A sensory neuron–expressed IL-31 receptor mediates T helper cell–dependent itch: Involvement of TRPV1 and TRPA1

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Background: Although the cytokine IL-31 has been implicated in inflammatory and lymphoma-associated itch, the cellular basis for its pruritic action is yet unclear.
Objective: We sought to determine whether immune cell–derived IL-31 directly stimulates sensory neurons and to identify the molecular basis of IL-31–induced itch.
Methods: We used immunohistochemistry and quantitative real-time PCR to determine IL-31 expression levels in mice and human subjects. Immunohistochemistry, immunofluorescence, quantitative real-time PCR, *in vivo* pharmacology, Western blotting, single-cell calcium imaging, and electrophysiology were used to examine the distribution, functionality, and cellular basis of the neuronal IL-31 receptor α in mice and human subjects.
Results: Among all immune and resident skin cells examined, IL-31 was predominantly produced by T_H2 and, to a significantly lesser extent, mature dendritic cells. Cutaneous and intrathecal injections of IL-31 evoked intense itch, and its concentrations increased significantly in murine atopy-like dermatitis skin. Both human and mouse dorsal root ganglia neurons express IL-31RA, largely in neurons that coexpress transient receptor potential cation channel vanilloid subtype 1 (TRPV1). IL-31–induced itch was significantly reduced in TRPV1-deficient and transient receptor channel potential cation channel ankyrin subtype 1 (TRPA1)–deficient mice but not in *c-kit* or proteinase-activated receptor 2 mice. In cultured primary sensory neurons IL-31 triggered Ca^{2+} release and extracellular signal-regulated kinase 1/2 phosphorylation, inhibition of which blocked IL-31 signaling *in vitro* and reduced IL-31–induced scratching *in vivo*.
Conclusion: IL-31RA is a functional receptor expressed by a small subpopulation of IL-31RA$^+$/TRPV1$^+$/TRPA1$^+$ neurons and is a critical neuroimmune link between T_H2 cells and sensory nerves for the generation of T cell–mediated itch. Thus targeting neuronal IL-31RA might be effective in the management of T_H2-mediated itch, including atopic dermatitis and cutaneous T-cell lymphoma. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: *Cytokine, atopic dermatitis, sensory nerve, skin, transient receptor potential channel*

Cytokines are critical contributors to various inflammatory skin diseases and cutaneous malignancies that are also pruritic, notably atopic dermatitis (AD) and cutaneous T-cell lymphoma.[1-5] How cytokines exert their pruritic effects and the extent to which there is direct or indirect involvement of sensory nerves that express specific cytokine receptors is currently unclear. Because many inflammatory and malignant pruritic skin diseases have an associated T_H2 cell signature, analysis of the interplay between T_H2 cells and sensory neurons will significantly enhance our understanding of the mechanisms underlying communication between the adaptive immune system and the nervous system to induce itch and therefore how to treat recalcitrant itch in human subjects.

Levels of IL-31, a T_H2 cell–derived cytokine, are increased in pruritic atopic skin and cutaneous T-cell lymphoma in human subjects,[3,6,7] and the cytokine induces severe pruritic atopic-like dermatitis (AD-like) in an IL-31 transgenic mouse model.[6] Moreover, neutralization of IL-31 in NC/Nga mice, an AD-like mouse model, reduced scratching and improved wound healing.[8]

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Supported by grants from the National Institutes of Health (NIH)/National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS; AR059402), Deutsche Forschungsgemeinschaft (DFG; Ste1014/2-2), IZKF Muenster (STE3/034/07), and CE.R.I.E.S. Paris (to M.S.); NIH NS14627 (to A.B.); NIH AR057194 (to E.C.); DFG Ce165/1-1 (to F.C.); DFG Ke1672/1-1 (to C.K.); and DFG (Ho2092/4-1) and DFG-FOR729 (Ho2092/5-2; to B.H.).

Disclosure of potential conflict of interest: C. Kempkes, M. Feld, and B. Homey have been supported by one or more grants from Deutsche Forschungsgemaeinschaft (the German Research Foundation). A. Ikoma is employed by Galderma Japan. S. R. Dillon has received research support from and is employed by ZymoGenetics (a wholly owned subsidiary of Bristol-Meyers Squibb) and owns stock/stock options in Bristol-Meyers Squibb. A. Basbaum has received research support from the National Institutes of Health (NIH). M. Steinhoff has received research support from the NIH/National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) and BMS/Zymogenetics. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication March 29, 2013; revised October 18, 2013; accepted for publication October 23, 2013.

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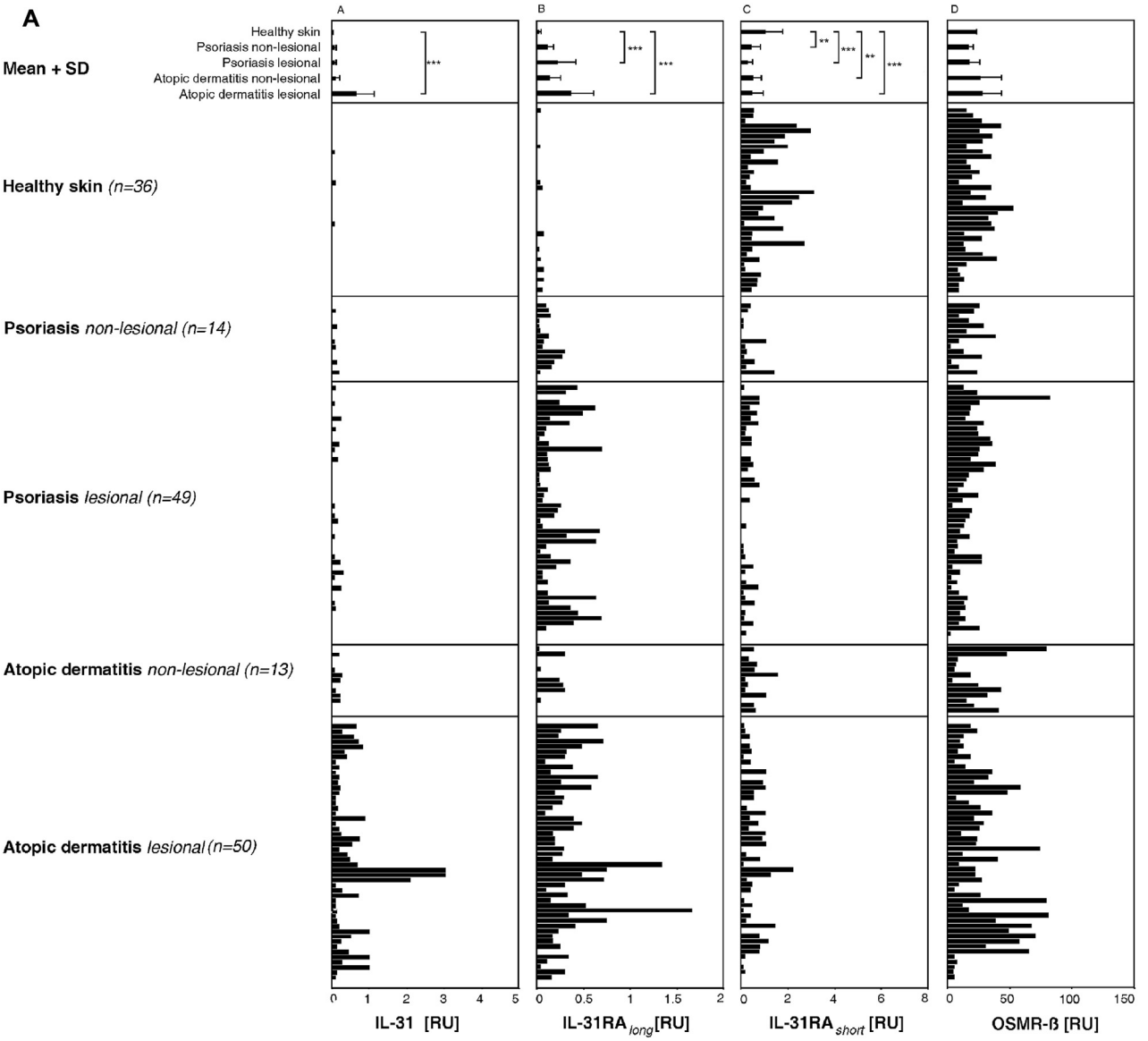
0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2013.10.048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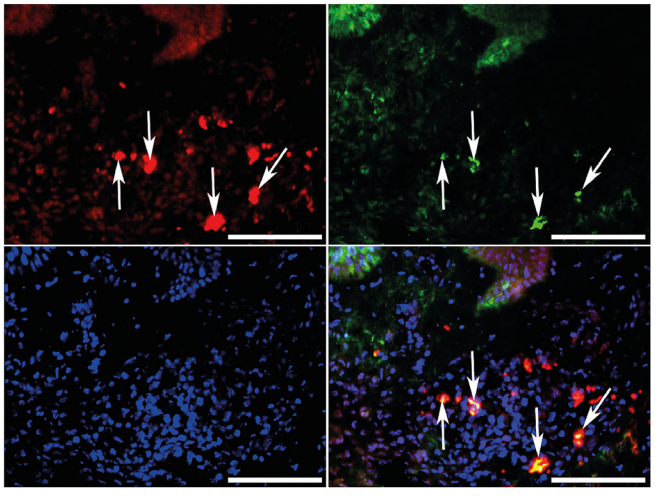
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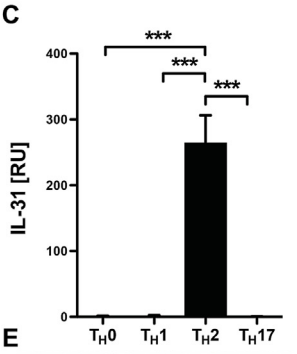
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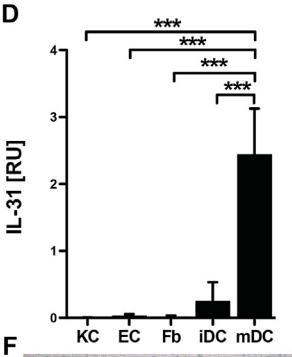
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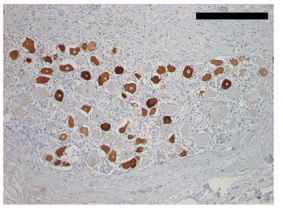
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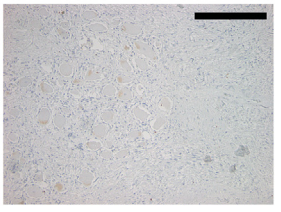
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Abbreviations used
AD: Atopic dermatitis
AITC: Allyl isothiocyanate
DRG: Dorsal root ganglia
ERK: Extracellular signal-regulated kinase
GRPR: Gastrin-releasing peptide receptor
HBSS: Hanks balanced salt solution
hpf: High-power field
IB4: Isolectin B4
KO: Knockout
MEK: Mitogen-activated protein kinase enzyme
Mrgpr: Mas-related G protein–coupled receptor
NPR-A: Natriuretic peptide receptor A
OSMRβ: Oncostatin M receptor β
OVA: Ovalbumin
PAR-2: Proteinase-activated receptor 2
qPCR: Quantitative real-time PCR
SC: Spinal cord
SEB: Staphylococcal enterotoxin B
TG: Trigeminal ganglion
TRPA1: Transient receptor channel potential cation channel ankyrin subtype 1
TRPV1: Transient receptor potential cation channel vanilloid subtype 1

IL-31 binds to IL-31RA, which exists as a short nonsignaling/inhibitory or a long signaling subunit.[9] To date, no long or short form of IL-31RA has been described in mice.

Although IL-31 receptor α is expressed in murine neuronal tissue,[10] detailed information about the neuroimmune link and functional relevance of IL-31RA$^+$ neurons is lacking.

Recent studies focused on the function of sensory neurons in itch.[11-13] Thus far, transient receptor potential cation channel vanilloid subtype 1 (TRPV1)$^+$ and a subpopulation of TRPV1$^+$/transient receptor channel potential cation channel ankyrin subtype 1 (TRPA1)$^+$ sensory neurons have been implicated to be required for pruritogen-induced itch signaling.[14-18] Whether cytokine-induced itch has a comparable neuronal basis is unknown. Therefore the aim of our study was determine the cellular basis of IL-31–induced itch.

METHODS

Materials

Recombinant mouse IL-31 was provided by ZymoGenetics (Seattle, Wash). For details, see the Methods section in this article's Online Repository at www.jacionline.org.

Patients

Patients and healthy control subjects were included after providing written informed consent within a study protocol approved by the ethics committees of the University Hospital Muenster, Heinrich-Heine-University Düsseldorf, and University Hospital Göttingen, Germany. For details, see the Methods section in this article's Online Repository.

Purification of naive CD4$^+$ T lymphocytes from adult blood and T helper cells

PBMCs were separated from buffy coats of healthy blood donor volunteers. For details, see the Methods section in this article's Online Repository.

Cell isolation and cell culture of human cells

For details, see the Methods section in this article's Online Repository.

Quantitative real-time PCR (TaqMan)

Quantitative real-time PCR (qPCR) was performed to analyze expression of IL-31, IL-31RA, and oncostatin M receptor β (OSMRβ) in lesional versus nonlesional skin of patients with AD versus healthy human subjects. For details, see the Methods section in this article's Online Repository.

Mouse model of AD and bacterial superantigen–induced skin inflammation

To determine IL-31 levels from AD-like skin lesions, we used 2 established mouse models, namely treatment with ovalbumin (OVA) or staphylococcal enterotoxin B (SEB), as described recently.[19,20] For details, see the Methods section in this article's Online Repository.

Pruritogen-induced scratching

For details, see the Methods section in this article's Online Repository.

Immunostaining of mouse dorsal root ganglia and spinal cord

Cryosections of murine spinal cord (SC; 10 μm) and dorsal root ganglia (DRG; 10 μm) were used. For details, see the Methods section in this article's Online Repository.

Primary DRG culture

Mice were anesthetized by means of intraperitoneal injection of pentobarbital perfused transcardially with Ca^{2+}-free and Mg^{2+}-free PBS. DRG neurons were cultured, as previously described.[21] For details, see the Methods section in this article's Online Repository.

Calcium imaging

Upper cervical to midcervical mouse DRG were enzymatically digested and processed for calcium imaging, as previously described.[21,22] For details, see the Methods section in this article's Online Repository.

Western blotting

DRG neurons from primary cell culture were homogenized by means of hot lysis in protein lysis buffer containing a protease and phosphatases inhibitor mixture (Roche Applied Science, Penzberg, Germany) and sonicated. Then cell debris was removed by means of centrifugation (14,000*g* at 4°C for 10 minutes). Samples were processed, as previously described.[21] For details, see the Methods section in this article's Online Repository.

RESULTS

Production of IL-31 by human T_H2 cells

Several studies have demonstrated that IL-31 is expressed by skin-homing T_H2 cells during inflammation, most notably in

FIG 1. IL-31 derives from human T_H2 cells, and IL-31RA is expressed on human DRG neurons. **A,** qPCR of IL-31, the IL-31RA long isoform, the IL-31RA short isoform, and OSMRβ. **B,** Colocalization of cutaneous lymphocyte–associated antigen *(red)* and IL-31 *(green)* in AD skin. *Scale bar* = 100 μm. **C,** Human T_H2 cells express IL-31 mRNA. **D,** Mature *(mDC)* dendritic cells express IL-31 mRNA. *EC,* Endothelial cells; *Fb,* fibroblast; *KC,* keratinocyte. **E,** IL-31RA immunostaining in human DRG neurons. *Scale bar* = 50 μm. **F,** Control. ***P < .001, Mann-Whitney *U* test.

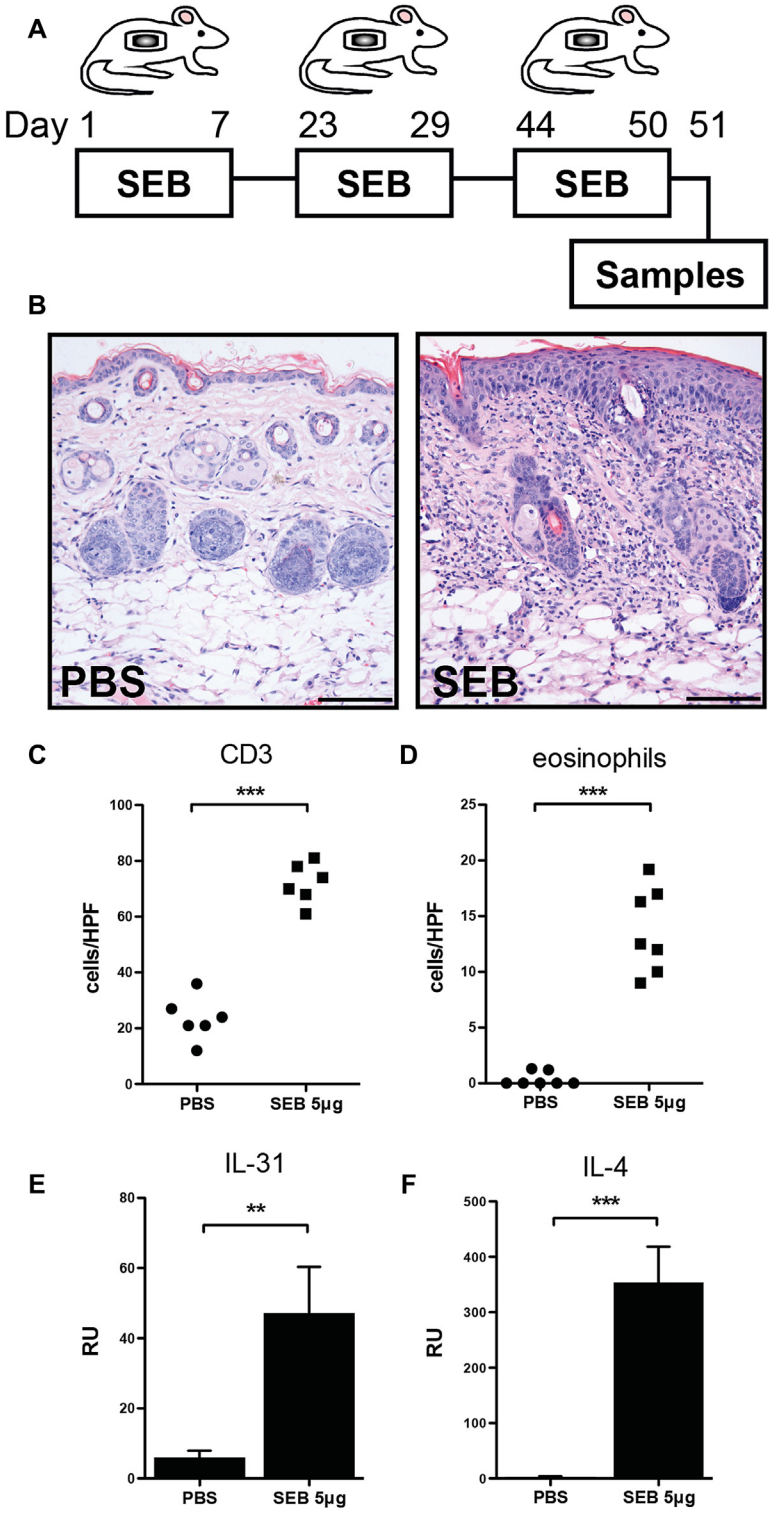


FIG 2. Superantigen-induced upregulation of IL-31 in an AD-like mouse model. **A,** Treatment regimen. **B,** Hematoxylin and eosin staining of vehicle (PBS)– and SEB-treated skin. *Scale bar* = 200 μm. **C** and **D,** Number of $CD3^+$ T cells (Fig 2, *C*) and eosinophils (Fig 2, *D*) in vehicle- versus SEB-treated skin. **E** and **F,** qPCR from skin samples reveals increased mRNA levels for IL-31 (Fig 2, *E*) and IL-4 (Fig 2, *F*) in SEB-treated skin. N = 8 mice per group. $^{**}P < .01$ and $^{***}P < .001$, Student *t* test. *Error bars* indicate SEMs.

patients with AD.[6,7,19,23] No study has systematically compared expression levels of IL-31 in all potentially relevant immune and permanent skin cells involved in AD. Using qPCR,[20] we compared expression levels of IL-31 and its receptor, IL-31RA, in various immune and permanent skin cells of patients with AD and psoriasis. Skin specimens were obtained from healthy

donors (n = 35), patients with AD (nonlesional, n = 13; lesional, n = 50), and patients with psoriasis (nonlesional, n = 14; lesional, n = 49). IL-31 mRNA transcript expression was increased (approximately 4-fold) in lesional skin of patients with AD compared with nonlesional or healthy skin (Fig 1, *A*). Lesional AD skin showed significantly higher levels of the IL-31RA long isoform compared with healthy skin ($P < .001$; Fig 1, *A*), whereas no statistical differences were observed for the inhibitory short isoform, which was largely expressed in healthy skin. In patients with psoriasis, the IL-31RA long isoform was also upregulated, although to a lesser extent than in lesional skin of patients with AD. Of note, although IL-31 was significantly upregulated in patients with AD, this was not the case for those with psoriasis. Compared with specimens from patients with AD, neither lesional nor nonlesional specimens from patients with psoriasis showed upregulation of either subform. OSMRβ was equally expressed in all examined samples (Fig 1, *A*).

We next colocalized IL-31 (green) with skin-infiltrating cutaneous lymphocyte–associated antigen–positive cells (red) in human lesional AD skin (Fig 1, *B*, colocalization, yellow cells and arrows). As expected in patients with AD, T_H2 cells were found almost exclusively in the dermis. Quantitative analysis of immunofluorescence revealed 62% ± 8.2% of skin-homing $CRTH2^+$ T_H2 cells to be positive for IL-31 (n ≥ 10 patients per group).

Next, we used qPCR from isolated human T-cell subtypes to compare IL-31 mRNA expression in the subsets of T cells and found that IL-31 was predominantly expressed by T_H2 cells and very unlikely to be derived from T_H0, T_H1, or T_H17 cells (Fig 1, *C*). We did not detect IL-31 mRNA in other immune or resident skin cells (keratinocytes, endothelial cells, and fibroblasts; Fig 1, *D*). The only other source in human skin appears to be mature dendritic cells, although at significantly lower levels compared with T_H2 cells (approximately 100-fold; Fig 1, *D*). Therefore we identified T_H2 cells as the major, if not exclusive, source of IL-31 in human atopic skin. Whether mature dendritic cells can also generate physiologically relevant quantities of IL-31 in patients with certain diseases is unknown.

Human DRG neurons express IL-31RA

Given the importance of IL-31 in pruritic skin diseases[1-4,6,19,23-25] and the detection of IL-31RA mRNA in human skin,[1-3] we next used immunohistochemistry to analyze the distribution of IL-31RA in DRG neurons obtained from human cadavers (Fig 1, *E*). We found 50.6% of small-diameter DRG neurons (<30 μmol/L) were $IL\text{-}31RA^+$, whereas all large-diameter DRG neurons (>50 μmol/L) were $IL\text{-}31RA^-$. Preabsorption control verifies specificity of the IL-31RA staining in human DRG neurons (Fig 1, *F*).

Upregulation of IL-31 in murine atopic-like dermatitis

We used topical application of the superantigen SEB (Fig 2, *A*) to produce an AD-like phenotype in mice (Fig 2, *B*).[7,20] The inflammatory infiltrate consisted of high numbers of $CD3^+$ T cells (Fig 2, *C*) and eosinophils (Fig 2, *D*) comparable with those seen in human AD. IL-31 mRNA was significantly upregulated in the skin of SEB-treated mice compared with that seen in vehicle-treated mice (Fig 2, *E*). IL-4, a second T_H2-associated cytokine, was also significantly upregulated in the skin ($P < .001$) after SEB treatment (Fig 2, *F*). In a second AD-like model, we used OVA (see Fig E1, *A-D*, in this article's Online Repository at www.jacionline.org) and also observed upregulation of IL-31 and IL-4, as observed in the SEB model. Thus IL-31 in both human AD and AD-like mouse models derives from cutaneous T_H2 cells and might activate IL-31RA on sensory nerves.

Intradermal IL-31 induces itch, but not pain, in murine skin

The underlying mechanism of IL-31–induced itch and effects of IL-31 on itch versus pain have not been studied yet. Fig 3, *A*, shows that IL-31 produces dose-dependent scratching after intradermal injection into the nape of the neck (50 ± 6.89 bouts/30 minutes with 1.575 nmol/40 μL, 90.67 ± 10.36 bouts/30 minutes with 3.15 nmol/40 μL, and 121.1 ± 12.79 bouts/30 minutes with 6.3 nmol/40 μL; vehicle produced only 15.2 ± 1.2 bouts/30 minutes; $P \leq .0001$).

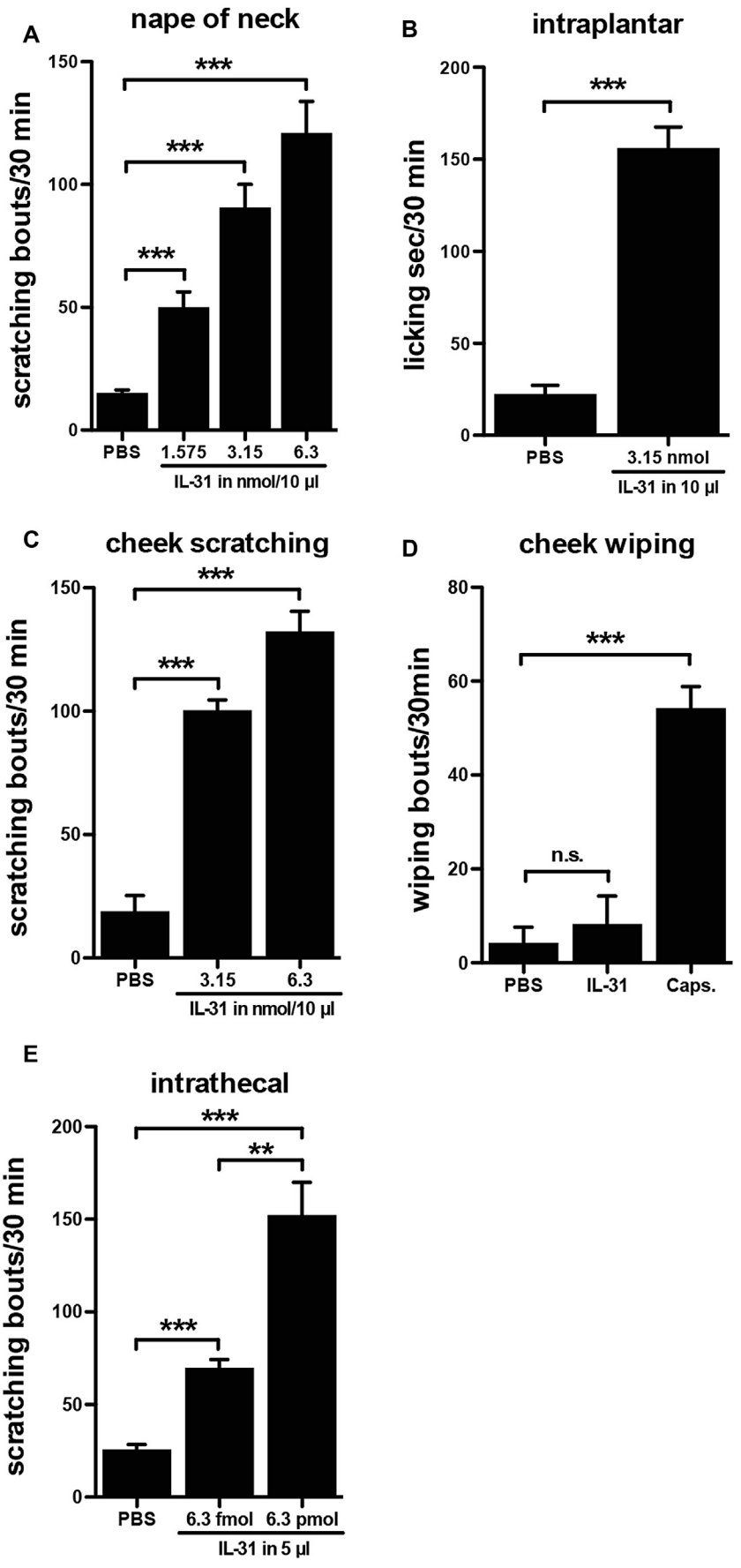
Intraplantar hind-paw injection (Fig 3, *B*) of IL-31 (3.15 nmol/10 μL) evoked profound paw licking (156.2 ± 11.39 seconds/30 minutes vs 22.6 ± 4.55 seconds/30 minutes with vehicle, $P \leq .0001$). IL-31 injection into the cheek (Fig 3, *C*)[26,27] provoked robust scratching (100.4 ± 4.16 bouts/30 minutes with 3.15 nmol/10 μL and 132.4 ± 8.13 bouts/30 minutes with 6.3 nmol/10 μL vs 18.8 ± 6.4 bouts/30 minutes with vehicle, $P = .002$). No differences were obtained for IL-31–induced wiping behavior compared with that seen with the control (8.25 ± 6.93 bouts/30 minutes with 3.15 nmol/10 μL IL-31 vs 4.25 ± 3.84 bouts/30 minutes with vehicle; Fig 3, *D*). As expected, capsaicin (a positive control for a painful stimulus) evoked significant wiping (54.25 ± 5.32 for 10 μg/10 μL; Fig 3, *D*).

Intrathecal IL-31 evokes itch in mice

We next examined whether itch can be provoked with an approach that bypasses the skin (Fig 3, *A-C*). To assess a possible direct action on central nervous system circuitry, including the central terminals of primary afferents, we injected IL-31 intrathecally (directly into the cerebrospinal fluid) at the lumbar level in mice, which induced caudally directed scratching (Fig 3, *E*). This was dose dependent, ranging from 69.83 ± 4.47 bouts/30 minutes (6.3 fmol/5 μL) to 152.3 ± 17.63 bouts/30 minutes (6.3 pmol/5 μL, $P < .0001$). These findings suggest that IL-31 can induce itch by directly targeting SC circuits, including the central terminals of primary afferents.

IL-31RA is localized in $TRPV1^+$ peptidergic murine DRG neurons

We used immunohistochemistry to localize IL-31RA in the DRG, trigeminal ganglion (TG), and SC. Consistent with our results from human DRG (Fig 1, *E*), we found IL-31RA immunoreactivity predominantly in small- to medium-diameter murine DRG neurons (Fig 4, *A*), which is equivalent to approximately 3.4% of the total neuron population; expression in TG was comparable (see Fig E2 in this article's Online Repository at www.jacionline.org). Importantly, there is complete coexpression of IL-31RA and TRPV1, a marker for capsaicin-responsive, peptidergic DRG neurons (Fig 4, *A*). However, only 16.2% ± 0.7% of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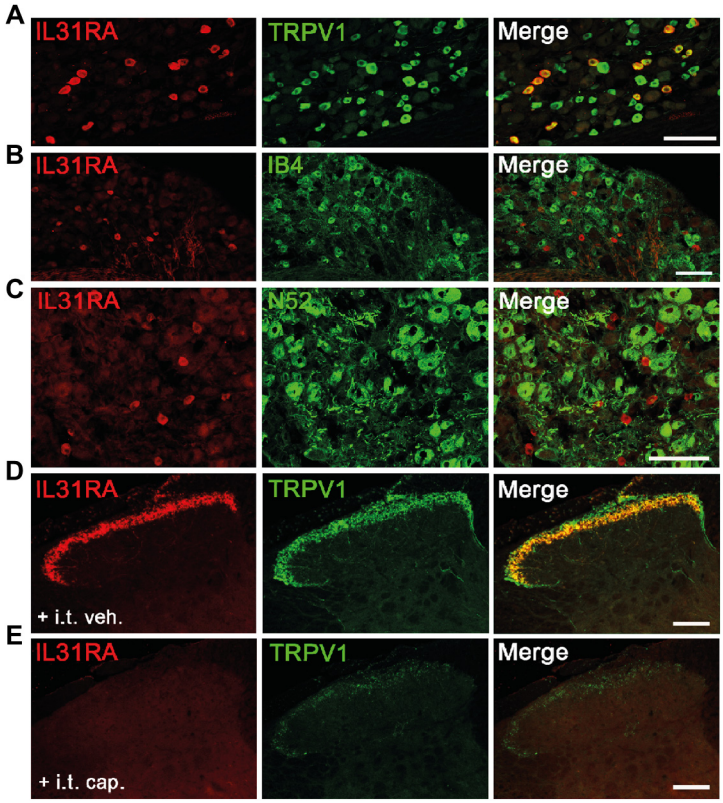

FIG 4. Localization of IL-31RA in murine DRG and SC. **A,** IL-31RA$^+$ *(red)* and TRPV1$^+$ *(green)* neurons partly colocalize. **B,** Minimal overlap of IL-31RA *(red)* and IB4$^+$ *(green)* subset of nonpeptidergic nociceptors. **C,** No overlap of IL-31RA$^+$ *(red)* and N52$^+$ unmyelinated neurons *(green)*. **D,** IL-31RA$^+$/TRPV1$^+$ in nerve terminals of the superficial dorsal horn. Intrathecal capsaicin *(i.t. cap.)*; **(E)** but not vehicle (Fig 4, *D*) ablated TRPV1$^+$ *(green)* and IL-31RA *(red)* immunoreactivity. *Scale bars* = 100 μm.

TRPV1$^+$ neurons are IL-31RA$^+$, and 6.7% ± 0.4% bound the lectin isolectin B4 (IB4), which marks the nonpeptidergic subpopulation of unmyelinated sensory neurons (Fig 4, *B*). We found no overlap of IL-31RA$^+$ neurons with N52 (a marker of cell bodies with myelinated axons; Fig 4, *C*). In the SC (Fig 4, *D*) we found a complete overlap of IL-31RA and TRPV1 in axon terminals and no evidence of postsynaptic expression of IL-31RA. The IL-31RA immunoreactivity was concentrated in outer lamina II, corresponding to the most ventral distribution of TRPV1 terminals. As expected, intrathecal injection of capsaicin, a neurotoxin that ablates central TRPV1 terminals,[28,29] produced a significant loss of both TRPV1$^+$ and IL-31RA$^+$ immunoreactive terminals in the dorsal horn (Fig 4, *E*). Importantly, specificity of the IL-31RA antibody was demonstrated by the absence of IL-31RA immunoreactivity in DRG neurons obtained from IL-31RA knockout (KO) mice (see Fig E3 in this article's Online Repository at www.jacionline.org). Thus a small subset of unmyelinated peptidergic (TRPV1$^+$) primary sensory neurons in DRG neurons and the trigeminal neurons express IL-31RA (Fig 4 and see Fig E2).

Neuronal mechanisms of IL-31–mediated itch

Previous studies in mice demonstrated that TRPV1- or TRPA1-expressing DRG neurons are important contributors to scratching behavior.[14-18,28-30] Whether transient receptor potential channels are involved in IL-31–mediated itch is unknown. We found that intrathecal capsaicin-treated mice markedly reduced IL-31–induced scratching (6.3 pmol/5 μL: 61 ± 13.7 bouts/30 minutes in intrathecal capsaicin-treated mice vs 133.3 ± 14.49 bouts/30 minutes in intrathecal vehicle-treated mice; Fig 5, *A*). We next injected IL-31 (6.3 nmol/40 μL) into the nape of the neck of TRPV1 KO mice and observed a significant reduction in scratching bouts (47.75 ± 2.56 bouts/30 minutes in TRPV1 KO vs 140 ± 23.97 bouts/30 minutes in WT littermates, $P = .0086$; Fig 5, *B*). These findings demonstrate that TRPV1 is itself critical to IL-31–evoked itch.

Because TRPA1 is required for Mas-related G protein–coupled receptor (Mrgpr)– and endothelin-1–mediated itch,[14,16,30,31] we also studied the consequence of TRPA1 deletion. Fig 5, *C*, shows that there is a significant reduction in IL-31 (6.3 nmol/40 μL)–induced scratching after nape of the neck injection in TRPA1

FIG 3. *In vivo* effects of IL-31 in mice. **A,** Injection of IL-31 into the nape of neck induced profound scratching. **B,** Intraplantar IL-31 significantly increased paw licking. **C** and **D,** Cheek injection of IL-31 only produced scratching (Fig 3, *C*) but no wiping (Fig 3, *D*). **E,** Intrathecal injection of IL-31 induced significant dose-dependent scratching compared with vehicle. N = 8 mice per group. $^{**}P < .01$ and $^{***}P < .001$, Student *t* test. *Error bars* indicate SEMs. *n.s.*, Not significant.

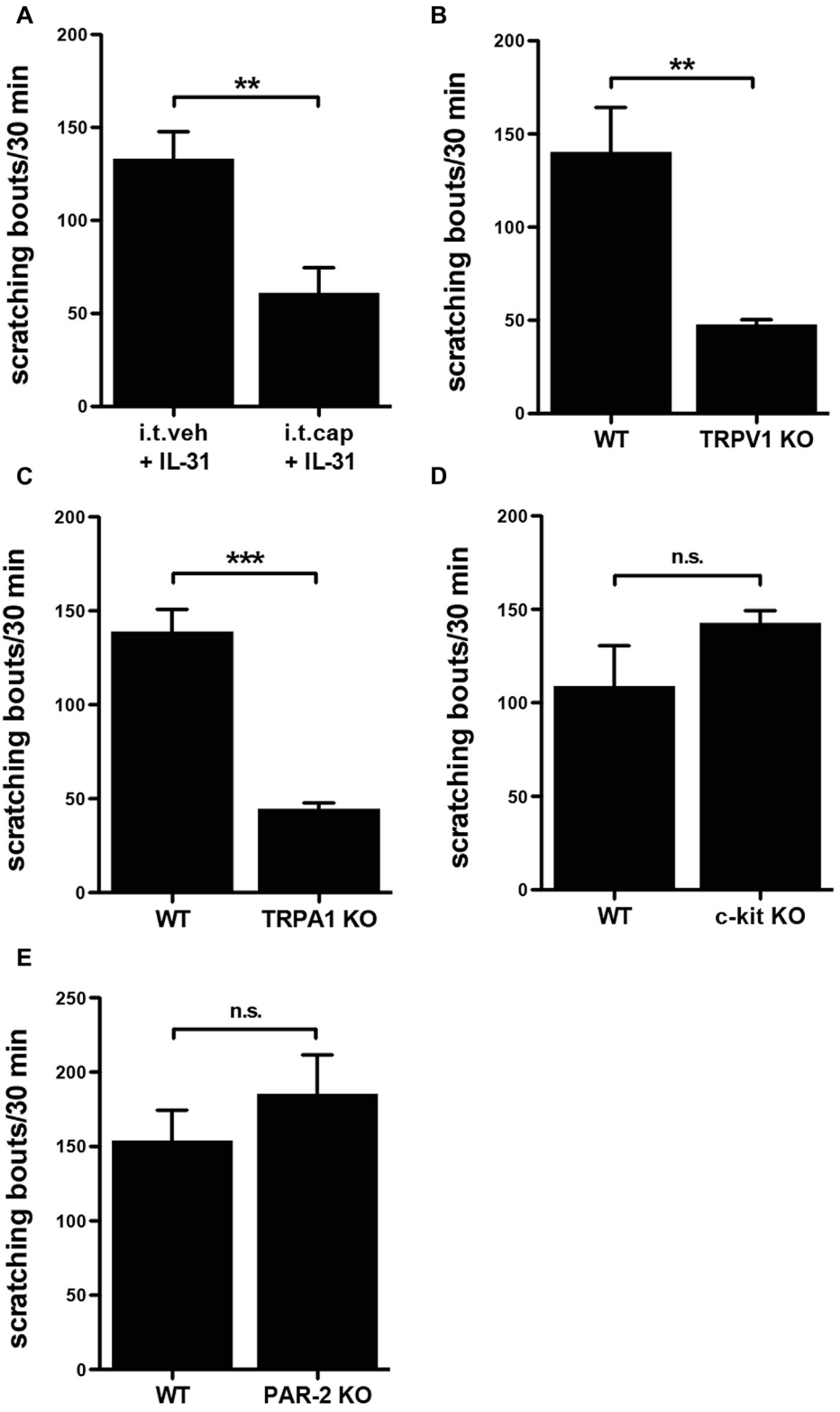

FIG 5. Neuronal requirement of IL-31–induced itch. **A,** Depletion of $TRPV1^{+}$ neurons by intrathecal capsaicin *(i.t. cap)* significantly decreased intrathecal IL-31–induced scratching. **B** and **C,** TRPV1 KO (Fig 5, *B*) and TRPA1 KO (Fig 5, *C*) mice show reduction in IL-31–induced scratching compared with WT littermates. **D** and **E,** *c-kit* mutant mice (Fig 5, *D*) and PAR-2 KO mice (Fig 5, *E*) showed equal scratching to WT mice after IL-31 injection. N = 8 mice per group. $^{**}P < .01$ and $^{***}P < .001$, Student *t*-test. *n.s.*, Not significant. *Error bars* indicate SEMs.

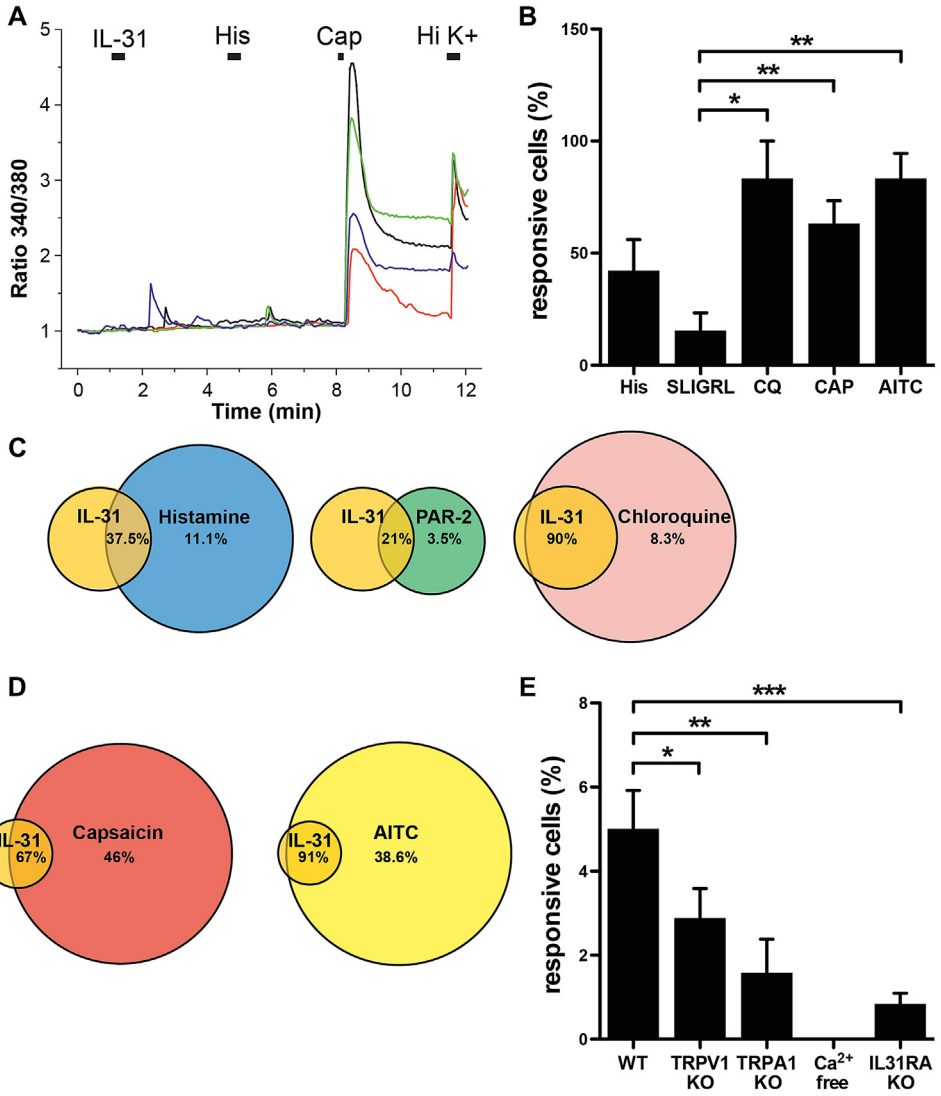

FIG 6. IL-31–induced calcium mobilization and characterization of IL-31–responsive DRG neurons. **A,** Neurons responding to IL-31 only *(blue),* histamine only *(green),* IL-31 and histamine *(black),* and neither IL-31 nor histamine *(red).* **B,** Percentages of IL-31–responsive neurons, which also respond to other compounds. **C** and **D,** Venn diagrams for DRG neurons in percentages. **E,** Percentages of IL-31–responsive neurons in different KO mice. N = 193 to 981 cells per group. For quantification, 10 to 30 dishes per group were used, and 20 to 50 cells per dish were counted. $*P < .05$, $**P < .01$, and $***P < .001$, unpaired *t* test. *Error bars* indicate SEMs.

KO mice (44.67 ± 3.17 bouts/30 minutes vs 139 ± 11.86 bouts/30 minutes in WT littermates). To address the possibility that IL-31–evoked itch is amplified by a mast cell release of TRPV1/TRPA1-dependent pruritogens, such as histamine or tryptase, we injected IL-31 into the necks of mast cell–deficient *c-kit* mutant and proteinase-activated receptor 2 (PAR-2) KO mice (6.3 nmol/40 μL). No differences were observed between *c-kit* mutant mice and their WT control animals (142.8 ± 6.48 bouts/30 minutes vs 109 ± 21.57 bouts/30 minutes; Fig 5, *D*). Also, we have not observed significant differences between PAR-2 KO (185.4 ± 26.25 bouts/30 minutes) and WT littermate mice (154 ± 20.59 bouts/30 minutes; Fig 5, *E*). Thus IL-31–induced itch is independent of mast cell degranulation or PAR-2–mediated itch.

Functional characterization of IL-31–responsive DRG neurons

To identify the functional properties of the IL-31RA population of pruriceptors, we imaged cervical (C3-C8) DRG cells for their Ca^{2+} responsiveness to IL-31 (Fig 6).[32,33] Consistent with the anatomic analysis, we found 2.1% (4/194) responded to 0.3 μmol/L, 3.0% (32/1054) responded to 1.0 μmol/L, and 4.0% (4/100) responded to 3.0 μmol/L IL-31 in a dose-responsive manner (Fig 6, *A*). A detailed analysis indicates heterogeneity in the responsiveness of DRG neurons: although some DRG neurons responded to IL-31 but not histamine, others responded to histamine but not IL-31, and others responded to both or neither. Many IL-31–responsive cells responded to capsaicin, which is consistent with the predominant TRPV1 expression

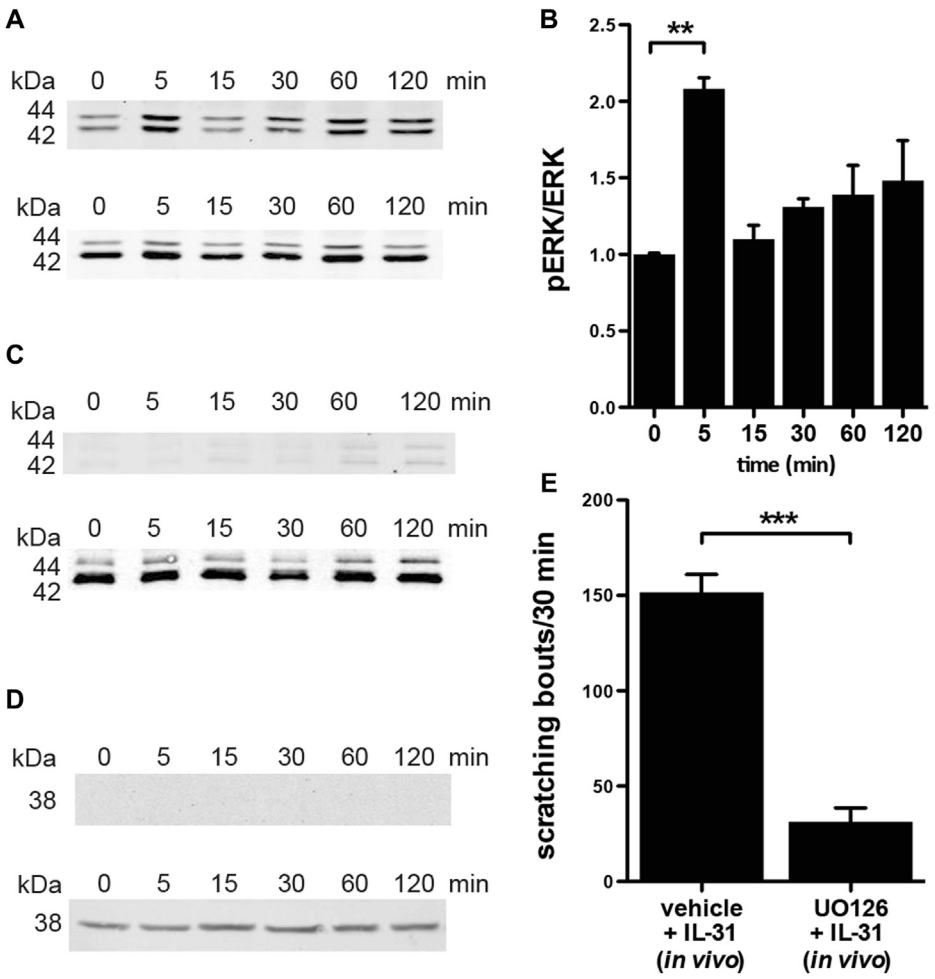

FIG 7. ERK1/2 phosphorylation in DRG is critical for IL-31–induced itch. **A** and **B,** Western blotting (Fig 7, *A*) and densitometric analysis (Fig 7, *B*) of murine cultured DRG neurons for phospho-ERK *(pERK)* 1/2 illustrate peak activation of ERK1/2 after 5 minutes. **C,** Pretreatment with the ERK1/2 inhibitor U0126 blocked IL-31–induced ERK1/2 activation. **D,** IL-31 stimulation does not lead to p38 phosphorylation in cultured DRG neurons. **E,** Intraperitoneal injection of U0126 before IL-31 blocked IL-31–evoked scratching. N = 8 mice per group. $^{**}P < .01$ and $^{***}P < .001$, Student *t* test. *Error bars* indicate SEMs.

seen in IL-31–responsive neurons (Fig 6, *A*). Moreover, we found that 11.1% of 495 tested cells responded to histamine, 3.5% of 575 cells responded to SLIGRL, 8.3% of 484 cells responded to chloroquine, and 38.6% of 484 cells responded to allyl isothiocyanate (AITC; mustard oil). Competence of viable cells was confirmed by a robust Ca^{2+} influx detected in all cells exposed to capsaicin or high K^+ levels (Fig 6, *A*). Thus IL-31 induced robust Ca^{2+} responses in DRG neurons that are also inducible by agonists to TRPA1, TRPV1, and chloroquine.

Fig 6, *B*, and the Venn diagrams in Fig 6, *C* and *D*, show the proportions of IL-31–responsive DRG neurons compared with IL-31–responsive neurons that also responded to histamine, SLIGRL, chloroquine, capsaicin, and AITC (Fig 6, *A* and *C*). Compared with histamine (37.5%) and SLIGRL (21%), a higher percentage of IL-31–responsive cells were activated by capsaicin (67%) and AITC (91%), respectively (Fig 6, *B* and *D*). Finally, IL-31–responsive DRG neurons were significantly reduced in TRPV1 and TRPA1 KO animals (Fig 6, *E*). The percentage of IL-31–responsive DRG neurons was significantly reduced in TRPV1 KO DRG neurons and even more in TRPA1 KO DRG neurons (Fig 6, *E*). Thus in contrast to histamine[15] or chloroquine,[16,17,32] for the first time, we show that IL-31–mediated calcium influx is, to some extent, dependent on TRPV1 and TRPA1 channels in murine DRG. Because IL-31 did not elicit calcium responses in the absence of extracellular calcium, we conclude that IL-31 triggers influx of calcium through these transient receptor potential transduction channels.

Contribution of extracellular signal-regulated kinase 1/2 to IL-31–mediated cell signaling in DRG neurons and IL-31–provoked itch

Although mitogen-activated protein kinase signaling pathways have been implicated in the processing of pain message by primary afferents,[33,34] their contribution to itch has not been studied. Fig 7, *A* and *B*, show that IL-31 stimulation of cultured murine DRG neurons induced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 that peaked at 5 minutes. The MEK inhibitor U0126 completely prevented IL-31–mediated phosphorylation of ERK1/2 *in vitro* (Fig 7, *C*). By contrast, IL-31 was without effect on the p38 signaling pathways in DRG neurons (Fig 7, *D*). Fig 7, *E*, shows that U0126 (30 mg/kg) injected

intraperitoneally 30 minutes before IL-31 injection into the neck (6.3 nmol/40 μL) significantly reduced scratching bouts (31.2 ± 7.46 vs 151.6 ± 9.52 in vehicle-treated mice, $P \leq .0001$). Thus ERK1/2, but not p38, is required for IL-31–induced itch.

Neural responses to IL-31 in the dorsal horn of the SC

To assess whether IL-31–induced itch activates pruritoceptive and/or nociceptive dorsal horn neurons, we used single-unit extracellular recordings to define the properties of IL-31–activated dorsal horn neurons (see Fig E4 in this article's Online Repository at www.jacionline.org). The majority of IL-31–responsive neurons were activated by pruritogens (histamine and SLIGRL) and noxious stimuli (heat and capsaicin; see Fig E4, *A* and *C*). The fact that a common population of neurons responds to both itch- and pain-provoking stimuli suggests that a circuit downstream of the IL-31RA$^+$/TRPV1$^+$/TRPA1$^+$ primary sensory neurons or a specific pattern of activity generated across subpopulations of responsive dorsal horn neurons determines the quality of the sensory perception (namely itch or pain).

DISCUSSION

The resistance of prevalent pruritic diseases to antihistamines, as exemplified by AD, argues strongly for the existence of histamine-independent pruritic pathways that are important targets for therapy of chronic itch.[11-13] We demonstrate that IL-31 induces itch by directly activating IL-31RA on TRPV1$^+$/TRPA1$^+$ sensory nerves in the skin. We show that T_H2 cells are the predominant cellular source of IL-31 and that the number and activation of T_H2 cells, as well as IL-31 levels, are increased in both patients and mouse models of AD. We conclude that T_H2 cells are the source of a novel, IL-31–triggered neuroimmune circuit that induces itch in patients with T_H2-dominated skin diseases by activating IL-31RA on sensory nerves.[5,35,36] Whether the central terminals of primary afferents are targeted in pruritic diseases in which the blood-brain barrier is compromised, such as multiple sclerosis, allowing for penetration of IL-31 into the central nervous system, remains to be determined.[37,38]

Although previous studies detected IL-31RA expression in DRG neurons,[10] the functional relevance of neuronal IL-31RA expression has not been explored. We found that IL-31RA is exclusively expressed by a subpopulation of TRPV1$^+$/TRPA1$^+$ DRG neurons. Of note, although the subset of IL-31RA$^+$ afferents (approximately 4% of DRG) is relatively small (Fig 6, *A*), IL-31RA activation of this population by means of either by intradermal or intrathecal IL-31 injection is clearly sufficient to evoke profound scratching in mice. These effects depend on a subset of TRPV1$^+$ afferents (TRPV1$^+$/IL-31RA$^+$), as well as on TRPA1 as a signal transducer, suggesting that both TRPV1 and TRPA1 are major contributors to IL-31–induced itch.[14,16-18,28,31,39]

Other studies reported colocalization of OSMRβ, a receptor subunit targeted by IL-31, in the nonpeptidergic $P_2X_3^+$ neuron population.[40-42] In contrast, we found IL-31RA immunoreactivity predominantly in the peptidergic TRPV1/TRPA1$^+$ neuron population (Fig 4). Importantly, we confirmed the specificity of our IL-31RA antibody through the absence of immunostaining in IL-31RA KO mice (see Fig E3). Intriguingly, although IL-31RA mRNA was significantly increased in lesional skin of patients with AD, this was not the case for OSMRβ mRNA, indicating a pivotal role of IL-31RA but not OSMRβ in IL-31–mediated itch (Fig 1, *A*).

The fact that IL-31 injection into the cheek induced itch but not pain suggests that itch and pain are triggered by different subsets of unmyelinated afferents and that subpopulations of afferents exist that are specialized in the itch domain.[18] Indeed, single-fiber recordings in human subjects described itch-specific unmyelinated afferents.[43] We also found that chloroquine, which exerts its action through the MrgprA3 subtype of the Mrgprs activates a very large percentage (90%) of IL-31–responsive DRG neurons. Because we found that TRPA1 is also involved in IL-31–induced itch, we conclude that the IL-31RA$^+$/TRPV1$^+$/TRPA1$^+$ subset of DRG neurons is responsible for IL-31–induced itch. Future studies will determine whether IL-31 induces the release of brain natriuretic peptide in murine central primary afferents or activates GRPR$^+$ and/or NPR-A$^+$ postsynaptic neurons.[22,44]

Our electrophysiologic analyses indicate that IL-31–responsive neurons in the dorsal horn can be activated by multiple pruritogens, which is consistent with a convergent itch transmission circuit.[11,15,18,22,44] Furthermore, although some pruritogen-responsive dorsal horn neurons are activated by noxious stimuli, our finding that the central terminals of the IL-31RA$^+$/TRPV1$^+$ afferents target the outer part of lamina II, rather than lamina I, suggests that the postsynaptic neurons engaged by the IL-31RA–expressing afferents are interneurons that are part of a circuit dedicated to itch. In this context interneurons that express GRPR, NPR-A, or both are ideally positioned to receive input from the IL-31RA$^+$/TRPV1$^+$ afferents and presumably from the afferents that respond to other pruritogens.[22,44,45] Together, we suggest that the IL-31RA$^+$ population of afferents provides a major input that triggers itch, but not pain, and that GRPR$^+$/NPR-A$^+$ interneurons might be targets of these axons. Despite this apparent convergence, however, specificity of itch provoked by different pruritogens can be maintained because different pruritogens engage a variety of signaling pathways in the same neuron.[13] The fact that itch or pain can be attenuated by inhibitors of ERK1/2 phosphorylation (present findings)[33,34] is also consistent with convergence of itch and pain transmission, although the locus of the ERK1/2 action could differ in itch- and pain-relevant circuits.

In conclusion, our results demonstrate that T_H2-derived IL-31 directly communicates with an IL-31RA$^+$/TRPV1$^+$/TRPA1$^+$ subpopulation of primary afferent neurons in the skin. We suggest that IL-31RA is a functional neural cytokine receptor involved in acute and chronic itch. In this respect IL-31RA represents the long hypothesized "missing link" in a direct neuroimmune crosstalk between T cells and sensory nerves in itch. This finding emphasizes that not only mast cells through histamine or tryptase release[46,47] but also T cells through cytokines can directly communicate with sensory nerves to induce itch. Thus blocking the effects of IL-31/IL-31RA might have a beneficial effect not only for the inhibition of inflammation but also to ameliorate directly the deleterious effects of T cell–mediated itch. The exceptionally high incidence of itch and AD worldwide and the fact that levels of both IL-31 and IL-31RA are increased[48-50] underscore the significance of our findings for the development of IL-31–directed antipruritic therapies.

We thank Janine Bilsborough for helpful discussions; Mirela Iodi-Carstens for assistance in electrophysiology; Christine Stadelmann-Nesser for human DRG; and Stephan Seeliger, Christian Mess, Ron Manlapaz, Robert Kubitza, Sabine Kellermann, Ulrike Wiesner, Ulrich Pippirs, Andrea Poppe, and Victoria Fong for expert technical assistance.

Clinical implications: We show that a functional cytokine receptor expressed by sensory nerves is involved in itch, leading to novel therapeutic strategies targeting neuronal cytokine receptors to treat T cell–mediated itch and AD.

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METHODS

Materials

The U0126 MEK inhibitor was purchased from AG Scientific (San Diego, Calif). The following antibodies were used: monoclonal rat anti-mouse IL-31RA (ZymoGenetics), biotinylated anti-IB4 (Vector Laboratories, Burlingame, Calif), and guinea pig anti-TRPV1 (generous gift of Dr D. Julius, University of California, San Francisco)[E1] and N52 (Sigma-Aldrich, St Louis, Mo).[E2] Secondary antibodies were purchased from Molecular Probes (Carlsbad, Calif).

Patients and biopsy specimens

The clinical investigation of patients was conducted according to the Declaration of Helsinki Principles. During autopsies, DRG slides were fabricated for examination. Patients with AD were identified according to the criteria defined by Hanifin and Rajka,[E3] which are based on typical clinical findings and histopathologic examination. The disease state was also monitored by using SCORAD scores. Visual analog scores (1-10) were used to assess pruritus intensity. Six-millimeter skin punch biopsy specimens were collected from nonlesional (n = 13) or lesional (n = 50) patients with AD, as well as from healthy skin (n = 52). Five to 10 skin samples from patients with AD with skin lesions and from healthy volunteers were used for immunohistochemistry. At least 3 slides were performed for each staining from each patient/donor.

Purification of naive $CD4^+$ T lymphocytes from blood and T helper cell differentiation

Peripheral blood naive $CD4^+$ T cells ($CD4^+CD45RA^+CD25^-CD45RO^-$) were isolated from PBMCs by using the $CD4^+$ T cell isolation kit II (Miltenyi Biotec) and FACSAria sorting, as described previously.[E4-E6] Naive T cells were cultured and stimulated with cytokines (T_H1: 10 ng/mL IL-12; T_H2: 25 ng/mL IL-4; T_H17: 10 ng/mL IL-1β, 20 ng/mL IL-6, 100 ng/mL IL-23, and 1 ng/mL TGF-β) in the presence of CD3/CD28 T Cell Expander (1 bead per cell; Invitrogen, Carlsbad, Calif)[E7] for 5 to 6 days. Cells (1×10^6 cells/mL) were restimulated for 24 hours with Dynabeads CD3/CD28 T Cell Expander (1 bead per cell).

Preparation and cultivation of primary cells

Normal human primary epidermal keratinocytes, dermal fibroblasts, and dermal microvascular endothelial cells were obtained from Clonetics (San Diego, Calif). Normal human primary epidermal keratinocytes, dermal fibroblasts, and dermal microvascular endothelial cells were cultured in keratinocyte growth medium, fibroblast growth medium, or endothelial cell growth medium (KGM-2, FGM-2, and EGM-2, respectively), as previously described.[E8] Human primary monocytes were isolated from purified PBMCs by using a monocyte isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Freshly isolated monocytes were cultured for 6 days in the presence of both GM-CSF (100 ng/mL) and IL-4 (50 ng/mL) for the generation of immature dendritic cells. Cells were matured with TNF-α (50 ng/mL) for an additional 3 days in the presence of GM-CSF and IL-4 to activate immature dendritic cells.[E9]

qPCR (TaqMan)

First, naive $CD4^+$ T lymphocytes were isolated by means of magnetic sorting with a human naive CD4 T cell isolation kit (Miltenyi Biotec) and an AutoMACS Separator, as described elsewhere.[E4,E5,E10] Immature and mature monocyte-derived dendritic cells were isolated, as previously described.[E11-E13] Keratinocytes, fibroblasts, and endothelial cells were obtained from Gibco (Invitrogen) and cultivated as described, according to the manufacturer's instructions. Samples were tested with primers for IL-31.

Second, skin biopsy specimens were homogenized in liquid nitrogen by using a Mikro-Dismembrator U (Braun Biotech, San Diego, Calif), and RNA was extracted with TRIzol reagent (Invitrogen). Samples from skin biopsy specimens were tested with primers for human IL-31, IL-31RA short form, IL-31RA long form, and OSMR. Four micrograms of RNA were treated with DNase I (Boehringer Ingelheim, Mannheim, Germany) and reverse transcribed. Primers for human IL-31 (NM_001014336.1) were as follows: forward, 5′-GCCCAGCCGCCAAAC-3′; reverse, 5′-GCTGTCTGATTGTCTTGAGATATGC-3′. Primers for human IL-31RA long form (NM_001242639.1) were as follows: forward, 5′-TAGTACCAGATCATCTGTGT-3′; reverse, 5′-TTAGACTTCTCCCTTGGTGTGC-3′. Primers for human IL-31RA short form (NM_001242637.1) were as follows: forward, 5′-TCAATTCCAGCATCTTGCAGTAC-3′; reverse, 5′-GCTGGCCATGACCTGAACA-3′. Primers for human OSMR (NM_003999.2) were as follows: forward, 5′-CCCAGTGCTACGTTCACGAA-3′; reverse, 5′-CCATGGGCAGTAGGATATGAATC-3′. TaqMan Ribosomal RNA Control Reagents were used to detect the 18S ribosomal RNA gene (Applied Biosystems, Foster City, Calif). RNA from skin biopsy specimens of SEB- and OVA-treated mice were extracted with TRIzol reagent (Invitrogen). One microgram of RNA was reversed transcribed by using SuperScript II (Invitrogen). Primers for murine IL-31 (NM_029594.1) were as follows: forward, 5′-CCACACAGGAACAACGAAGCCT-3′; reverse, CCCGGTCCAGGCTGAAACACG-3′. Primers for murine IL-4 (NM_021283.2) were as follows: forward, 5′-GGGCTTCCAAGGTGCTTCGCA-3′; reverse, 5′-TCCAGGCATCGAAAAGCCCGA-3′. Primers for murine glyceraldeyde-3-phosphate dehydrogenase (GAPDH; housekeeping gene; NM_008084.2) were as follows: forward, 5′-GCCTTCTCCATGGTGGTGAA-3′; reverse, 5′-GCACAGTCAAGGCCGAGAAT-3′. Twenty-five nanograms of cDNA was amplified per reaction, either in the presence of SYBR green master mix or in the presence of TaqMan universal master mix (Applied Biosystems). Gene-specific PCR products were measured with an ABI PRISM 7000 Sequence Detection Systems (Applied Biosystems; stage 1, 50°C for 2 minutes; stage 2, 95°C for 10 minutes; and stage 3, 95°C for 15 seconds and 60°C for 1 minute, repeated 40 times). Gene expression was related to the housekeeping gene and are presented as relative units of expression.

Immunofluorescence in human skin

After 3 washes in PBS for 10 minutes, sections were immunostained with primary antibodies against human IL-31RA (goat IgG, R&D Systems; 10 μg/mL for 90 minutes at 37°C) and cutaneous lymphocyte–associated antigen (rat IgM, BD PharMingen; 25 μg/mL; 180 minutes at 37°C), PGP 9.5 (mouse IgG; AbD Serotec, Oxford, United Kingdom; 1:1000, overnight at 4°C), or goat IgG as isotype control, followed by a rabbit anti-goat, anti-rat, or anti-mouse fluorescent secondary antibody (Molecular Probes; 1:200, 30 minutes at room temperature). Cells were examined by using a Zeiss Axiovert inverted microscope with a 63x/1.2 C-Apochromat water immersion lens (Carl Zeiss, Thornwood, NY).

Immunostaining of human DRG tissue for IL-31R

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated to examine IL-31RA immunoreactivity in human DRG tissue. Slides were pretreated with Target Retrieval Solution (DAKO, Glostrup, Denmark) and heated in a humidified oven for 40 minutes at 90°C and then washed several times in PBS. Sections were then incubated with an anti–IL-31RA antibody (1:1000, R&D Systems) at 4°C overnight. For visualization, we used a 2-component kit, according to the manufacturer's recommendations (goat-on-rodent HRP-Polymer; Biocare Medical, Concord, Calif). Absorption controls were performed as follows: IL-31RA protein (R&D Systems) was preincubated with the primary anti–IL-31RA antibody (1:100) for 48 hours at 4°C, followed by the standard immunostaining protocol, as described above.

Mice

C57BL/6 WT and mast cell–deficient kit/kit (age 6-8 weeks) mice and littermate WT control animals were purchased from Jackson Laboratory (Bar Harbor, Me). PAR-2 KO mice (generous gift of Shaun Coughlin, University of California, San Francisco) and TRPV1 KO mice (generous gift of David Julius, University of California, San Francisco) were bred from heterozygous pairs and from the offspring WT littermates. Homozygous PAR-2 or TRPV1 KO mice were used for experiments. IL-31RA KO mice were obtained from ZymoGenetics. All experiments were approved by the Institutional Animal

Care and Use Committee of the University of California, San Francisco, and conducted in accordance with the National Institutes of Health's "Guide for the care and use of laboratory animals."

Mouse model of AD and bacterial superantigen-induced skin inflammation

Six-week-old female mice (8 per group) were epicutaneously treated with OVA (OVA group), SEB (SEB group), or vehicle (PBS group) after achievement of isoflurane anesthesia (Univentor 400 Anesthesia Unit; Abbott Laboratories, Abbott Park, Chicago, Ill). The backs of the mice were shaved with an electronic razor and tape stripped with adhesive tape to induce a standardized skin injury. Stripping included adhering a piece of tape to the shaved skin 4 times, after which it was removed against the direction of the hair. Two different concentrations of SEB (Sigma-Aldrich), 0.5 and 5 μg, were topically applied to a 1 × 1–cm patch of sterile gauze alone (in 100 μL of PBS) or with OVA. We used 100 μg of OVA (grade V, Sigma-Aldrich) in 100 μL of PBS for epicutaneous sensitization. The gauze was secured to the shaved skin with transparent adhesive tape (Tegaderm; Owens and Minor, Richmond, Va) for 1 week (first sensitization week). Two weeks later (second sensitization week), mice were again tape stripped, and an identical patch was reapplied to the same skin site. The last epicutaneous sensitization (third sensitization week) was similarly given 2 week later. Mice received a total of three 1-week patch exposures separated by 2-week intervals, totaling 7 weeks. Mice were killed, and multiple 4-μm sections from treated skin were obtained for histologic analyses. Individual inflammatory cell types (eosinophils, mast cells, and total numbers of infiltrating cells) were counted in 10 to 20 high-power fields (hpfs) at ×1000 magnification and expressed as cells per hpf. The numbers of eosinophils and mast cells and total numbers of infiltrating cells were determined. CD3 (PharMingen) staining was performed on 4-μm frozen sections after acetone fixation by using a biotin-conjugated secondary antibody anti-rat IgG (Vector Laboratories). Cells were counted in 10 hpfs at ×400 magnification and expressed as cells per hpf, with means and SEMs. RNA was extracted from cryopreserved skin specimens to perform cytokine expression analyses by using qPCR With TaqMan Gene Expression. Primers and probes for IL-4 (Mm00445259) and IL-31 (Mm01194496) were designed by Applied Biosystems. Finally, serum samples were used to determine total and specific IgE levels by using ELISA.

Pruritogen-induced scratching

The right cheeks of the experimental mice were shaved 1 day before injection of pruritogens or capsaicin (10 μL).[E14] Mice were placed in Plexiglas cylinders and videotaped for 30 minutes by using a mirror placed underneath the transparent table. Intraplantar injections were performed at a maximal volume of 20 μL into the left hind paw. Nape of the neck injections (20-40 μL) were conducted in mice shaved 1 day before the experiments. Mice were placed in Plexiglas cylinders, and scratching behavior was monitored as described above. Intrathecal injection of IL-31 (5 μL, 6.3 pmol-5 fmol) was made in awake, lightly restrained mice at the level of the pelvic girdle with a luer-tipped Hamilton syringe with a 30-gauge needle. For intrathecal capsaicin (10 μg) injection, mice were anaesthetized with 1.5% isoflurane.[E15]

Immunostaining of mouse DRG and SC tissue for IL-31RA

The staining protocol was performed according to the ZymoGenetics protocol. After antigen retrieval was performed with a ready-to-use citrate buffer (HK086-9K; Biogenex, Freemin, Calif) at 80°C for 20 minutes, slices were cooled down for 20 minutes on ice and washed 3 times with 1× PBS. Sections were incubated with both anti–IL-31RA 1:2,000 and anti-TRPV1 1:2,000, anti–IL-31RA 1:2,000 and IB4-biotin 1:1,000, or IL31RA and N52 (1:10,000) for 2 nights at 4°C in Ventana dilution buffer (Fisher Scientific, Waltham, Mass). Next, sections were washed in 0.1 mol/L PBS (3× 10 minutes), followed by a 1-hour incubation at room temperature with appropriate secondary antibodies (all 1:800) conjugated with goat anti-rat Alexa Fluor 594 (Molecular Probes) for IL-31RA, anti–guinea pig 488 (Molecular Probes) for TRPV1, and anti-streptavidin 488 for IB4-Biotin (Molecular Probes). After washes in PBS, slides were placed under cover slips by using Prolong gold (Invitrogen).

Primary DRG culture

Harvested DRG neurons were incubated with Hanks balanced salt solution (HBSS; Invitrogen) containing 1.3 mg/mL papain (Sigma) and 0.65 mg/mL L-cysteine (Sigma) for 10 minutes at 37°C and then incubated with HBSS containing 3 mg/mL collagenase (Sigma) for 10 minutes at 37°C. Digests were washed with complete medium consisting of Eagle minimal essential medium with Earle BSS medium supplemented with 10% (vol/vol) horse serum (Sigma), 2.0 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from PAA Laboratories, Pasching, Austria), 1× GIBCO MEM Vitamin Solution (Invitrogen), and 1× N1 medium supplement (Sigma). The strain cell suspension was filtered through a 100-μm cell strainer and cultured overnight in dishes with laminin-coated glass cover slips (Sigma) containing complete media. Incubation with inhibitors was performed throughout the experiments, as indicated for 30 minutes before IL-31 stimulation. Cells were stimulated with the same volume of vehicle or IL-31 (100 ng per volume).

Calcium imaging

Upper cervical to midcervical DRG tissues removed from mice were enzymatically digested at 37°C for 10 minutes in HBSS containing 20 U/mL papain (Worthington Biochemical, Lakewood, NJ) and 6.7 mg/mL L-cysteine (Sigma), followed by 10 minutes at 37°C in HBSS containing 3 mg/mL collagenase (Worthington Biochemical). The ganglia were then mechanically triturated by using fire-polished glass pipettes. Cells were pelleted; suspended in Eagle minimal essential medium with Earle BSS (Gibco) containing 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 1× vitamins (Gibco), and 10% horse serum (Quad Five, Ryegate, Mont); plated on poly-D-lysine–coated glass cover slips; and cultured for 16 to 24 hours. Cells were incubated in Ringer solution (pH 7.4; 140 mmol/L NaCl, 4 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 4.54 mmol/L NaOH) with 10 μmol/L Fura-2 AM and 0.05% of Pluronic F-127 (Invitrogen). Cover slips were mounted on a custom aluminum perfusion block and viewed through an inverted fluorescence microscope (Nikon Eclipse TS100, Melville, NY). Fluorescence was excited by UV light at 340 and 380 nm alternately, and the emitted light was collected by using a CoolSnap camera attached to a Lambda LS lamp and a Lambda optical filter changer (Sutter Instrument Company, Novato, Calif). Ratiometric measurements were made using the computer software Simple PCI (Compix, Cranberry Township, Pa) every 3 seconds. Approximately 40 cells were observed per dish and subjected to the identical stimulus sequence. Solutions were delivered by a solenoid-controlled 8-channel perfusion system (ValveLink, AutoM8) at a flow rate at 6 mL/min. IL-31 (0.3, 1, and 3 μmol/L) and histamine (100 μmol/L), the PAR-2 agonist SLIGRL-NH2 (100 μmol/L), or chloroquine (300 μmol/L) were delivered, usually in this order. After applications of pruritogens, 1 μmol/L capsaicin or 100 μmol/L AITC and 144 mmol/L potassium were applied in this order. Stimulus duration was 30 seconds (10 seconds for capsaicin and AITC). Ratios were normalized to prestimulus baseline. Cells were judged to be responsive if the ratio value increased by greater than 10% of the resting level after chemical application. Stimulus duration was 30 seconds (10 seconds for capsaicin). Ratios were normalized to prestimulus baseline. Cells were judged to be responsive if the ratio after chemical application increased by greater than 10% of the resting level.

Western blotting

DRG neurons from primary cell culture were homogenized in hot protein lysis buffer containing a protease and phosphatase inhibitor mixture (Roche Applied Science, Penzberg, Germany) and sonicated, and then cell debris was removed by means of centrifugation (14,000*g* at 4°C for 10 minutes). Samples were boiled in sample buffer (50 mmol/L Tris-HCL [pH 6.8], 2% [wt/vol] SDS, 0.1% [wt/vol] bromophenol blue, 10% [vol/vol] glycerol, and 2.5% [vol/vol] 2-mercaptoethanol) for 5 minutes, separated by SDS-PAGE (12% acrylamide), and blotted onto a nitrocellulose membrane (Amersham

Biosciences). Membrane blocking was performed with the LI-COR Blocking System (LI-COR, Lincoln, Neb) for 1 hour. Primary antibodies (mouse phospho-p38/rabbit p38 or mouse phospho-ERK/rabbit p-ERK) were applied overnight at 4°C. After 5 washes with PBS/Tween, the membrane was incubated with anti-mouse or anti-rabbit secondary antibody tagged with fluorophores (LI-COR) for 2 hours at room temperature. The detection was performed on a LI-COR Scanner.

Electrophysiology

Anesthesia was induced with pentobarbital sodium (60 mg/kg administered intraperitoneally) and maintained by means of intermittent supplemental injections to achieve a level of approximately 10 to 20 mg/kg/h. The lumbosacral SC was exposed by means of laminectomy, and a tungsten microelectrode (FHC, Bowdoin, Me) recorded extracellular single-unit activity. A chemical search strategy was used to isolate units in the superficial dorsal horn.[E16] In this study IL-31 protein (100 μg/μL) was used.

By using a 30-gauge needle connected to a Hamilton microsyringe, we microinjected the cytokine (approximately 0.2 μL, approximately 20 μg of IL-31) into the plantar skin (intradermally). The SC recording electrode targeted the superficial dorsal horn (<300 μm from surface). We searched for cells that had ongoing activity. If no unit was isolated, the procedure was repeated 30 minutes or more later at a different site on the plantar skin surface or on the opposite side. We then waited 30 minutes or more until firing decreased to a steady low level. The microinjection needle was left in place, and IL-31 was reinjected in a volume of 1 μL. Responsive units (>30% increase above baseline firing) were studied further. In some units IL-31 was injected again in a volume of 1 μL 30 minutes later to test for tachyphylaxis.

A second 30-gauge needle containing either histamine (50 μg/1 μL) or the PAR-2 agonist SLIGRL-NH2 (50 μg/1 μL) was then inserted intradermally into the hind paw. It was left in position for several minutes until any ongoing activity evoked by needle insertion had waned. The second chemical was then injected, and the neuron's activity was recorded for another 30 minutes. The same procedure was then repeated with the other chemical, such that the order of presentation of histamine and the PAR-2 agonist was counterbalanced across experiments. After this, we usually tested the cell's responsiveness to light brushing with a cotton wisp, followed by pinching with forceps. Units were classified as wide dynamic range if they responded at a higher firing rate to pinch than to light touch (and also noxious heat, if tested). They were classified as nociceptive specific if they responded to pinch (and noxious heat if tested) but not to light touch. No mechanically insensitive units were studied. Some units were tested for responsiveness to noxious heat (48°C-56°C for 10 seconds) and cooling (down to 0°C over 60 seconds) delivered by a computer-controlled Peltier thermode (NTE-2A; Physitemp, Clifton, NJ). After testing the response to natural stimulation, we tested responses to topical application of mustard oil (Sigma; 75% in mineral oil, 2 μL), followed by intradermal injection of capsaicin (Sigma; 3.3 mmol/L per 1 μL). Only 1 neuron was studied per animal. Action potentials were recorded to a computer and analyzed by using Chart software (AD Instruments, Colorado Springs, Colo). Neuronal activity was usually quantified as the number of action potentials per minutes and displayed in peristimulus time histogram format with 1-second bins. To assess tachyphylaxis, mean responses to the first and second injections of IL-31 were quantified as number of action potentials per minute over a 10-minute period and compared by using the paired *t* test, with a *P* value of less than .05 set as significant. At the end of the experiment, an electrolytic lesion was made through the recording microelectrode. The SC was fixed in 10% buffered formalin, and 50-μm sections were cut and mounted on slides for microscopic verification of the lesion/recording site.

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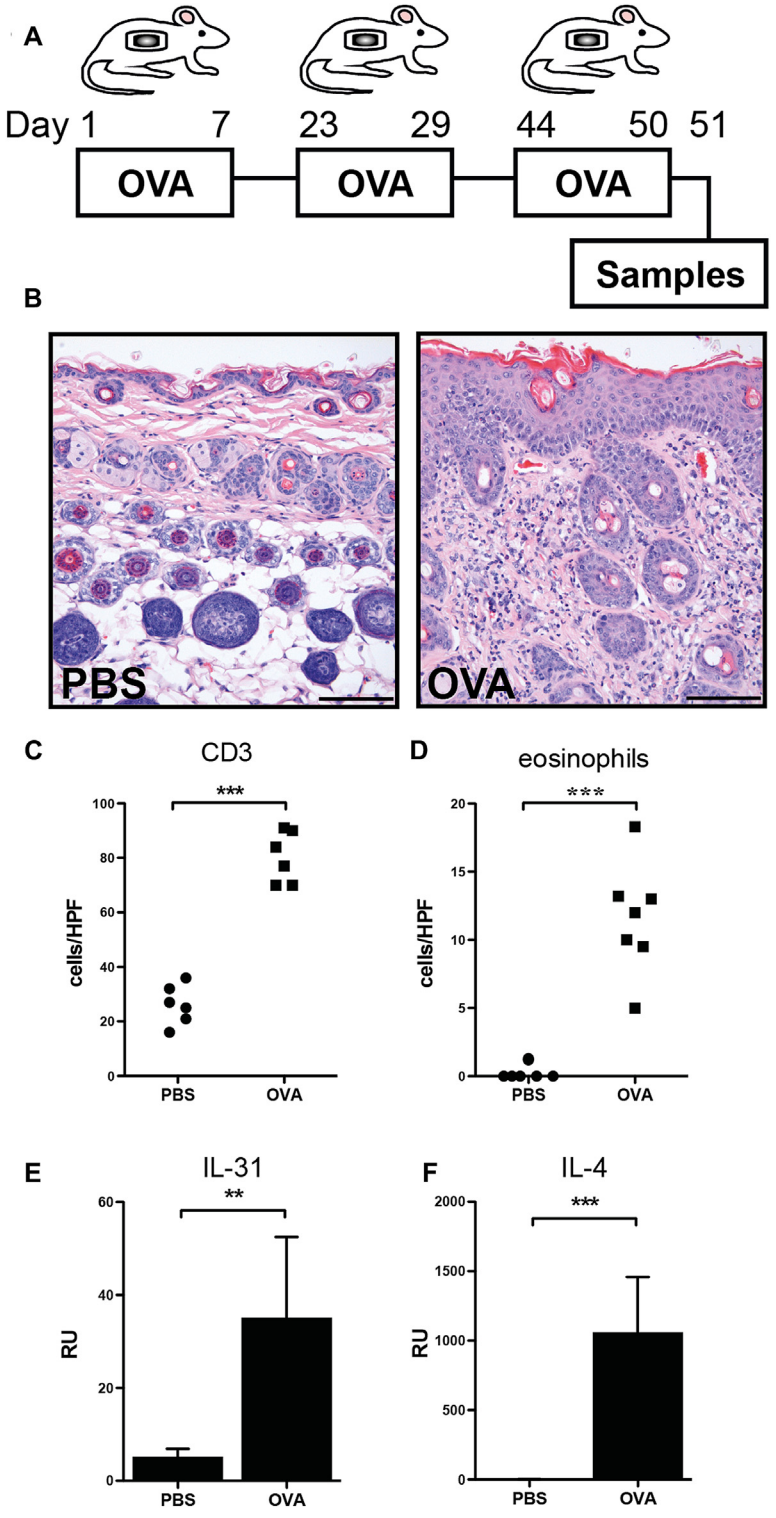

FIG E1. OVA-induced skin inflammation in mice. **A,** Topical treatment of mice during a maximum period of 51 days with OVA (100 μg in PBS) or vehicle was performed as indicated in the time scale diagram. **B,** Histologic features of hematoxylin and eosin–stained skin sites topically treated with either OVA or vehicle. *Scale bar* = 200 μm. **C,** Quantitative analysis for $CD3^+$ cells after repeated topical exposure to OVA or vehicle reveals higher numbers of $CD3^+$ cells in the OVA-treated compared with the vehicle-treated group. **D,** Eosinophil infiltration was significantly higher in OVA-treated skin compared with the vehicle (PBS) control group. **E** and **F,** qPCR analysis of IL-31 (Fig E1, *E*) and IL-4 (Fig E1, *F*) in skin biopsy specimens after OVA treatment reveals significantly increased expression for both T_H2-related cytokines compared with the vehicle control. $^{**}P < .01$ and $^{***}P < .001$, Student *t* test (SEM).

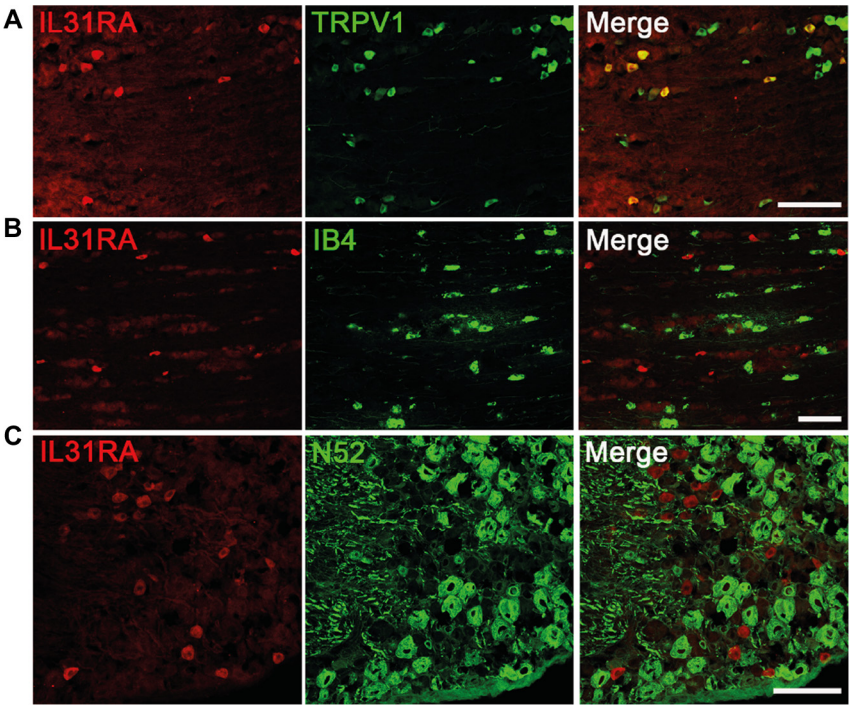

FIG E2. Colocalization of IL-31RA with neuronal markers in murine TG tissue. **A,** All IL-31RA$^+$ neurons *(red)* colocalized with TRPV1$^+$ neurons *(green)*. **B,** IL-31RA$^+$ neurons *(red)* showed minimal overlap with IB4$^+$ neurons *(green)*. **C,** No colocalization of IL-31RA$^+$ neurons *(red)* with N52 *(green)*, a marker for myelinated neurons. *Scale bar* = 100 μm.

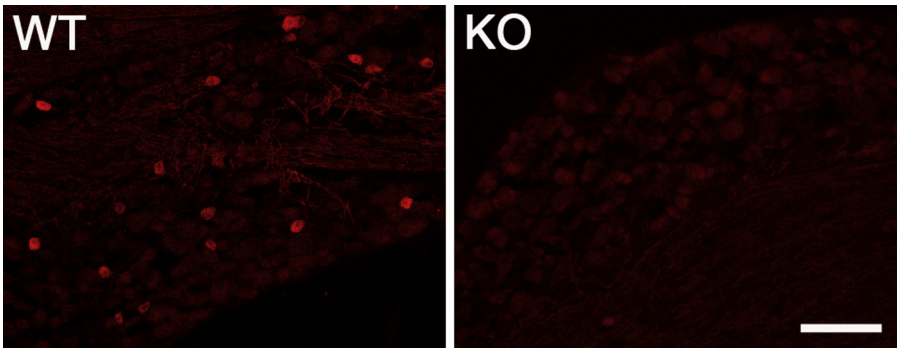

FIG E3. Specificity of the IL-31RA antibody was tested in DRG neurons of IL-31RA KO and WT mice. IL-31RA$^+$ immunoreactivity was detected in DRG neurons of WT mice. No signal was detectable in IL-31RA KO DRG tissue. *Scale bar* = 100 μm.

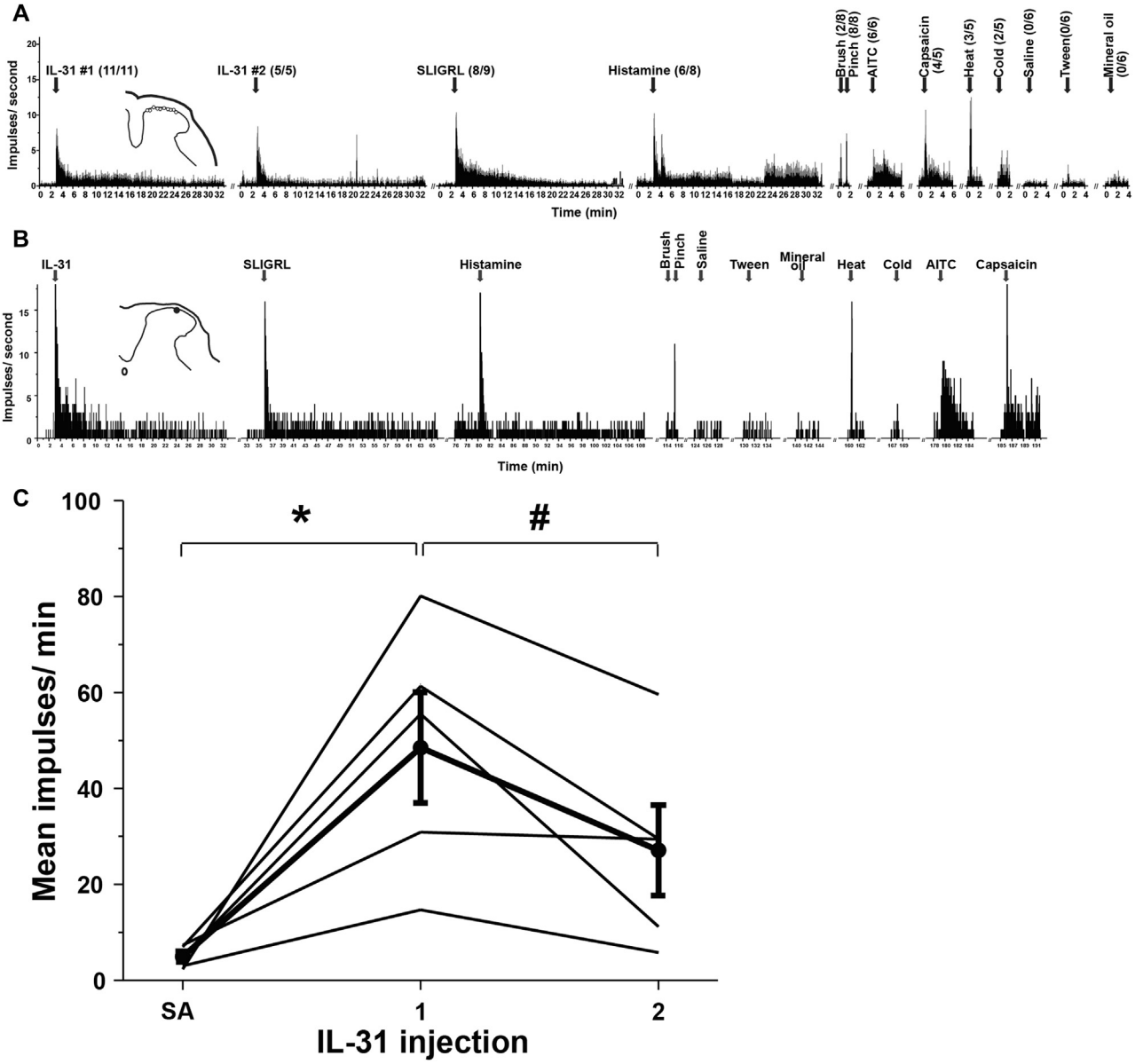

FIG E4. Intradermal IL-31 activates murine dorsal horn nociresponsive neurons. All cells were isolated by using the IL-31 search strategy described in the Methods section. **A,** Mean peristimulus time histograms show, from left to right, unit responses to 2 successive intradermal IL-31 injections into the hind paw without testing the receptive field. Units that had a chemical receptive field were then classified by an electrode readjusted to identify firing units to IL-31. IL-31 injection was followed by single injections of SLIGRL-NH_2, histamine, brush, pinch, AITC, capsaicin, noxious heat, cooling, or vehicle. Numbers in parentheses provide the number of responders in relation to the total number of neurons tested. The *left inset* shows histologically recovered recordings sites *(circles)* compiled on a representative section of the lumbar enlargement. *Error bars (gray)* indicate SEMs. **B,** Peristimulus time histogram (bin width: 1 s) shows a representative single dorsal horn neuron response to intradermal IL-31 (left peristimulus time histogram), followed by SLIGRL-NH_2, histamine, graded mechanical, AITC, capsaicin, heat, cold and vehicles (saline, Tween-80, and mineral oil). This neuron responded to all stimuli but not the vehicles. The *left inset* shows the recording site *(dot)* in the lumbar superficial dorsal horn. **C,** Quantification. *Thin lines* plot each unit's spontaneous activity (*SA*; recorded for 3 minutes) and response to 2 successive intradermal microinjections of IL-31 (analyzed for 10 minutes) at 30-minute interval. The *thick line* shows the mean of 5 units tested. *Error bars* indicate SEMs. *Mean response ($P < .05$, paired *t* test) to the first injection of IL-31 is significantly different from SA. #Mean response ($P < .05$) to the second injection of IL-31 is significantly different from the first response.