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The Neuropeptide Y Y_2 Receptor Is Coexpressed with Nppb in Primary Afferent Neurons and Y_2 Activation Reduces Histaminergic and IL-31-Induced Itch[S]

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ABSTRACT

Itch stimuli are detected by specialized primary afferents that convey the signal to the spinal cord, but how itch transmission is regulated is still not completely known. Here, we investigated the roles of the neuropeptide Y (NPY)/Y_2 receptor system on scratch behavior. The inhibitory Y_2 receptor is expressed on mouse primary afferents, and intrathecal administration of the Y_2 agonist peptide YY $(PYY)_{3–36}$ reduced scratch episode frequency and duration induced by compound 48/80, an effect that could be reversed by intrathecal preadministration of the Y_2 antagonist BIIE0246. Also, scratch episode duration induced by histamine could be reduced by $PYY_{3–36}$. In contrast, scratch behavior induced by α-methyl-5HT, protease-activated receptor-2–activating peptide SLIGRL, chloroquine, topical dust mite extract, or mechanical itch induced by von Frey filaments was unaffected by stimulation of Y_2. Primary afferent neurons expressing the *Npy2r* gene were found to coexpress itch-associated markers such as natriuretic peptide precursor b, oncostatin M receptor, and interleukin (IL) 31 receptor A. Accordingly, intrathecal $PYY_{3–36}$ reduced the scratch behavior induced by IL-31. Our findings imply that the NPY/Y_2 system reduces histaminergic and IL-31-associated itch through presynaptic inhibition of a subpopulation of itch-associated primary afferents.

SIGNIFICANCE STATEMENT

The spinal neuropeptide Y system dampens scratching behavior induced by histaminergic compounds and interleukin 31, a cytokine involved in atopic dermatitis, through interactions with the Y_2 receptor. The Y_2 receptor is expressed by primary afferent neurons that are rich in itch-associated neurotransmitters and receptors such as somatostatin, natriuretic peptide precursor b, and interleukin 31 receptors.

Introduction

More than 350 years ago itch was defined as an "unpleasant sensation that elicits the desire or reflex to scratch" by the German physician Samuel Hafenreffer (Ikoma et al., 2006). Although acute itch serves a purpose by initiating scratching that will remove irritants that can cause harm, persistent itch severely affects the quality of life. Itch-inducing, or pruritogenic, substances are detected by specific receptors expressed by primary afferent neurons (Schmelz et al., 1997; Liu et al., 2009). Neurotransmitters such as natriuretic polypeptide b (Nppb) transmit the signal further to the dorsal horn of the spinal cord (Mishra and Hoon 2013), where the gastrin-releasing peptide receptor (Grpr) system is subsequently activated (Sun et al., 2009; Aresh et al., 2017), relaying the signal further to projection neurons that convey the information to the brain (Mu et al., 2017).

The neurotransmitter neuropeptide Y (NPY) is expressed by spinal GABAergic interneurons located in laminae I–IV of the dorsal horn (Rowan et al., 1993; Bourane et al., 2015). Under normal conditions, expression of *Npy* mRNA is below the detection limit in dorsal root ganglia neurons (Usoskin et al., 2015) but becomes significantly upregulated after peripheral nerve injury (Wakisaka et al., 1991). Spinal NPY interneurons are activated by nociceptive input (Liu et al., 2010; Polgar et al., 2013) and NPY binds to the NPY receptor family, whose Y_1 receptor is expressed on excitatory somatostatin-expressing interneurons in the spinal cord and in a few primary afferent neurons (Zhang et al., 1999). Activation of the Y_1 receptor results in reduced histaminergic and mechanical itch transmission (Gao et al., 2018), and conversely ablation or silencing of NPY interneurons results in increased itch and alloknesis upon mechanical stimuli (Bourane et al., 2015), indicating that the NPY/Y_1 system regulates both mechanical and histaminergic itch.

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ABBREVIATIONS: BIIE0246, (2*S*)-5-(diaminomethylideneamino)-*N*-[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]-2-[[2-[1-[2-oxo-2-[4-(6-oxo-5,11-dihydrobenzo[c][1]benzazepin-11-yl)piperazin-1-yl]ethyl]cyclopentyl]acetyl]amino]pentanamide; DRG, dorsal root ganglion; 5HT, serotonin; IL-31, interleukin-31; LTMR, low-threshold mechanoreceptors; Nppb, natriuretic polypeptide b; NPY, neuropeptide Y; PYY, peptide YY; SLIGRL, protease-activated receptor-2–activating peptide; Sst, somatostatin.

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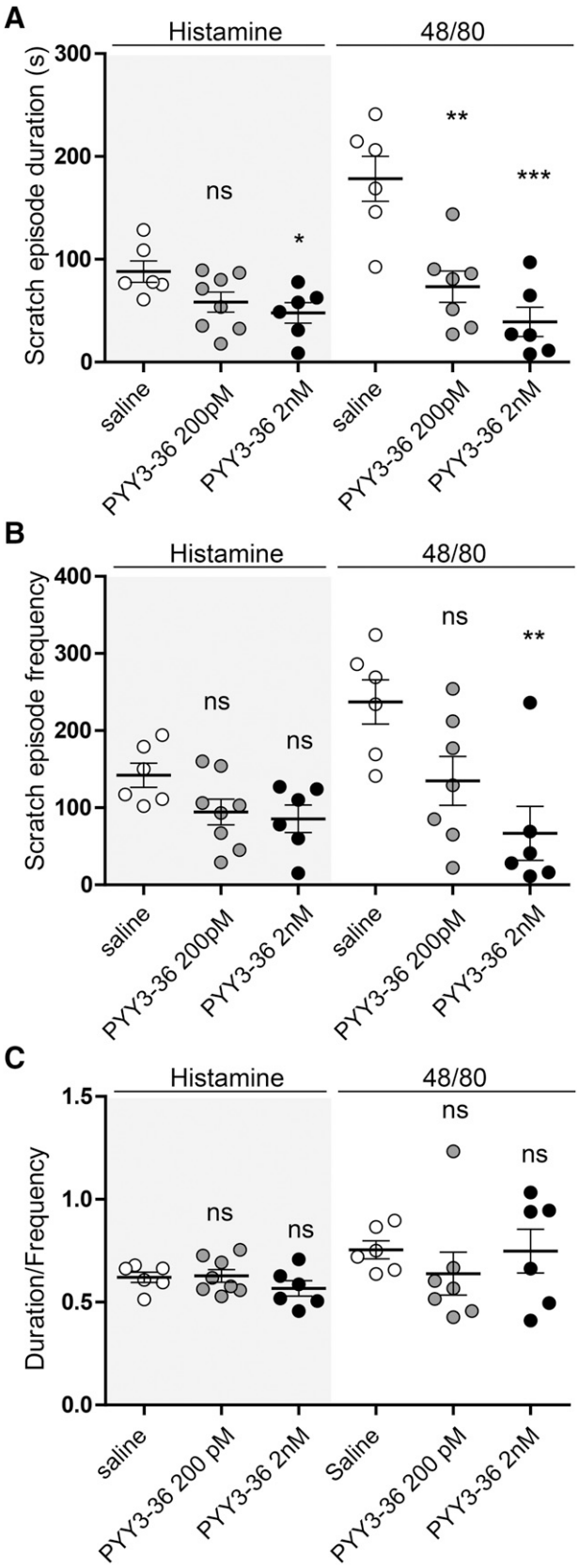

Fig. 1. Activation of the NPY/Y2 system reduces histaminergic itch. Effect of $PYY_{3–36}$ (200 pM or 2 nM) or saline on histamine (100 μg)- or 48/80 (100 μg)-induced (A) scratch episode duration (total time spent scratching), (B) scratch episode frequency (number of scratch episodes), and (C) duration/frequency (mean length of scratch episodes). Each individual animal is plotted in the diagrams. ns > 0.05, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (A–C) $PYY_{3–36}$ (2 nM or 200 pM). One-way ANOVA with Bonferroni's Multiple Comparison Test (GraphPad Prism).

In the spinal neuronal network, the Y_2 receptor is found in central terminals of primary afferent neurons (Brumovsky et al., 2005; Usoskin et al., 2015; Li et al., 2016; Zeisel et al., 2018) and dorsal rhizotomy completely abolishes Y_2 immunoreactivity in the spinal cord (Brumovsky et al., 2005); however, a recent analysis has also shown weak *Npy2r* mRNA expression in some excitatory (Glut10, Glut11) and inhibitory (Gaba7, Gaba10, Gaba11) neuronal subtypes of dorsal horn interneurons (Häring et al., 2018). Activation of Y_2 has been shown to have a protective role against the development and maintenance of peripheral neuropathic pain (Solway et al., 2011). However, the role of the Y_2 receptor in itch is unknown. Y_2 couples via the inhibitory G protein subunits α_I and α_o (http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=306) and activation of the receptor decreases the firing frequency by activating G-protein-linked inwardly rectifying potassium (GIRK) conductances and depressing calcium currents in Y_2-expressing neurons (Acuna-Goycolea et al., 2005; Ghamari-Langroudi et al., 2005). As Y_2 is expressed presynaptically on primary afferent neurons, we hypothesized that selective activation of Y_2 could have a dampening effect on itch transmission. In the present analysis we used a pharmacological and bioinformatical approach to address the role of Y_2 in itch.

Materials and Methods

Animals. Animal procedures were approved by the local ethical committee in Uppsala (Uppsala djurförsöksetiska nämnd) and followed the Directive 2010/63/EU of the European Parliament and of the Council, The Swedish Animal Welfare Act (Djurskyddslagen: SFS 1988:534), The Swedish Animal Welfare Ordinance (Djurskyddsförordningen: SFS 1988:539), and the provisions regarding the use of animals for scientific purposes: DFS 2004:15 and SJVFS 2012:26. All behavioral analyses were performed in a controlled environment at 20–24° C, 45%–65% humidity, and during the light 12-hour day/night cycle.

Itch Behavior, Generally. Adult male C57BL6 mice (>7 weeks old) were placed and acclimated for 5–10 minutes in a transparent plastic chamber (820 cm^3) with bedding before they were sedated using isoflurane and injected intrathecally with Y_2-related substances. The chemical-induced itch behavior (described below) was recorded for 60 minutes with a digital camera, and mechanical itch (described below) was also recorded using a video camera while the experimenter stimulated the mice mechanically using 0.07-gf (gram force) von Frey filaments. One scratch episode was defined as lifting up either hind paw followed by scratching toward the pruritogen-injected or mechanically stimulated area from the time point when the paw was lifted until it was placed back on the ground. Scratch episode frequency was defined as number of scratch episodes during the defined time span, scratch episode duration was defined as total time spent scratching during the defined time span, and the duration/frequency ratio provided the mean length of a scratch episode. The itch behavior was scored manually using the software AniTracker v1.0.

Chemical Itch. Adult male C57BL6 mice (>7 weeks old) were sedated using isoflurane, after which the mice were slowly injected intrathecally (L5–L6) with 5 μl of the agonist peptide YY $(PYY)_{3–36}$ 2 nM (8.1 pg/ml) or 200 pM (0.81 pg/ml), selective for Y_2 (http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=306) (Bachem, Bubendorf, Switzerland), or the Y_2 antagonist BIIE0246 ((2*S*)-5-(diaminomethylideneamino)-*N*-[2-(3,5-dioxo-1,2-diphenyl-1,2,4-

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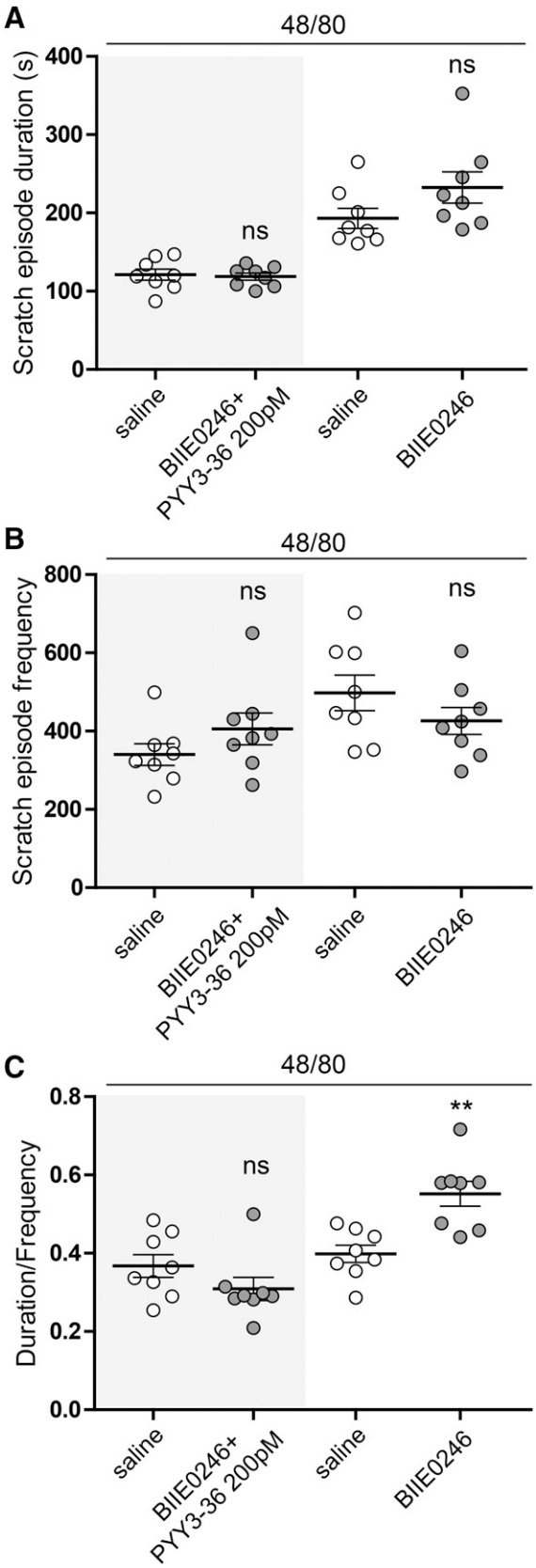

Fig. 2. The Y_2 antagonist BIIE0246 blocks the effect of PYY3-36 on scratch behavior. Effect of BIIE0246 (1 μM) (0.9 μg/ml) or saline administered 10 minutes before PYY_{3-36} (200 pM), or BIIE0246 alone, on 48/80 (100 μg)-induced (A) scratch episode duration (total time spent scratching), (B) scratch episode frequency (number of scratch episodes), and (C) duration/frequency (mean length of scratch episodes). Each individual animal is plotted in the diagrams. ns > 0.05, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (A) BIIE0246 + PYY_{3-36} (200 pM) unpaired *t* test, passed D'Agostino and Pearson normality test; (B and C) BIIE0246 + PYY_{3-36} (200 pM), Mann-Whitney test; (A–C) BIIE0246 (1 μM) unpaired *t* test, passed D'Agostino and Pearson normality test (GraphPad Prism).

triazolidin-4-yl)ethyl]-2-[[2-[1-[2-oxo-2-[4-(6-oxo-5,11-dihydrobenzo[c][1]benzazepin-11-yl)piperazin-1-yl]ethyl]cyclopentyl]acetyl]amino]pentanamide) (http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=306) 1 μM (0.9 μg/ml; Tocris Bioscience, Bristol, UK) or saline (9 mg/ml; Apoteket, Sweden) using a 25-μl Hamilton syringe. After injection, mice were acclimated for 10 minutes in their respective home cage for recovery before administration of a 50-μl intradermal injection in the nape of the neck of: compound 48/80 (mast cell degranulator, condensation product of *N*-methyl *p*-methoxyphenethylamine with formaldehyde) (13 mM, 100 μg/50 μl; Sigma, St. Louis), histamine (18 mM, 100 μg/50 μl; Sigma), α-methyl serotonin (3.2 mM, 30 μg/50 μl; Sigma), SLIGRL (3 mM, 100 μg/50 μl; Bachem), IL-31 [12 μM, 9.5 ng/50 μl, PeproTech, Sweden (US)] or chloroquine (CQ; 10 mM, 160 μg/50 μl; Sigma) and then transferred to a transparent cage supplied with bedding. Scratching behavior was recorded for 60 minutes using a digital camera. The investigators were not present in the room during the recordings and were blinded to the intrathecal treatment given. The itch behavior was later scored manually using the software AniTracker v1.0 and the results were displayed as the mean ± S.E.M.

To evaluate the selectivity of 200 pM PYY_{3-36} for Y_2, 1 μM (0.9 μg/ml) Y_2-selective antagonist BIIE0246 (http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=306); Tocris Bioscience) or saline (control) was given intrathecally 10 minutes before PYY_{3-36} or saline using a 25-μl Hamilton syringe.

Atopic Dermatitis-Like Itch. Adult female Nc/Nga mice (9–10 weeks; Charlies River, Japan) were shaved dorsally and 200 mg of depilatory cream (Veet hair removal cream, Apotea, Sweden) was applied on the shaved area and removed after 2 minutes incubation. Atopic dermatitis (AD)-like symptoms were initially induced by application of 100 mg Biostir dust mite extract (Biostir Inc., Osaka, Japan) on the dorsal skin and the back sides of the ears, after which the mice were returned to their home cage. From the second dust mite treatment onwards, the mice were subjected to the following treatment twice a week for a maximum of 5 weeks: 150 μl of 4% sodium dodecyl sulfate (dissolved in saline or phosphate-buffered saline) was applied to the hairless surface to enable the mite extract to reach stratum corneum (if hair had regrown it was removed as described above), after which the surface was dried with a hair dryer (cold mode). The animal was then returned to the home cage for 2–3 hours, after which 100 mg of Biostir AD ointment was applied to the surface. During the dust mite treatment, the mice developed atopic dermatitis-like symptoms and when the severity of the symptoms reached grade 7–8, according to the grading system/photos from the supplier (Biostir), the mice were enrolled in the behavior study. Basal level of itch behavior was recorded after mimicking the intrathecal injection procedure but without performing the injection, i.e., the mice were handled under anesthesia for 4–5 minutes and then recovered for 10 minutes in home cage. On the second day, and at the same time of day as the baseline recording, mice were given an intrathecal injection of either 5 μl of saline or 2 nM PYY_{3-36} under anesthesia induced by 3%–4% isoflurane. When completely recovered from sedation, the mice were transferred to a transparent cage supplied with bedding. Scratching behavior was recorded for 60 minutes using a digital camera. The itch behavior was later scored manually using the software AniTracker v1.0, and the results were displayed as the mean ± S.E.M. Observers were blinded to the intrathecal treatment given when they performed video analyses.

Mechanical Itch. Adult male C57BL6 mice (>7 weeks) were sedated using 3%–4% isoflurane and slowly injected intrathecally

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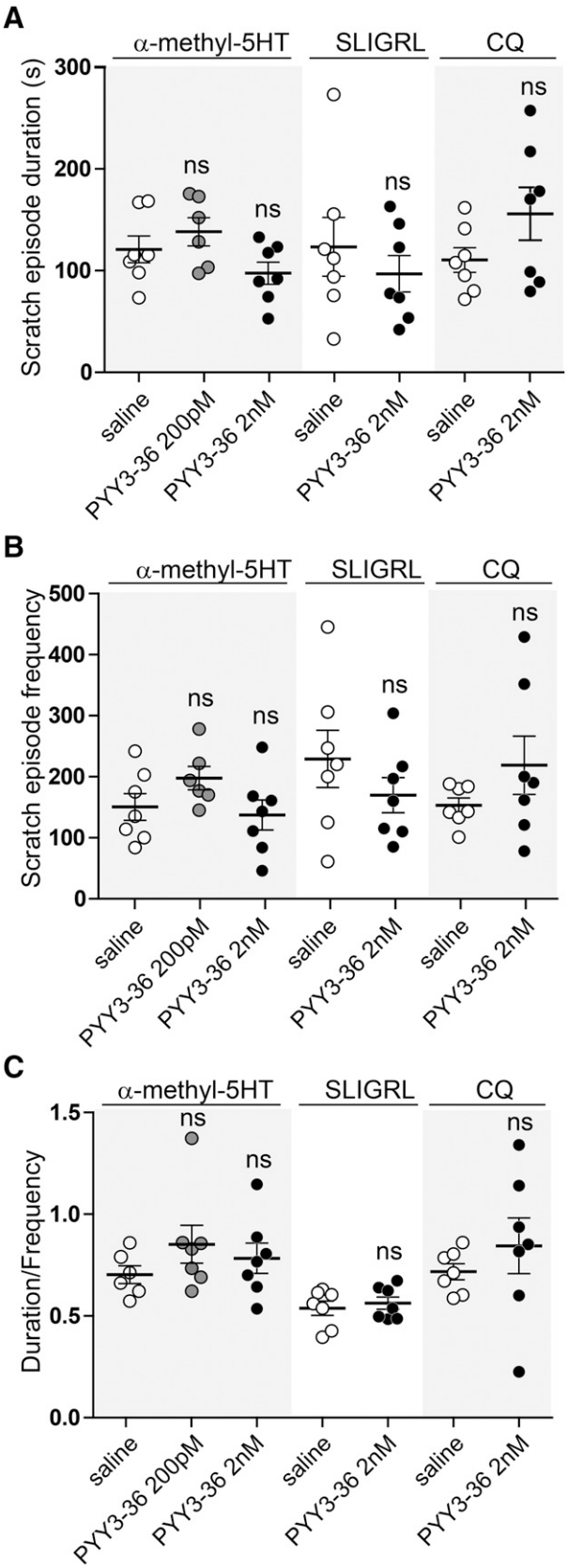

Fig. 3. Activation of the NPY/Y2 system does not affect nonhistaminergic itch induced by α-methyl-5HT, SLIGRL, or chloroquine (CQ). Effect of PYY_{3-36} (200 pM or 2 nM) or saline on nonhistaminergic α-methyl-5HT, SLIGRL, or CQ-induced (A) scratch episode duration, (B) scratch episode frequency, and (C) duration/frequency. Each individual animal is plotted in the diagrams. ns > 0.05, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (A–C), α-Methyl-5HT, one-way ANOVA with Bonferroni's multiple comparison test (GraphPad Prism). (A–C) SLIGRL and CQ, Mann-Whitney test (GraphPad Prism).

(L5–L6) with 5 μl of PYY_{3-36} (2 nM) or saline (9 mg/ml) using a 25-μl Hamilton syringe. Mice were then acclimated for 10 minutes in a transparent cage with bedding and recorded with a digital camera. After acclimation, 0.07-gf von Frey filaments (AgnThos, Lidingö, Sweden) were applied to the nape of the neck. A behavior response upon von Frey stimuli would consist of either a shake of the fur (recorded as one shake episode) or a scratch by either hind paw (recorded as a scratch episode). Each mouse was given three repeats of five consecutive mechanical stimuli at the frequency of 1/s by a 0.07-gf von Frey filament. The mechanical itch behavior was reported as the mean number of scratch/shake episodes per repeat (per five von Frey stimuli); frequency), mean time spent scratching/shaking per repeat (per five von Frey stimuli; duration), and mean length of shake or scratch episodes (duration/frequency). The results were displayed as the mean ± S.E.M. Shaking of the fur behavior has been shown to relate well to pruritogen-induced scratching (Jinks and Carstens 2002).

Statistical Analysis. Gaussian distribution was tested using D'Agostino and Pearson normality test (GraphPad Prism, CA). Unpaired *t* test (GraphPad Prism) was used for the data sets (comparison between two different treatments) that passed the normality test, and Mann-Whitney test (GraphPad Prism) was used for the other datasets. Comparison among three different treatments was performed using a one-way analysis of variance (ANOVA) with Bonferroni posttest (GraphPad Prism). As the control level of scratch behavior induced by 48/80 differed between experiments, we only compared data within each experiment. The differences observed may be related to variances between different batches of C57BL6 mice and/or batch differences in the prepared solutions of compound 48/80.

Single-Cell Computational Analysis of Npy2r-Expressing Dorsal Root Ganglion Neurons. Single-cell gene expression data from dorsal root ganglion (DRG) neurons was accessed from Li et al. (GEO accession: GSE63576), Usoskin et al. (GSE59739), and Zeisel et al. (mousebrain.org, l6_r3_spinal_cord_neurons.loom) (Usoskin et al., 2015; Li et al., 2016; Zeisel et al., 2018). Total reads for each cell were normalized to the median read count of all cells, and the counts were further logarithmized [single-cell analysis in Python (SCANPY), log1p] (Wolf et al., 2018). Top 5000 highly variable genes (HVG) were detected for all datasets (SCANPY, filter_gene_dispersion) (Zheng et al., 2017), and the shared genes were used to transform and merge the datasets scaled to unit variance and zero mean (SCANPY, scale) using mutual nearest neighbor transformation (SCANPY, mnn_correct) (Haghverdi et al., 2018). Both the Häring and Zeisel datasets had annotated cell types, which have been summarized by Gatto et al. (2019), and the cell types in these two datasets were mapped according to the functional subtypes of Gatto. A linear support vector classification algorithm (scikit-learn, LinearSVC) (Pedregosa et al., 2011) was trained to predict Gatto cell types using Usoskin and Zeisel gene expression as predictor variables and Gatto cell types as target labels. Cell-type labels were thereafter predicted for Li cells. Cell-type labels were then mapped back to a merged and unscaled dataset containing all genes for expression analysis. A dotplot was constructed grouped by the Gatto cell types (SCANPY, dotplot).

Differentially expressed genes were calculated for the Itch Nppb population against the remaining cells of the dataset, with overestimated variance *t* test as scoring values (SCANPY, rank_genes_groups). Full python code can be found at https://github.com/JonETJakobsson/Npy2r-neurons-in-DRG.

Results

The Y_2 Receptor Regulates Histaminergic and IL-31-Induced Itch. To investigate the role of the NPY/Y_2

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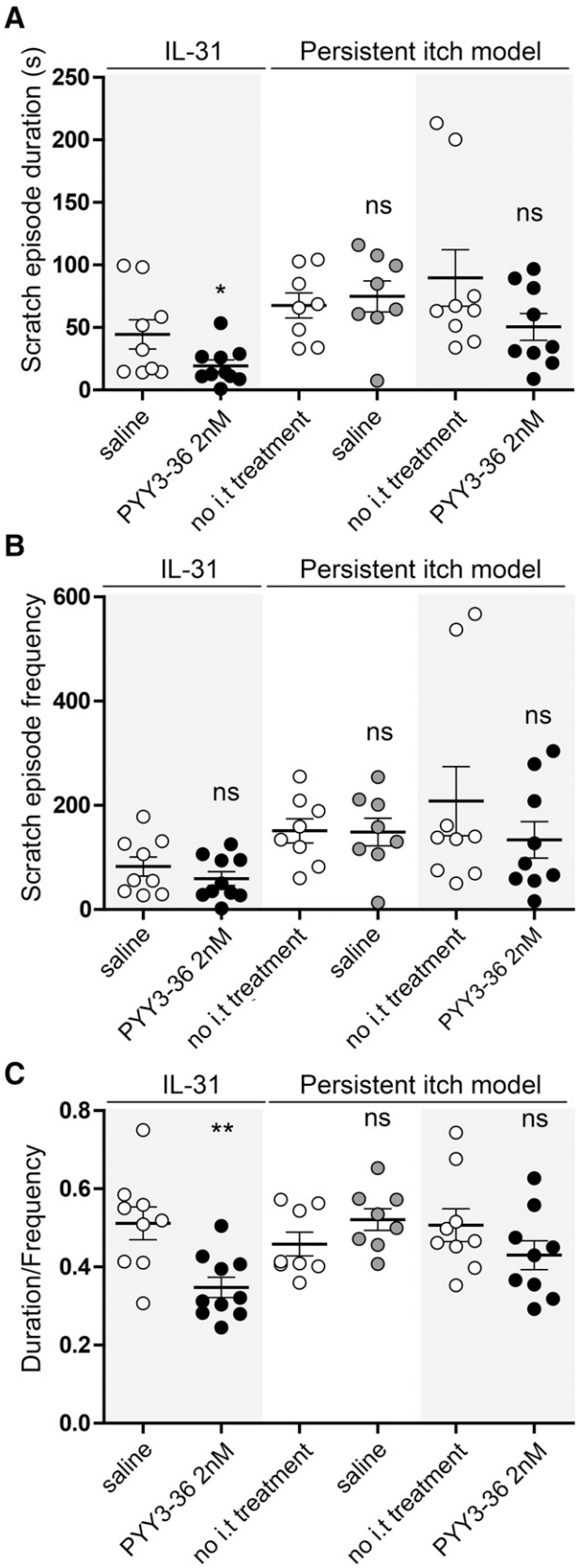

Fig. 4. The NPY/Y2 system regulates IL-31-induced itch but not persistent atopic dermatitis-associated itch. Effect of $PYY_{3–36}$ (2 nM) or saline on IL-31-induced or persistent atopic dermatitis–associated (Nc/Nga) (A) scratch episode duration, (B) scratch episode frequency and, (C) duration/frequency. Each individual animal is plotted in the diagrams. ns > 0.05, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (A) IL-31, Mann Whitney test (GraphPad Prism). (B–C) IL-31, unpaired *t* test, passed D'Agostino and Pearson normality test (GraphPad Prism). (A–C) Persistent itch model Nc/Nga, paired *t* test, passed D'Agostino and Pearson normality test (GraphPad Prism).

system in chemical itch, mice were injected intrathecally with the selective Y_2 agonist $PYY_{3–36}$ (http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=306) or saline followed by an intradermal injection of a pruritogen in the nape of the neck. Intrathecal injection of $PYY_{3–36}$ (200 pM) had no effect on histamine-induced scratch episode duration or frequency ($P > 0.05$), whereas injection with a higher concentration of $PYY_{3–36}$ (2 nM) attenuated the duration ($P < 0.05$) (Fig. 1A) but not the frequency of histamine-induced scratch episodes ($P > 0.05$), although a trend toward fewer scratch episodes was observed (Fig. 1B). The mean lengths of scratch episodes did not differ between $PYY_{3–36}$ (200 pM, 2 nM) and saline-treated mice ($P > 0.05$) (Fig. 1C). Hence, activation of Y_2 dampens histamine-induced itch by reducing the total time spent scratching.

Compound 48/80 is a mast cell degranulator that mainly induces release of histamine, prostaglandins, cytokines, and mast cell proteases (Moon et al., 2014), resulting in itch (Liu et al., 2016). Intrathecal injection of $PYY_{3–36}$ (200 pM) reduced the duration of compound 48/80-induced scratch episodes ($P < 0.01$), whereas the frequency of scratch episodes was similar to that of saline-treated mice ($P > 0.05$) (Fig. 1, A and B). However, when mice were intrathecally injected with the higher concentration of $PYY_{3–36}$ (2 nM), both the duration and the frequency of 48/80-induced scratch episodes were significantly reduced ($P < 0.001$, $P < 0.01$) (Fig. 1, A and B). The mean length of scratch episodes was not changed, neither in 200 pM- ($P > 0.05$) nor in 2 nM $PYY_{3–36}$-treated mice ($P > 0.05$) compared with saline-treated mice (Fig. 1C), showing that Y_2 activation regulates 48/80-induced scratching behavior by reducing both the number of scratch episodes (the perception/initiation of itch) and the total time spent scratching.

To verify the specificity of the Y_2 agonist $PYY_{3–36}$ and its effect on histaminergic itch, the selective Y_2 antagonist BIIE0246 (1 μM) (http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=306) was intrathecally injected 10 minutes before injection of $PYY_{3–36}$ (200 pM). Both the duration and the frequency were reversed to a level similar to that of saline-treated mice upon intradermal injection of compound 48/80 ($P = 0.77$, $P = 0.16$), verifying the selectivity of $PYY_{3–36}$ for the Y_2 receptor (Fig. 2). When only BIIE0246 was intrathecally injected prior to compound 48/80 administration, the mean length of scratch episodes (duration/frequency) was significantly increased compared with saline-treated mice ($P = 0.0015$), showing that the Y_2 antagonist BIIE0246 increased the scratch response, most probably by blocking the binding of endogenous NPY (Fig. 2C).

To evaluate if the NPY/Y_2 system also regulates nonhistaminergic scratch behavior, each of the nonhistaminergic pruritogens, α-methyl-serotonin (5HT; 30 μg), protease-activated receptor-2–activating peptide (SLIGRL; 100 μg), and the antimalarial drug chloroquine (160 μg), were injected intradermally after an intrathecal injection of $PYY_{3–36}$ (2 nM) or saline. There was no difference in scratch episode duration ($P > 0.05$, $P = 0.71$, $P = 0.26$) (Fig. 3A), number of scratch episodes ($P > 0.05$, $P = 0.26$, $P = 0.32$) (Fig. 3B), or the mean length of scratch episodes ($P > 0.05$, $P = 0.71$, $P = 0.26$)

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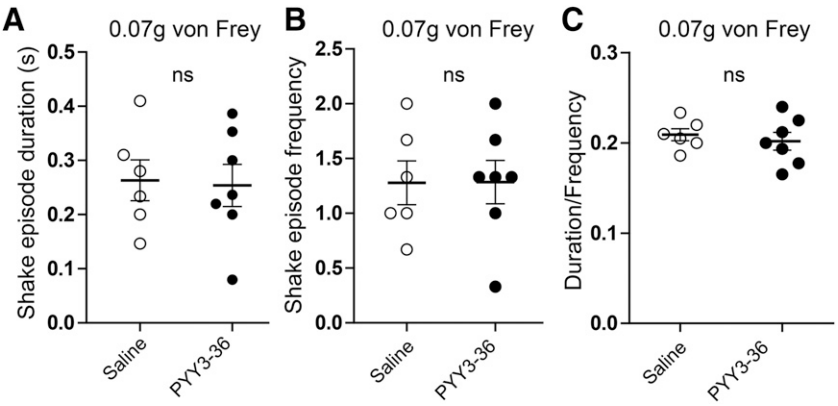

Fig. 5. Activation of the NPY/Y_2 system does not affect mechanical itch. Effect of $PYY_{3–36}$ (2 nM) or saline (A) on shake episode duration induced by five applications of 0.07 gf von Frey filament, (B) on shake episode frequency, or (C) on mean length of shake episode. Each individual animal is plotted in the diagrams. (A–C) Mann-Whitney *t* test (GraphPad Prism).

(Fig. 3C) between $PYY_{3–36}$ and saline after injection of each of the nonhistaminergic pruritogens.

Interleukin (IL)-31 has been implicated in the pathophysiology of atopic dermatitis (Dillon et al., 2004; Nobbe et al., 2012; Kato et al., 2014), and to test the role of the NPY/Y_2 system in the regulation of IL-31-associated itch, mice were given an intradermal injection of IL-31 (12 μM) 10 minutes after an intrathecal injection of $PYY_{3–36}$ (2 nM) or saline. Compared with saline-treated mice, both scratch duration and mean length of scratch episodes were decreased in mice pretreated with $PYY_{3–36}$ ($P = 0.0279$ and $P = 0.0035$, respectively), whereas scratch episode frequency was unaltered (Fig. 4), showing that Y_2 regulates IL-31-induced itch by reducing the total time spent scratching and by shortening the length of the scratch episodes.

To evaluate the involvement of the NPY/Y_2 system in more persistent forms of itch, a translational model for atopic dermatitis was established using the Nc/Nga strain. Intrathecal injection of vehicle did not affect scratch episode duration ($P = 0.61$), number of scratch episodes ($P = 0.93$), or mean length of scratch episodes ($P = 0.22$), compared with the scratch behavior prior to the injection (Fig. 4). Likewise, intrathecal injection of the Y_2 receptor agonist $PYY_{3–36}$ (2 nM) did not affect scratch episode duration ($P = 0.14$), number of scratch episodes ($P = 0.33$), mean length of scratch episodes ($P = 0.07$) (Fig. 4), compared with the scratch behavior prior to the injection, although a trend toward decreased scratch behavior was observed. Thus, although the NPY/Y_2 system can reduce scratching induced by IL-31, an interleukin involved in atopic dermatitis, the NPY/Y_2 system did not have a clear effect in reducing scratch behavior in a translational model of atopy. Atopic dermatitis is also associated with IL-4, IL-5, and IL-13 (Oetjen and Kim, 2018), which may be reflected in the different treatment outcomes.

The Y_2 Receptor Does Not Regulate Acute Mechanical Itch Behavior. To investigate the role of Y_2 in mechanical itch, mice were given an intrathecal injection of $PYY_{3–36}$ (2 nM) or saline, and their acute behavioral response following application of a 0.07 gf von Frey filament to the nape of the neck was monitored. There was no difference in shake duration ($P = 1.0$) (Fig. 5A), shake frequency ($P = 0.88$) (Fig. 5B), or mean length of shake episode ($P = 0.67$) (Fig. 5C) between $PYY_{3–36}$- and saline-injected mice, showing that NPY/Y_2 is not involved in acute mechanical itch transmission. Scratch episode duration, frequency, and mean length of scratch episode were also monitored, but few scratch responses were observed and no differences were observed between the treatments (Supplemental Fig. 1).

Npy2r Primary Afferent Neurons Coexpress Itch-Associated Genes. To relate the observed itch phenotype to gene expression patterns among *Npy2r*-expressing DRG neurons, the DRG single-cell gene expression datasets from Usoskin et al. (2015), Zeisel et al. (2018), and Li et al. (2016) were merged and aligned (Fig. 6A), resulting in a combined set of 2406 DRG neurons (622 from Usoskin, 1580 from Zeisel, and 204 from Li). The hypothesized cell function labels assigned by Gatto were mapped to Usoskin and Zeisel cells. Cell-type labels for neurons in the Li dataset were predicted and assigned Gatto cell-type labels (Fig. 6B). All cell types had cells from each dataset, with the exception of Cool cells, which were almost exclusively from Zeisel dataset (Fig. 6C). Ninety percent of all Itch Nppb neurons, 38% of all Aδ-nociceptors, and 17% of all C-nociceptors expressed *Npy2r* (Fig. 6D). The other cell types had less than 10% expression of *Npy2r*. As the Itch Nppb population had by far the highest *Npy2r* expression, further analysis was focused on this population.

The top differentially expressed genes for the Itch Nppb population were found to include several itch-related genes, such as the neuropeptides *Nppb* and somatostatin (*Sst*), the interleukin 31 receptors *Il31ra* and *Osmr,* the leukotriene receptor *Cystrl2*, the serotonin receptor *Htr1f,* and the NPY receptor *Npy2r* (Dillon et al., 2004; Angelova-Fischer and Tsankov 2005; Nobbe et al., 2012; Mishra and Hoon, 2013; Stantcheva et al., 2016) (Fig. 6D; Table 1). Furthermore, the Itch Nppb population showed expression of other genes related to itch transmission, such as those involved in histaminergic signaling [histamine receptor (*Hrh1*) (low), phospholipase C beta 3 (*Plcb3*), transient receptor potential cation channel subfamily V member 1 (*Trpv1*)] and the SLIGR/SLIGRL receptor PAR2 (*F2rl1*) (low) (Nystedt et al., 1995; Shimada et al., 2006; Liu et al., 2011). Receptors not present or absent in the Itch Nppb population were serotonin receptors (*Htr2a* and *Htr7*), histamine receptors (*Hrh3* and *Hrh4*), BAM8-22/SLIGRL receptor [*Mrgprx1* (also known as *Mrgprc11*)] (Liu et al., 2009; Liu et al., 2011), and the chloroquine receptor *Mrgpra3* (Liu et al., 2009) (Fig. 6D). Thus, the Itch Nppb population of primary afferents displayed enriched expression of several genes involved in pruriceptive transmission, including transducers for histaminergic and IL-31-mediated itch.

Discussion

The results presented here show that the Y_2 receptor is expressed in Itch Nppb primary afferent neurons and that

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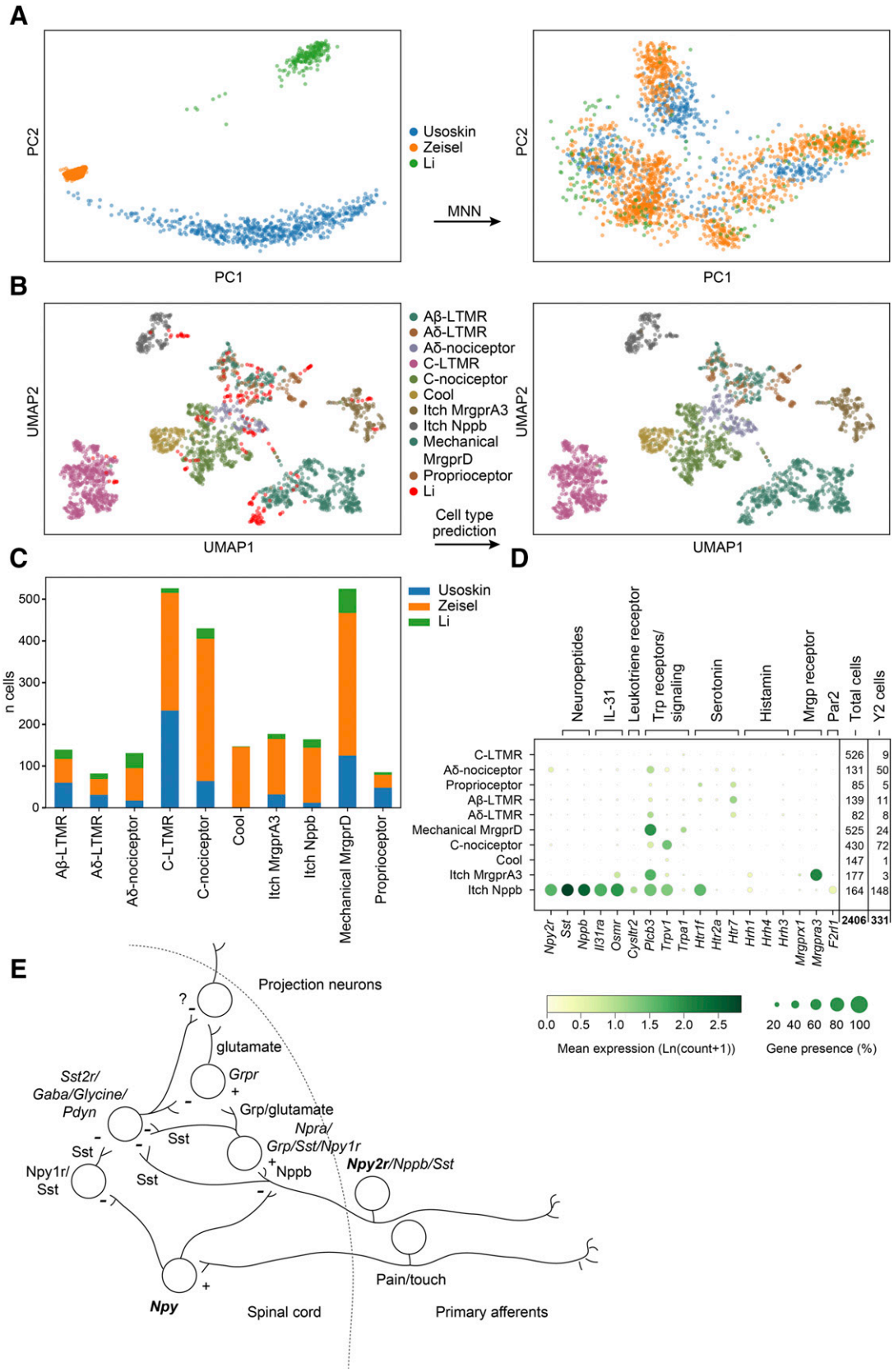

Fig. 6. *Npy2r* primary afferent neurons express itch-associated genes. (A) Datasets were transformed using mutual nearest neighbor transformation. The left PCA plot shows the top two principal components of the scRNA seq neurons from the Usoskin et al., Zeisel et al., and Li et al. dataset, respectively. The right plot represents the top two principal components of neurons after scaling and transformation. (B) Gatto cell-type labels available to Usoskin and Zeisel datasets were used to train a linear support vector classification algorithm that was used to predict cell-type labels of Li neurons. To the left is a uniform manifold approximation and projection (UMAP) plot with Gatto cell types for all neurons in the Usoskin and Zeisel datasets and

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TABLE 1

Differentially expressed genes in the Itch Nppb population

Top 20 differentially expressed genes compared with all other sequenced primary afferent populations. Log fold change represents the log 2-fold change for each gene, Score represents the z-score from P value calculation, and P value was adjusted using the Benjamini-Hochberg method (https://genomebiology.biomedcentral.com/articles/10.1186/s13059-017-1382-0).

Gene	Log Fold Change	Score	*P* Value
Nts	7.6	17.5	1.3E−36
Sst	7.9	15.5	1.3E−30
Il31ra	7.5	15.1	3E−29
Ada	5.7	13.7	2.1E−26
Osmr	5.3	13.6	3.5E−27
Jak1	3.9	13.4	1.1E−28
Nppb	7.4	12.8	2.1E−23
Fam210b	4.2	12.3	5.9E−23
Pxmp2	3.1	12.0	1.3E−23
Htr1f	5.2	11.9	6.4E−22
Npy2r	5.5	11.6	1.3E−20
Tesc	3.4	11.4	4.6E−22
Adk	3.5	11.2	2.9E−21
9230105E05Rik	10.5	11.2	1.1E−18
Mical1	3.6	11.1	5.5E−21
Scg2	3.6	11.1	5.2E−21
Htr1a	4.7	11.0	2.1E−19
Cmtm7	4.7	10.7	1.6E−18
Tspan8	3.7	10.5	3.4E−19
Gm525	6.8	10.4	1E−16

activation of this inhibitory G protein-coupled receptor reduces histamine-, 48/80-, and IL-31-induced itch, whereas the scratch behavioral responses induced by the other tested nonhistaminergic pruritogens or mechanical stimuli were unaffected by the NPY/Y_2 system.

Y2-Primary Afferent Neurons Are Implicated in the Sensations of Touch, Pin-Prick Pain, and Itch. In previous studies, *Npy2r* primary afferents were shown to mediate the sensation of pinprick pain (Arcourt et al., 2017) and be related to low threshold mechanical responsiveness (Li et al., 2011). Arcourt and coworkers (2017) used optogenetics to activate *Npy2r*-Cre-expressing neurons in the dorsal root ganglia, using a bacterial artificial chromosome (BAC) transgene, which resulted in a nocifensive behavior that they related to the selective activation of *Npy2r* nociceptors that showed partial coexpression of (TrkA) and calcitonin gene-related peptide (CGRP) and formed free nerve endings in the skin. Li and coworkers (2011), on the other hand, used a *Npy2r*-GFP mouse line and reported that *Npy2r*-GFP primary afferents are Aβ RA–low-threshold mechanoreceptors (LTMR) that form longitudinal lanceolate endings associated with hair follicles. Thus, previous analyses were inconclusive regarding the role of Y_2 primary afferents in somatosensation.

Our analysis of 2406 primary afferent neurons, from three different scRNA-seq datasets (Usoskin et al., 2015; Li et al., 2016; Zeisel et al., 2018), showed that *Npy2r* mRNA is expressed in just 8% (11/139) of Aβ–LTMR, 10% (8/82) of Aδ-LTMR, and 2% (9/526) of C-LTMR and that most *Npy2r*-expressing cells could be found in the itch-associated class (Itch Nppb 45% (148/331) and pain-associated classes C-nociceptor 22% (72/331) and Aδ-nociceptor 15% (50/331) according to the Gatto nomenclature (Fig. 6D). Moreover, an analysis describing the role of the Runx1 transcription factor in itch-related primary afferents showed that the dorsal root ganglia of *Runx1;Wnt1-Cre* conditional knockout mice were completely ablated of *Npy2r* mRNA and various itch-associated receptors and transducers, including *Nppb*, *Il31ra*, *Osmr*, and *Mrgpra3*, and consequently displayed diminished responses to most pruritogens tested, including 48/80, α-methyl-5HT, IL-31, and chloroquine (Qi et al., 2017), indicating a role for the NPY/Y_2 system in pruritogenic processes. Indeed, our analyses showed that activation of the inhibitory receptor Y_2 dampens histamine-, 48/80-, or IL-31-induced itch. Additionally, Itch Nppb neurons express genes associated with histamine signaling (*Hrh1*, *Trpv1*, *Plcb3*) and IL-31 signaling (*Il31ra*, *Osmr*), and low/absent levels of mRNA for the chloroquine receptor *MrgprA3* or the main SLIGRL receptor *MrgprX1 (MrgprC11)*, showing that NPY and Y_2 are indeed involved in specific regulation of histaminergic and IL-31-evoked itch. Thus far, Y_2 has been associated with Aβ rapidly adapting (RA)-LTMR with respect to touch [(Li et al., 2011) transgenic line], pin-prick pain [(Arcourt et al., 2017) transgenic line], and itch [(Qi et al., 2017) as well as the pharmacological experiments reported here, transgenic line]. Future analysis, using transgenic tools that enable selective targeting of the different subclasses of *Npy2r* mRNA-expressing primary afferent neurons (Itch Nppb, C/Aδ-nociceptors, and Aβ/Aδ/C- LTMR, respectively), would enable us to link each population to a specific sensory role.

A potential caveat when using pharmacological tools is their limited anatomic specificity. Because low expression of *Npy2r* also can be detected in 5.5% of spinal dorsal horn neurons, with 77.6% of these belonging to inhibitory and 22.4% to excitatory subpopulations (Häring et al., 2018), we cannot rule out that Y_2 could have functions in the modulation of pruriceptive signaling also within the spinal cord. However, the expression level of *Npy2r* is limited and the overlap

Li dataset in red. Right figure represents the neurons after cell-type prediction. (C) Distribution of neurons from different datasets in cell-type populations. Height of bars represents number of cells. (D) Dotplot of itch-related genes grouped by cell type. Percent of cells in a population that expresses a gene is represented by the dot size. The mean expression of a gene in the population is represented by the dot color. (E) Simplified schematic drawing that indicates how we suggest that the NPY/Y_1/Y_2 system inhibits chemical itch transmission. Circles denote populations of neurons. Extended from Mishra and Hoon (2013), Kardon et al. (2014), and Gao et al. (2018).

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between *Npy2r* and key itch-associated markers in the spinal cord are low. Only 3.5% of *Npy2r* neurons express *Grpr* or *Bhlhb5 (Bhlhe22)*, whereas 17.6% express Grp, but only 1.1% express both the *Npr1* (the Nppb receptor) and *Grp* transcripts, thus representing the Nppb/itch-associated part of the Grp population (Mishra and Hoon 2013; Häring et al., 2018). Also, dorsal rhizotomy completely abolishes Y_2 immunoreactivity in the spinal cord (Brumovsky et al., 2005), suggesting that Y_2 is predominately located to the central terminals of primary afferents projecting to the spinal cord and that NPY/Y_2 inhibits itch mainly through the interaction with itch-associated primary afferents.

Itch Nppb Primary Afferents Coexpress Somatostatin and Nppb. The neuropeptides *Nppb* and *Sst* are highly coexpressed in the Itch Nppb population (Gatto et al., 2019) (Fig. 6D). Nppb is a well established mediator of itch (Mishra and Hoon, 2013; Pitake et al., 2018), and activation of Sst primary afferents induces scratching (Huang et al., 2018), whereas ablation of primary afferent Sst neurons decreases scratching induced by IL-31, histamine, chloroquine, and LY344864 (a $5HT_{1F}$ agonist) (Stantcheva et al., 2016), arguing that *Npy2r*/*Nppb*/*Sst* -rich primary afferents mediate itch (Fig. 6E).

Five somatostatin receptor subtypes are found in mammals, and they have similar signaling pathways (http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=61). Among these, the Sst2a receptor is almost exclusively expressed by inhibitory neurons in the spinal cord (Häring et al., 2018). This ligand-receptor interaction leads to neuronal hyperpolarization (Yin et al., 2009), and targeting Sst2a with a specific agonist increases, whereas a specific antagonist decreases, histamine-, Nppb-, and Grp-induced scratching, suggesting a disinhibition mechanism (Huang et al., 2018). As *Sst*, *Nppb*, and *Npy2r* display extensive overlap in primary afferent neurons, this suggests that the Itch Nppb neurons may promote scratching both by driving itch circuits via excitation and disinhibition (Fig. 6E).

Spinal NPY Interneurons Can Dampen Itch in Several Possible Ways. Spinal NPY interneurons are activated by noxious mechanical, chemical, temperature, and touch stimuli (Liu et al., 2010; Polgar et al., 2013; Bourane et al., 2015) and hence could represent neuronal population–enabling counter-stimuli, for instance, scratch or noxious heat, to inhibit itch (Fig. 6E). Ablation of spinal NPY neurons results in alloknesis to mechanical stimuli and spontaneous scratching, indicating that NPY neurons indeed gate/inhibit itch (Bourane et al., 2015). We have recently shown that selective activation of spinal Y_1-expressing neurons reduces the duration and frequency of mechanically induced scratching and the duration of histamine- or 48/80-induced scratching, resulting in shorter scratch episodes (Gao et al., 2018). The Y_1 receptor is mainly expressed by excitatory interneurons (Häring et al., 2018), showing partly overlapping Sst expression (Zhang et al., 1999). Sst in turn inhibits inhibitory (disinhibition) class B basic helix-loop-helix protein 5 (Bhlhb5) neurons implicated in the regulation of itch (Ross et al., 2010; Kardon et al., 2014) (Fig. 6E). Hence, our present data indicate that NPY can reduce itch (scratching behavior) both through Y_1-mediated inhibition of spinal circuits, which reduces the length of the episodes of scratching (counter-stimuli) (Gao et al., 2018), and through Y_2-mediated inhibition of itch-transmitting primary afferents, which results in a reduction of both the initiation of scratching/perception of itch (fewer scratch episodes) and the duration of the behavior (Fig. 6E). The NPY/Y_1/Y_2 system thus constitutes an elaborate circuit that controls itch transmission both by reducing the perceived itch and by making the counter-stimuli (scratch) more efficient in relieving itch.

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Authorship Contributions

Participated in research design: Lagerström, Gao, Ma, Jakobsson, Xu, Larhammar, Weman.

Conducted experiments: Gao, Ma, Jakobsson, Weman.

Contributed new reagents or analytic tools: Larhammar, Xu.

Performed data analysis: Gao, Ma, Jakobsson, Weman, Lagerström.

Wrote or contributed to the writing of the manuscript: Gao, Ma, Jakobsson, Weman, Larhammar, Xu, Lagerström.

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Supplemental data

The Neuropeptide Y Y_2 receptor is co-expressed with Nppb in primary afferent neurons and Y_2 activation reduces histaminergic and IL-31-induced itch

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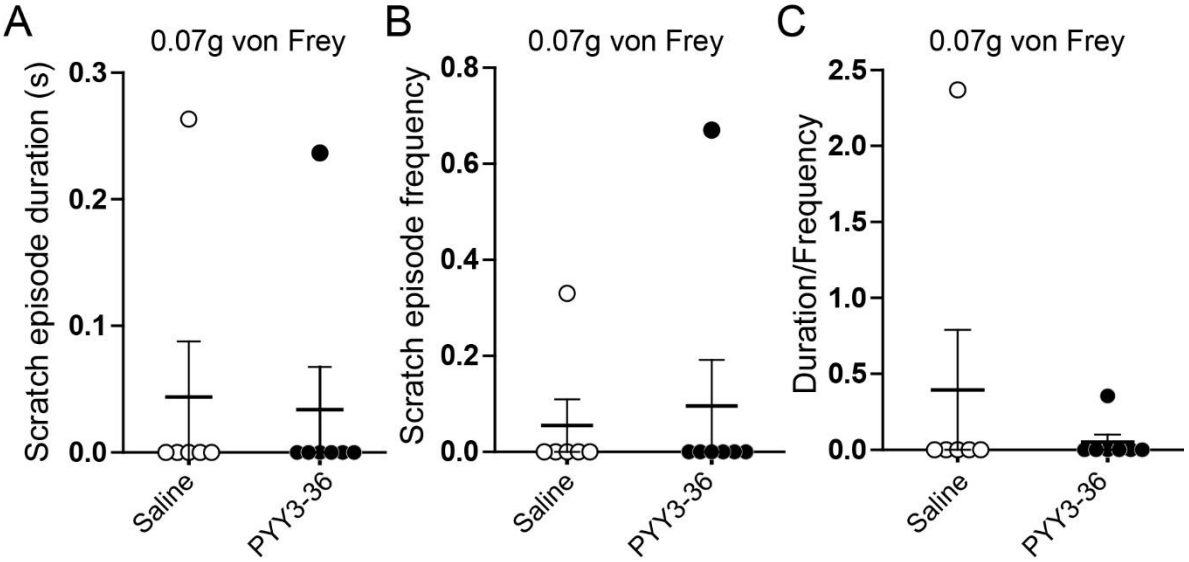

Figure S1. Activation of the NPY/Y_2 system does not affect mechanical itch. (A) The effect of PYY3-36 (2 nM) or saline on mean scratch episode duration induced by five applications of 0.07 g von Frey filament to the nape of the neck (P=0.91), **(B)** mean scratch episode frequency (P=0.91) or **(C)** duration/frequency (P=0.91). (A-C) Mann-Whitney t-test (GraphPad Prism, CA).