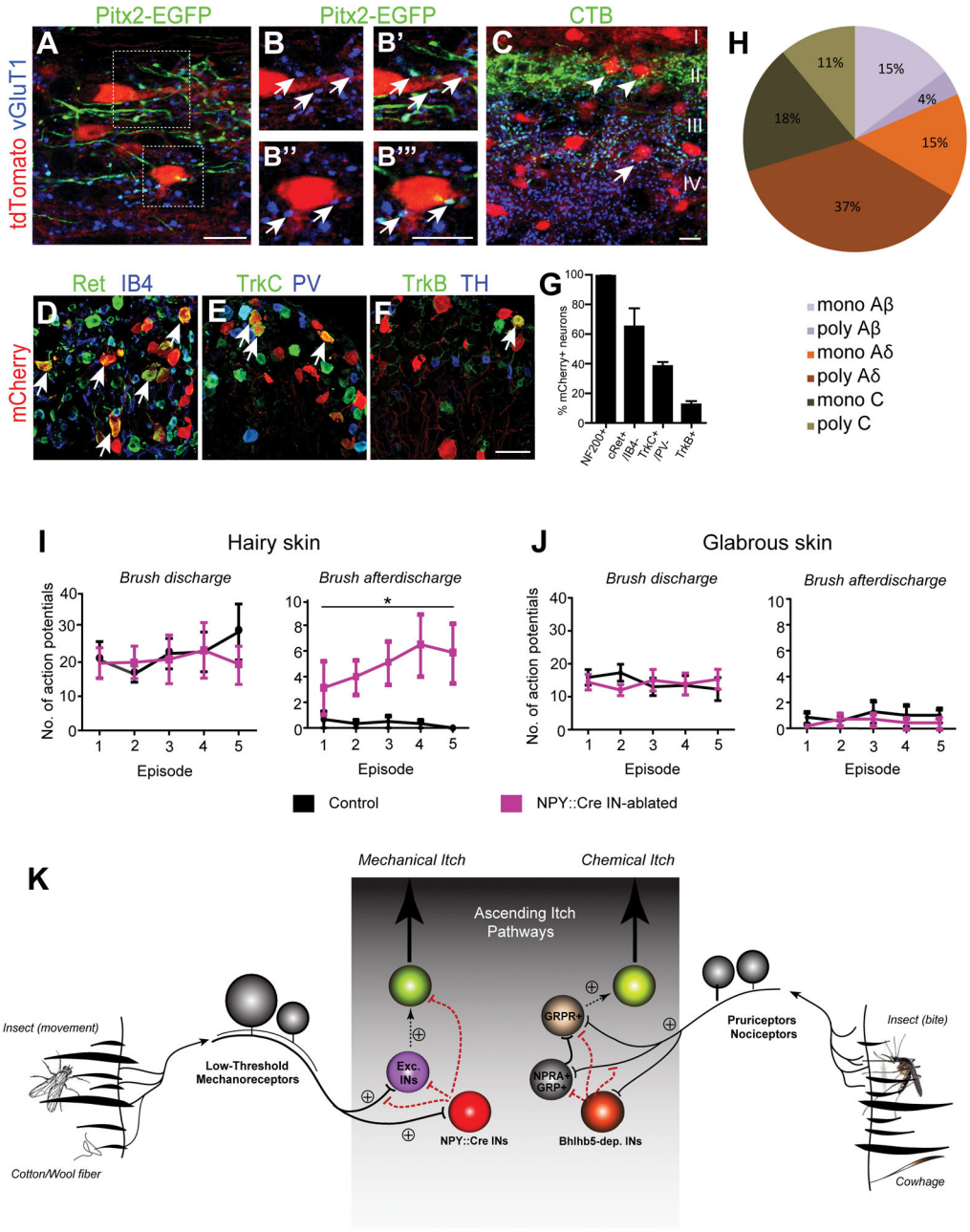

Fig. 4. NPY::Cre INs form a feedforward inhibitory pathway from hairy skin to suppress mechanical itch

(A–B) Sections from lumbardorsal spinal cord of *NPY::Cre; R26*$^{\text{LSL-tdTomato}}$; *Pitx2*-EGFP mice showing Pitx2-EGFP^{+} LTMs synaptic terminal afferents (GFP^{+}/vGluT1^{+}) in close apposition to NPY::Cre-TdTomato^{+} INs. Higher magnification images are shown in B to B ‴. **(C)** Sections from lumbar dorsal spinal cord of *NPY::Cre; R26*$^{\text{LSL-tdTomato}}$ mice 2 days post injection of CTB in hairy skin stained with indicated markers. **(D–G)** Transynaptic labeling of LTMs following selective infection of NPY::Cre INs with pseudotyped EnvA-mCherry rabies virus. Representative sections through the dorsal root ganglion of a P13 *NPY::Cre; Lbx1*$^{\text{FlpO}}$; *R26*$^{\text{ds-HTB}}$ mouse stained with the indicated markers of LTM subtypes

(D–F). Arrows indicate double labeled neurons.**(G)** Quantification of sensory neuronal markers in relation to the total number of mCherry^{+} neurons. Data: mean ± sem, n=3 mice. **(H)** Classification of potentials in NPY::Cre INs induced by dorsal root stimulation. Of the 27 recorded cells, 3 cells with a monosynaptic Aβ input displayed a second monosynaptic Aδ (1 cell) and polysynaptic C (2 cells) input. **(I and J)** *In vivo* extracellular recordings from lumbar dorsal spinal cord neurons in response to mechanical stimulation of hairy and glabrous skin. **(I)** Increase in the mean of afterdischarge firing in NPY::Cre IN-ablated compared to control mice in hairy skin receptive fields (control: 0.4 ± 0.3 spikes/sec, n=7 cells; NPY::Cre IN-ablated: 4.9 ± 1.4 spikes/sec, n=7 cells, two way ANOVA, P=0.03). The number of spikes fired during active brush was unchanged (control: 22.3 ± 4.2 spikes/sec, n=7 cells; NPY::Cre IN-ablated: 20.1 ± 5.7 spikes/sec, n=7 cells). **(J)** Afterdischarge firing in NPY::Cre IN-ablated compared to control mice in glabrous skin receptive fields (control: 0.9 ± 0.3 spikes/sec, n=5 cells; NPY::Cre IN-ablated: 0.5 ± 0.2 spikes/sec, n=7 cells). The number of spikes fired during active brush was unchanged (control: 14.1 ± 2.4 spikes/sec, n=5 cells; NPY::Cre IN-ablated: 15.3 ± 2.8 spikes/sec, n=7 cells). **(K)** Model for the mechanical itch pathway: light touch stimuli on hairy skin stimulates low-threshold mechanoreceptors (LTMs) to evoke mechanical itch. This itch pathway is gated by other LTMs via their activation of inhibitory NPY::Cre INs. The mechanical itch circuit is independent of the chemical itch pathways transduced by NPRA/GRP/GRPR (26), which are gated by inhibitory Bhlhb5-dependent INs. Scale bars: 10 μm (A–C), 50 μm (D–F).