

# HTR7 Mediates Serotonergic Acute and Chronic Itch

## Highlights

- Activation of peripheral HTR7 receptors triggers itch but not pain behaviors
- HTR7 promotes opening of the inflammatory ion channel TRPA1
- Serotonin- and SSRI-evoked itch is mediated by HTR7 and TRPA1
- HTR7 and TRPA1 are required for the development of atopic dermatitis in mice

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## In Brief

Aberrant serotonin signaling has long been linked to acute and chronic itch disorders. Morita et al. now show that HTR7 is a mediator of serotonergic and SSRI-evoked itch and is required for the development of chronic itch in a mouse atopic dermatitis model.

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# HTR7 Mediates Serotonergic Acute and Chronic Itch

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## SUMMARY

Chronic itch is a prevalent and debilitating condition for which few effective therapies are available. We harnessed the natural variation across genetically distinct mouse strains to identify transcripts co-regulated with itch behavior. This survey led to the discovery of the serotonin receptor HTR7 as a key mediator of serotonergic itch. Activation of HTR7 promoted opening of the ion channel TRPA1, which in turn triggered itch behaviors. In addition, acute itch triggered by serotonin or a selective serotonin reuptake inhibitor required both HTR7 and TRPA1. Aberrant serotonin signaling has long been linked to a variety of human chronic itch conditions, including atopic dermatitis. In a mouse model of atopic dermatitis, mice lacking HTR7 or TRPA1 displayed reduced scratching and skin lesion severity. These data highlight a role for HTR7 in acute and chronic itch and suggest that HTR7 antagonists may be useful for treating a variety of pathological itch conditions.

## INTRODUCTION

Chronic itch is a debilitating disorder that results from a number of skin diseases such as atopic dermatitis, as well as systemic conditions including kidney failure, cirrhosis, and some cancers. Although itch from allergy or bug bites is often treatable with antihistamines, most forms of chronic itch are resistant to antihistamine treatment (Mollanazar et al., 2015). Further, an estimated 10%–20% of the population will suffer from chronic itch at some point in their lifetime (Matterne et al., 2011; Metz and Ständer, 2010). Consequently, understanding the molecular basis of chronic itch is of significant clinical interest. Itch signals are transduced via a subset of primary afferent sensory neurons that innervate the skin. A number of studies have identified cells and receptors that transduce acute itch signals (Bautista et al., 2014; Garibyan et al., 2013; Mollanazar et al., 2015; Ross, 2011). However, the molecular mechanisms underlying the

development, maintenance, and transmission of chronic itch signals remain largely unknown. Likewise, a number of unanswered questions about acute itch transduction persist, including the peripheral mechanisms by which itch is induced by the pruritogen serotonin.

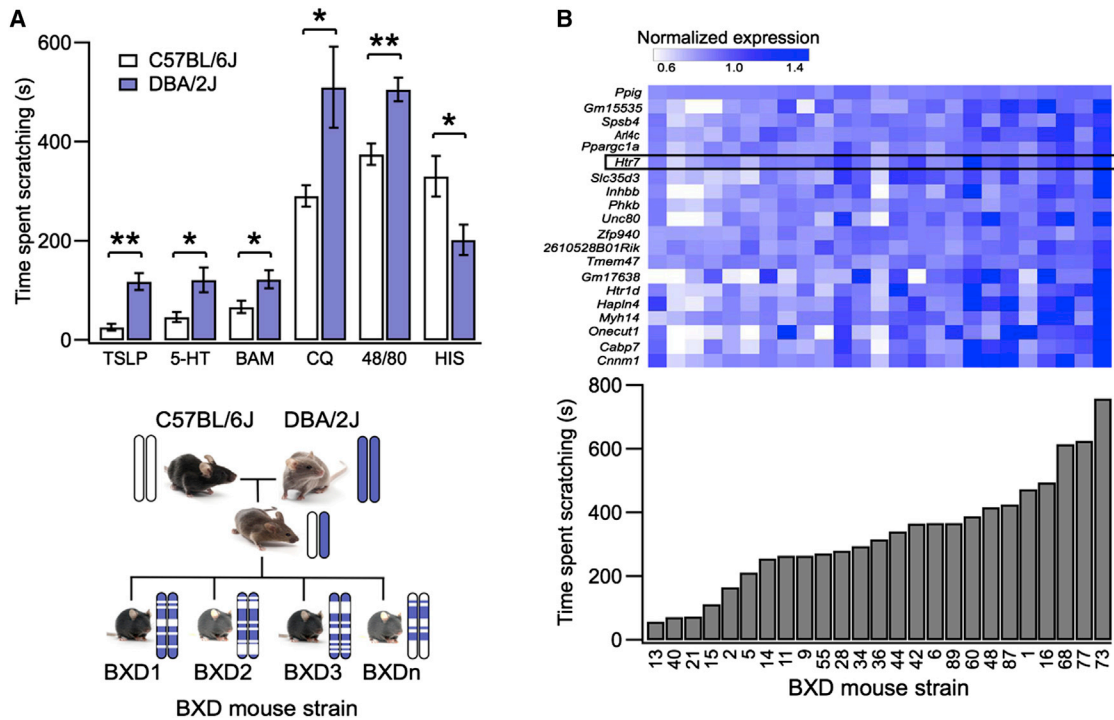
Peripheral serotonin, or 5-hydroxytryptamine (5-HT), is a component of the “inflammatory soup” and has been identified as a potent inducer of itch (Bautista et al., 2014; Hoon, 2015) and pain (Bardin, 2011; Julius and Basbaum, 2001; Kayser et al., 2007; Zeitz et al., 2002). Indeed, in humans, aberrant 5-HT signaling in the skin is linked to itch in atopic dermatitis (Huang et al., 2004; Soga et al., 2007), as well as in other disorders including allergy (Liu et al., 2013; Lundberg et al., 1999), uremia (Kerr et al., 1992), cholestasis (Schwörer et al., 1995), and psoriasis (Nordlind et al., 2006). Although numerous studies have highlighted the importance of 5-HT in acute and chronic itch (Han et al., 2013; Huang et al., 2004; Imamachi et al., 2009; Liu et al., 2013; Mishra and Hoon, 2013), the 5-HT receptor subtypes that transduce serotonergic itch signals have remained enigmatic.

Here, we examined itch behaviors and sensory neuron gene expression across genetically distinct mouse strains. We observed a correlation between acute itch behavior and expression of the 5-HT receptor, HTR7. This first clue to the function of HTR7 in the periphery served as motivation for an extensive characterization of its role in serotonergic and chronic itch. We found that HTR7 is expressed in a subset of primary afferent sensory neurons that innervate the skin and promote acute itch but not pain behaviors. HTR7 is functionally coupled to the irritant receptor TRPA1, and together they trigger neuronal excitation and mediate acute serotonergic- and SSRI-evoked itch. We also established that HTR7 and TRPA1 are key mediators of chronic itch in a mouse model of atopic dermatitis. Our finding is the first demonstration of a 5-HT receptor that mediates both acute and chronic itch in the periphery.

## RESULTS

### Examining Natural Variation in Itch Behaviors and Gene Expression

Previous studies have shown that genetically distinct inbred mouse strains display variable somatosensory behaviors such



**Figure 1. A Survey of Mouse Natural Variation Reveals *Htr7* as a Candidate Itch Transducer in DRG Neurons**

(A) The mouse strains C57BL/6J and DBA/2J display differential scratching behavior to various pruritogens: TSLP, thymic stromal lymphopoietin (15 nM); 5-HT, 5-hydroxytryptamine (1 mM); BAM, bovine adrenal medulla peptide 8-22 (3.5 mM); CQ, chloroquine (40 mM); 48/80, compound 48/80 (4 mM); HIS, histamine (27 mM). Student's *t* test; \**p* < 0.05; \*\**p* < 0.01; *n* = 4–8/strain. Error bars represent SEM. At bottom, schematic representation of the BXD recombinant inbred strains from a cross between C57BL/6J and DBA/2J mouse strains.

(B) Heatmap visualization of the 20 genes whose expression in dorsal root ganglia (DRG) neurons correlates most highly with itch behavior (time spent scratching, 30 min) across BXD mouse strains; each row reports normalized gene expression from one strain (white, low relative to gene median; dark blue, high) ordered based on CQ itch sensitivity (bottom). Black box highlights *Htr7*. See also Tables S1 and S2.

as pain and itch (Green et al., 2006; Nair et al., 2011). We set out to identify transcripts that were co-regulated with itch behaviors across such mouse strains. We first surveyed the effects of a battery of pruritogens in C57BL/6J (BL6) and DBA/2J (DBA) mice (Figure 1A). As itch is mediated by both histamine-dependent and -independent itch circuits, we examined a number of pruritogens that target each pathway. Because we sought to compare responses to a single pruritogen across strains, rather than between pruritogens, we chose concentrations for each pruritogen that elicited reliable scratching behaviors in the literature (Green et al., 2006; Kuraishi et al., 1995, 2000; Liu et al., 2012; Wilson et al., 2011, 2013b). All histamine-independent compounds tested—the anti-malarial drug chloroquine, compound 48/80, and the endogenous pruritogens 5-HT, thymic stromal lymphopoietin (TSLP), and bovine adrenal medulla peptide 8-22 (BAM 8-22)—evoked more avid scratching in the DBA strain than in BL6 (Figure 1A). In contrast, histamine-evoked itch was more avid in the BL6 strain than in DBA (Figure 1A; Green et al., 2006).

To ask whether these parental itch phenotypes were heritable, we next assayed itch behaviors in a panel of inbred progeny strains from a cross between BL6 and DBA (Taylor et al., 1973) (BXD; Figure 1A). We were particularly interested in histamine-independent itch responses because most forms of

chronic itch are histamine independent. We chose to use the itch response to chloroquine as a representative of all histamine-independent pruritogens, for a number of reasons. First, chloroquine evokes histamine-independent itch in both mice and humans (Green et al., 2006; Liu et al., 2009). Second, the subpopulation of neurons that expresses the chloroquine receptor *MrgprA3* mediates multiple forms of histamine-independent acute and chronic itch, and selective activation of these neurons triggers itch but not pain behaviors (Han et al., 2013). Finally, given that the DBA parental strain exhibited stronger itch behaviors to all non-histaminergic pruritogens tested (even those known to signal via distinct cellular pathways; Figure 1A), we hypothesized that this strain harbors one or more alleles that promote multiple forms of histamine-independent itch, for which chloroquine could serve as a proxy. We observed a wide range of itch responses in progeny strains from the BXD cross upon chloroquine injection (Figure 1B), far beyond the spread attributable to measurement error alone (broad-sense heritability = 0.65), reflecting a quantitative relationship between genetic background and this complex somatosensory behavior.

We considered that the DNA variation across strains that drove itch behaviors would also perturb expression of many genes, including some involved in distinct itch pathways. This

expectation was based on the principle that a naturally occurring DNA sequence variant underlying a given phenotype (e.g., disease or behavior) often affects the expression of hundreds of genes of related but distinct function (Schadt, 2005; Cookson et al., 2009). Indeed, it is axiomatic that genes of many subtypes, though of broadly similar biological function, can be co-regulated transcriptionally. For example, nutrient restriction upregulates genes that are protective for starvation as well as those dispensable for this behavior but required for other related stress responses (Giaever and Nislow, 2014). We thus hypothesized that there could be transducers in any of a multitude of itch pathways among the transcripts co-regulated with chloroquine itch across mouse strains. To identify such transcripts, we transcriptionally profiled dorsal root ganglia (DRG), which contain the neurons responsible for detecting itch, touch, and pain stimuli, in each BXD progeny strain. We identified 72 genes whose expression correlated positively across strains with itch behavior, and 37 negatively correlated with itch ( $|r| > 0.55$ ; Figure 1B and Table S1); this set of transcriptional correlates of itch was enriched in a number of somatosensory and cell signaling categories, including 5-HT signaling (Table S2).

### HTR7 Is a Candidate Itch Transducer

Among the top transcriptional correlates of chloroquine itch, we noted two 5-HT receptors, HTR7 and HTR1d. 5-HT has long been linked to chronic itch and pain (Bardin, 2011; Bautista et al., 2014; Hoon, 2015; Julius and Basbaum, 2001; Zeitz et al., 2002), and somatosensory neurons express ten distinct subtypes of 5-HT receptors (Bardin, 2011; Manteniotis et al., 2013) with the exact role of each subtype in somatosensation yet to be defined. We thus singled out for functional characterization HTR7, the receptor whose expression was most highly correlated with itch behavior (Figure 1B). *Htr7* expression was 2-fold higher in the most sensitive progeny strain than in the least responsive progeny (Figure 2A), and 1.3-fold higher in the more itch-prone DBA parental strain than in BL6 ( $BL6 = 1,031.67 \pm 78.19$  reads,  $DBA = 1,305.00 \pm 18.19$  reads;  $p < 0.05$ ;  $n = 3$  mice/strain).

### HTR7 Is Expressed and Functional in Sensory Neurons that Innervate the Skin

Somatosensory ganglia contain an array of neurons that mediate itch, touch, and pain. We expected that, if HTR7 played a role in itch transduction, we should observe expression in a subset of the small-diameter DRG neurons that detect noxious stimuli and innervate the skin. In validation of our RNA-seq measurements, we detected *Htr7* transcripts in human and mouse DRG with RT-PCR (Figure 2B). Using in situ hybridization, we detected the *Htr7* transcript in 11.2% of DRG neurons, the majority of which were small-diameter neurons (average size =  $17.7 \mu\text{m}$ ; Figure 2C). Next, we probed hairy skin using antibodies against HTR7 and TRPA1 and found that HTR7 protein was expressed in  $23.6\% \pm 6.9\%$  of TRPA1-positive fibers that innervate the skin (Figure 2D). Although we also observed HTR7 immunoreactivity in the epidermis, such staining was non-specific, as it manifested in both wild-type and HTR7 knockout mice (Figure S1A). We next used ratiometric calcium imaging to investigate whether HTR7 was functional in cutaneous primary afferent sensory neurons.

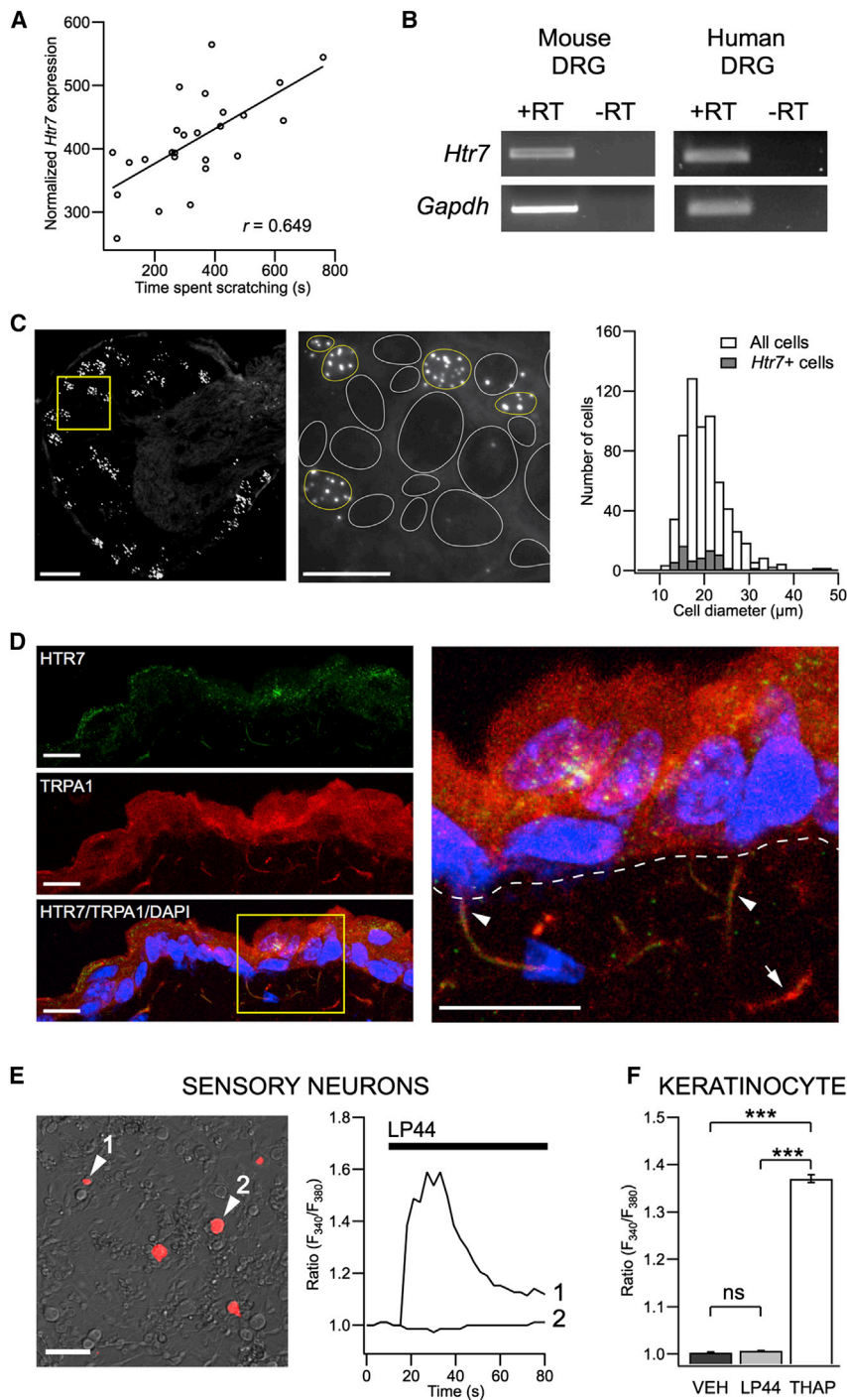
We first labeled cutaneous afferents via intradermal injection of the retrograde tracer cholera toxin-B-594 (CTB-594) and cultured dissociated sensory neurons 24 hr post-injection. Application of the HTR7-selective agonist, 4-[2-(Methylthio)phenyl]-N-(1,2,3,4-tetrahydro-1-naphthalenyl)-1-iperazinehexanamide hydrochloride (LP44), to cultured DRG neurons triggered a rise in intracellular calcium in  $8.3\% \pm 0.76\%$  of labeled cutaneous afferents ( $37.9\% \pm 5.2\%$  of all neurons were labeled red), suggesting that HTR7 is functional in neurons that innervate the skin (Figure 2E).

The LP44-evoked neuronal response was dose dependent with an  $EC_{50}$  at  $85.7 \pm 3.0 \mu\text{M}$  (Figure S1B). In contrast, LP44 failed to elicit a rise in intracellular calcium in primary mouse keratinocytes (Figure 2F), suggesting that HTR7 is not functional in keratinocytes. Overall, these data show that HTR7 is expressed and functional in a subset of small-diameter sensory neurons that innervate the skin and mediate itch and/or pain.

Previous studies have shown that neurons that transduce itch and pain signal via the TRPA1 and TRPV1 ion channels (Imamachi et al., 2009; Karai et al., 2004; Mishra and Hoon, 2013; Wilson et al., 2011, 2013a). We found that all LP44-sensitive neurons were responsive to the TRPA1 agonist allyl isothiocyanate (AITC) and to the TRPV1 agonist capsaicin (Figures 3A–3C). LP44-positive neurons were also sensitive to other pruritogens, including 5-HT (100%), chloroquine (63.6%), histamine (27.3%), and BAM8-22 (13.6%), but not TSLP (Figure 3C). These data show that HTR7 is functionally expressed in a heterogeneous subpopulation of serotonergic neurons that may subserve distinct roles in itch or inflammation. LP44 also evoked membrane depolarization and action potential firing in DRG neurons (Figure 3D). Thus, HTR7 activation induces neuronal excitability in a subset of TRPV1- and TRPA1-positive neurons. To determine whether HTR7, TRPA1, and TRPV1 are required for LP44-evoked excitation, we evaluated responses in neurons isolated from mice lacking these receptors. LP44-evoked calcium responses were significantly attenuated in DRG neurons isolated from *Htr7*<sup>-/-</sup> and *Trpa1*<sup>-/-</sup> mice, but not in *Trpv1*<sup>-/-</sup> neurons (Figure 3E). Similar results were observed in trigeminal ganglia (TG; data not shown). These results suggest that HTR7 is functionally coupled to TRPA1 and that the two receptors work together to mediate LP44-evoked excitation in a subset of itch and/or pain neurons.

### HTR7 Activates TRPA1 via Adenylate Cyclase Signaling

HTR7 is a Gs-coupled GPCR that activates adenylate cyclase (AC) via both  $G\alpha$  and  $G\beta\gamma$  in a variety of cell types (Adham et al., 1998; Lovenberg et al., 1993; Tang and Gilman, 1991). Previous studies have also shown that both AC and  $G\beta\gamma$  signaling activate the ion channel TRPA1 (Schmidt et al., 2009; Wilson et al., 2011). In sensory neurons, we found that inhibition of AC and  $G\beta\gamma$  signaling with 2',3'-dideoxyadenosine and gallein, respectively, attenuated LP44-evoked calcium signals, whereas U-73122, an inhibitor of PLC signaling, had no effect (Figure 3F). We next asked whether HTR7 could activate heterologous TRPA1 channels expressed in HEK293 cells. LP44-evoked currents were observed in HEK293 cells transfected with both hHTR7 and hTRPA1, but not in cells expressing hHTR7 alone (Figures 3G–3I); AITC triggered a similar current of larger



### Figure 2. HTR7 Is Expressed in Sensory Neurons that Innervate the Skin

(A) *Htr7* expression in DRG neurons correlates strongly with CQ itch across BXD strains. Each point represents one BXD strain.

(B) PCR amplification of *Htr7* transcripts shows expression in mouse and human DRG.

(C) Left: in situ hybridization of an *Htr7* probe in mouse DRG. Scale bar, 100  $\mu\text{m}$ . Middle: a higher magnification of the region enclosed by the yellow box in the left panel. *Htr7*-positive cell bodies are highlighted in yellow, *Htr7*-negative cell bodies in white. Scale bar, 50  $\mu\text{m}$ . Right: *Htr7* is expressed mostly in small-diameter cells ( $n = 5$  sections, 642 cells).

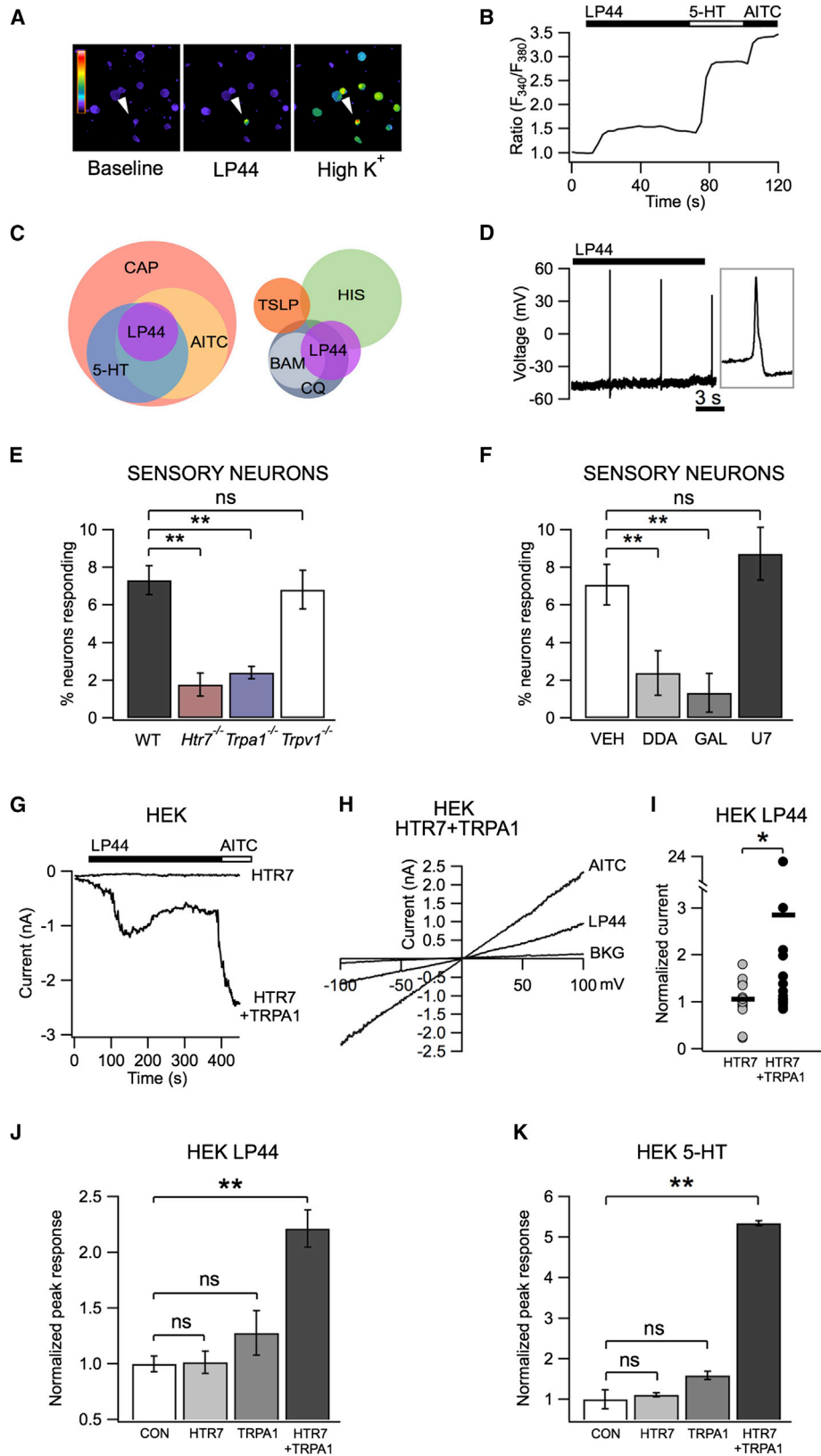
(D) Immunostaining of hairy skin shows expression of HTR7 in a subset of TRPA1-positive sensory neuronal fibers. Left: immunostaining of hairy skin with antibodies against HTR7 and TRPA1, counter stained with DAPI. Right: a higher magnification of region enclosed by the yellow box in the left panel. Arrowheads mark HTR7- and TRPA1-positive neuronal fibers; the arrow marks an HTR7-negative, TRPA1-positive neuronal fiber. Dotted line demarcates dermal-epidermal boundary based on DAPI staining. Scale bar, 10  $\mu\text{m}$ .

(E) Left: retrogradely labeled cutaneous afferents with cholera toxin subunit B (CTB) were cultured and LP44 (100  $\mu\text{M}$ ) responses were assayed using calcium imaging. Scale bar, 100  $\mu\text{m}$ . Right: LP44 promotes calcium influx in labeled cutaneous afferent 1, but not 2.

(F) LP44 does not activate primary mouse keratinocytes; quantification of peak LP44-evoked  $\text{Ca}^{2+}$  responses in mouse primary keratinocytes to vehicle (VEH), LP44, and thapsigargin (THAP). One-way ANOVA, Tukey-Kramer post hoc test; ns,  $p \geq 0.05$ ; \*\*\* $p < 0.001$ . Error bars represent SEM. See also Figure S1.

magnitude in cells expressing both hHTR7 and hTRPA1 (Figures 3G and 3H). Likewise, LP44 evoked significant increases in intracellular calcium in HEK293 cells expressing both HTR7 and TRPA1, but not in cells expressing either receptor alone (Figure 3J). HTR7 and TRPA1 together, but neither alone, also conferred 5-HT sensitivity to HEK293 cells (Figure 3K). Thus, HTR7 and TRPA1 receptors are functionally coupled and mediate response to both LP44 and 5-HT.

2004), others found that systemic application of an HTR7 agonist had a modest attenuating effect on inflammatory pain (Brenchat et al., 2009, 2012), and most recently, injection of an HTR7 agonist into the anterior cingulate cortex attenuated mechanical hypersensitivity (Santello and Nevian, 2015). To investigate whether specific activation of peripheral HTR7 by LP44 triggered itch and/or pain behaviors, we used the mouse cheek model of itch, in which pruritic agents elicit scratching with the hindlimb,



(legend on next page)

and nociceptive agents cause wiping with the forepaw (LaMotte et al., 2014). Injection of LP44 into the cheek of wild-type mice evoked scratching behaviors that were not observed following vehicle injection (Figure 4A). HTR7 is the sole mediator of LP44-evoked scratching as pharmacological inhibition (SB-269970; Figure 4A, gray) or genetic ablation (Figure 4B, red) of HTR7 significantly attenuated LP44-evoked scratching to levels similar to vehicle. We also observed significantly more scratching in the DBA mouse strain than in BL6 (Figure S1C), mirroring the higher *Htr7* expression in DBA sensory ganglia. Given the requirement of TRPA1 for LP44-evoked signaling in neurons (Figure 3E), we hypothesized that TRPA1 would likewise be required for LP44-evoked behaviors. Indeed, animals lacking *Trpa1*, but not *Trpv1*, exhibited attenuated LP44-evoked scratching (Figures 4C and 4D). Echoing our finding of differences between DBA and BL6 mice, we observed some variation in LP44 response across distinct wild-type strains (compare wild-type mice in Figures 4B–4D); analysis of any one mutant strain was thus carried out with respect to its isogenic wild-type. *Htr7* and *Trpa1* were required for LP44-evoked itch in both the cheek and neck itch models in line with the requirement of these receptors in LP44-evoked excitability in both TG and DRG neurons. Taken together, these data show that both HTR7 and TRPA1 are required for LP44-evoked itch behaviors.

Does LP44 act on HTR7 and TRPA1 receptors that are expressed on sensory neurons, or on non-neuronal cells in the skin to induce itch behaviors? We first asked whether sensory neurons are required for LP44-evoked itch. To address this question, we used the TRPV1 agonist, resiniferatoxin (RTX), which when injected results in defunctionalization of TRPV1- and TRPV1/TRPA1-positive neurons (Karai et al., 2004; Mishra and Hoon, 2013; Wilson et al., 2013a). LP44-evoked scratching was significantly decreased in RTX-treated mice, to levels observed in vehicle-treated mice (Figure 4E). These findings show that LP44 induces itch via TRPV1<sup>+</sup> sensory neurons but do not reveal the site of transduction. Previous studies suggest

that HTR7 and TRPA1 may be expressed in mast cells (Idzko et al., 2004; Oh et al., 2013), which release a variety of compounds that can activate sensory neurons and trigger itch. Thus, we next asked whether HTR7-evoked itch is mast cell dependent. We compared HTR7-evoked itch behaviors in a mouse strain lacking mast cells (*Kit<sup>W-sh</sup>*) to wild-type controls (Figure 4F). LP44 triggered considerable scratching in both strains, suggesting that HTR7-mediated responses do not require mast cells, or pruritogens/cytokines released by mast cells, to trigger itch behaviors. TRPA1 has also been proposed to be expressed in keratinocytes; though whether it is expressed and functional in mouse keratinocytes remains to be determined. However, LP44-evoked itch behaviors in *K14-Cre;Trpa1<sup>fl/fl</sup>* mice and *Trpa1<sup>fl/fl</sup>* mice were indistinguishable (Figure 4G), indicating that TRPA1 in keratinocytes does not underlie this phenotype; these findings are consistent with our observation that keratinocytes exhibited no calcium response to LP44 (Figure 2F). Overall these data show that neither mast cells nor TRPA1-mediated signaling in keratinocytes are required for acute itch triggered by HTR7 activation and suggest that HTR7 in sensory neurons may mediate LP44-evoked itch.

We next sought to establish whether activation of HTR7 could elicit pain. Unlike 5-HT, which has been shown to trigger both itch-evoked scratching and nocifensive wiping behaviors (Akiyama et al., 2010), we observed no wiping in LP44-injected animals (Figure 4H). Likewise, injection of LP44 into the hindpaw did not cause thermal hypersensitivity, in stark contrast to 5-HT injection, which induced a significant decrease in the thermal withdrawal latency (Figure 4I). Overall, these data make clear that acute activation of HTR7 by LP44 evokes itch but not pain behaviors.

### HTR7 Mediates Serotonergic Itch

We next set out to evaluate the role of HTR7 in serotonergic itch. Most studies measuring 5-HT-evoked itch and pain behaviors have used relatively high doses (1–94 mM; Akiyama et al., 2010; Kuraishi et al., 2000; Nojima and Carstens, 2003; Zeitz

### Figure 3. HTR7 Signals through Gβγ, Adenylate Cyclase, and TRPA1 to Promote Neuronal Excitation

(A) Fura-2-loaded DRG neurons treated with LP44 (100 μM) and KCl (75 mM). Arrowhead depicts an LP44-responsive neuron. Pseudocolor bar represents Fura-2 ratio.

(B) Representative trace shows a neuron that responds to LP44 (100 μM), 5-HT (100 μM), and allyl isothiocyanate (AITC; 100 μM).

(C) Left: LP44-responsive neurons are all sensitive to 5-HT (100 μM), AITC (100 μM), and capsaicin (1 μM). Right: the majority of these neurons overlap with the population of chloroquine-sensitive neurons (64%), and smaller subsets overlap with chloroquine/BAM8-22-sensitive (6%) or histamine-sensitive neurons (27%). No overlap is observed in the CQ-insensitive, TSLP-positive population.

(D) Current-clamp recording showing action potential firing evoked by LP44 (100 μM) in a representative DRG neuron. Inset, single action potential.

(E) Percentage of calcium responders to LP44 (100 μM) is reduced in neurons from *Htr7<sup>-/-</sup>* and *Trpa1<sup>-/-</sup>* mice, but not *Trpv1<sup>-/-</sup>* mice, relative to wild-type (WT).

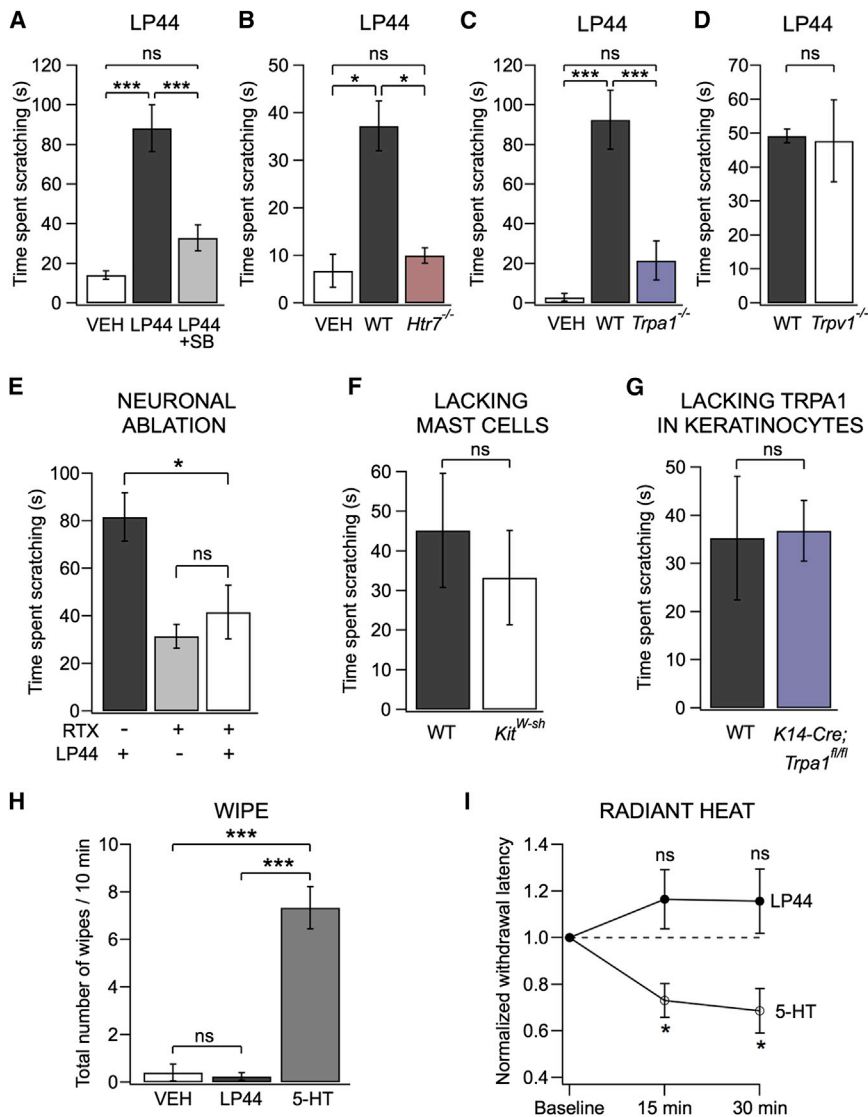
(F) Percentage of calcium responders to LP44 (100 μM) is reduced in neurons pretreated with the adenylate cyclase blocker 2',3'-dideoxyadenosine (DDA, 50 μM) or the Gβγ blocker gallein (GAL, 100 μM), but not the PLC blocker, U73122 (U7, 1 μM), relative to vehicle treatment (VEH). One-way ANOVA, Tukey-Kramer post hoc test; ns,  $p \geq 0.05$ ; \*\* $p < 0.01$ ;  $n = 3$  mice/genotype or treatment,  $n \geq 200$  cells/genotype or treatment.

(G) An inward current is evoked by LP44 (100 μM) in HEK293 cells transfected with HTR7 and TRPA1 but not with HTR7 alone using voltage-clamp recording. (H) Representative current-voltage trace of HEK293 cell transfected with HTR7 and TRPA1 in the absence (background, BKG) or presence of LP44 (100 μM) or AITC (100 μM).

(I) HTR7 and TRPA1 are both required for LP44-evoked currents in transfected HEK cells; quantification of LP44-induced peak currents (normalized to vehicle treatment) in HEK293 cells transfected as in (G). Each point represents one cell. Fisher's exact test; \* $p < 0.05$ ;  $n = 16$  cells/transfection.

(J) HTR7 and TRPA1 are both required for LP44-evoked calcium influx in transfected HEK cells; quantification of peak LP44-evoked  $Ca^{2+}$  response in HEK293 cells transfected with pcDNA3 (CON), HTR7, and/or TRPA1.

(K) HTR7 and TRPA1 are both required for 5-HT-evoked calcium influx in transfected HEK cells; quantification of peak 5-HT-evoked (100 μM)  $Ca^{2+}$  response in HEK293 cells transfected with pcDNA3 (CON), HTR7, and/or TRPA1. One-way ANOVA, Tukey-Kramer post hoc test; ns,  $p \geq 0.05$ ; \*\* $p < 0.01$ ;  $n = 3$ –5 transfections. Error bars represent SEM.



#### Figure 4. HTR7 Triggers Robust Acute Itch Behaviors, but Not Pain

(A) LP44 (2 mM), but not vehicle (VEH; 20 μl, 2% DMSO in PBS) injection induces scratching behaviors in the cheek model of itch. This response is blocked by the pre-injection of HTR7 antagonist SB-269970 (SB, 5 mM).

(B) Scratching evoked by LP44 injection is attenuated in *Htr7*<sup>-/-</sup> mice relative to *Htr7*<sup>+/+</sup> wild-type mice (WT).

(C) Scratching evoked by LP44 injection is attenuated in *Trpa1*<sup>-/-</sup> mice relative to *Trpa1*<sup>+/+</sup> wild-type mice (WT).

(D) LP44-evoked itch behavior is similar in *Trpv1*<sup>+/+</sup> (WT) and *Trpv1*<sup>-/-</sup> mice.

(E) TRPV1-positive neuronal ablation by resiniferatoxin (RTX) eliminates LP44-evoked scratching.

(F) LP44-evoked itch behaviors are similar in mast cell deficient mice (*Kit*<sup>W-sh</sup>) and wild-type control mice (WT).

(G) LP44-evoked itch behaviors are similar in *K14-Cre;Trpa1*<sup>fl/fl</sup> mice and *Trpa1*<sup>fl/fl</sup> control mice.

(H) LP44 (2 mM) does not evoke wiping behavior, while 1 mM 5-HT causes robust wiping. VEH, vehicle.

(I) Thermal hypersensitivity in mice injected with 5-HT (10 μM) and LP44 (2 mM). One-way ANOVA, Tukey-Kramer post hoc test; ns, p ≥ 0.05; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; n = 5–9 mice/genotype/behavior. Error bars represent SEM. See also Figure S1.

et al., 2002). Yet the elevated 5-HT levels in chronic itch patients and in mouse models have been measured to be in the micromolar range (Kerr et al., 1992; Liu et al., 2013; Lundberg et al., 1999; Soga et al., 2007). To test for distinctions between these two 5-HT dose regimes, we evaluated the behavioral effects of each in mice. Upon injection of 1 mM 5-HT in wild-type animals, we observed both scratching and wiping behaviors (Figures 5A and 5B), consistent with previous reports (Akiyama et al., 2010; Moser and Giesler, 2014). By contrast, injection of 100 μM 5-HT in wild-type mice elicited robust scratching but little wiping (Figures 5A and 5B). Together, these data show that 5-HT has distinct physiological effects at different concentrations and that 100 μM 5-HT preferentially activates itch pathways. HTR7 does not mediate behavioral responses to high 5-HT levels, as 1 mM 5-HT evoked itch and pain behaviors in *Htr7*<sup>-/-</sup> mice indistinguishable from those of wild-type animals (Figure 5C, red). However, the itch-specific effects of 100 μM 5-HT were mediated by HTR7: *Htr7*<sup>-/-</sup> animals exhibited a significant decrease

itch behaviors as compared to wild-type littermates, in response to 100 μM 5-HT (Figure 5E, blue).

Previous studies have shown that SSRIs increase 5-HT levels (Blardi et al., 2002) and that elevated 5-HT levels promote itch sensations and scratching in humans (Cederberg et al., 2004; Rausl et al., 2013). Some studies have shown that SSRIs can alleviate certain forms of chronic itch and pain (Anjaneyulu and Chopra, 2004; Browning et al., 2003; Zyllicz et al., 2003), while others have reported that SSRIs can induce severe itch and rash (Cederberg et al., 2004; Sannicandro et al., 2002; Warnock and Morris, 2002; FDA, 2014). We thus asked whether intradermal injection of SSRIs increased local 5-HT levels to trigger itch behaviors in mice. Injection of the SSRI sertraline induced a rapid increase in skin 5-HT (Figure 5F) and induced itch-evoked scratching in wild-type animals (Figure 5G). Mirroring the results of 100 μM 5-HT injection (Figures 5D and 5E), we observed a significant attenuation of SSRI-evoked scratching in *Htr7*<sup>-/-</sup> and *Trpa1*<sup>-/-</sup> mice as compared to wild-type



littermates (Figure 5H; no significant differences in behavior were observed between *Htr7*<sup>+/+</sup> and *Trpa1*<sup>+/+</sup> mice). Thus, HTR7 and TRPA1 together mediate some forms of serotonergic and SSRI-mediated itch.

We also examined the contribution of HTR7 to itch behaviors to a number of non-serotonergic pruritogens. Histamine, compound 48/80 and chloroquine all induced comparable itch behaviors in *Htr7*<sup>-/-</sup> and wild-type mice (Figure 5I). *Htr7*<sup>-/-</sup> mice also exhibited normal radiant heat- and AITC-evoked pain behaviors (Figures 5J and 5K). Overall, our findings make clear that HTR7 is a mediator of serotonergic itch, but not other forms of itch or pain.

### HTR7 and TRPA1 Are Required for the Development of Chronic Itch

Altered 5-HT signaling is associated with a variety of chronic itch conditions in humans, including atopic dermatitis (Huang et al., 2004; Soga et al., 2007). We next asked whether HTR7 plays a role in chronic itch in vivo, using a mouse model of atopic dermatitis. Previous studies have shown that topical treatment with a vitamin D analog, MC903, induces an atopic dermatitis-like phenotype that includes secretion of the atopic dermatitis cytokine, TSLP, immune cell infiltration, skin hyperplasia, and the development of eczematous lesions (Li et al., 2006). However, several additional hallmarks of human atopic dermatitis (AD), including high 5-HT levels in the skin and intense itch-evoked scratching, have yet to be quantified in this mouse model. To further validate this mouse model of human atopic dermatitis, we used a treatment course of 7 consecutive days of topical application of MC903 to the mouse cheek and monitored itch behaviors for a total of 12 days; we also measured 5-HT levels and skin lesion severity immediately following the final treatment.

Consistent with previous reports (Li et al., 2006), we found that wild-type mice treated with MC903 developed eczematous-like lesions that worsened over time in redness (erythema), dryness (xerosis), and scabbing (excoriation; Figures 6A and 6B). As is true for human atopic dermatitis patients, we found that MC903-treated mice displayed high levels of 5-HT in eczematous skin (Figure 6C), concomitant with scratching behaviors that began on the third day of treatment and increased in intensity, even after the 7-day treatment period (Figure 6D). These data show for the first time that the itch behaviors and 5-HT levels associated with human atopic dermatitis are recapitulated in this atopic dermatitis model.

*Htr7*<sup>-/-</sup> mice treated with MC903 exhibited less severe lesions and scratched significantly less than the wild-type strain across the entire 12-day experiment (Figures 6B and 6D, red). Consistent with the requirement for TRPA1 in HTR7-evoked acute itch (Figure 3H), lesion severity and scratching were also significantly reduced in *Trpa1*<sup>-/-</sup> mice (Figures 6B and 6D, blue). No significant differences in itch behavior or lesion severity were observed between *Htr7*<sup>+/+</sup> and *Trpa1*<sup>+/+</sup> wild-type animals of each strain and thus we combined all data for comparison to knockout animals. Previous studies have implicated HTR7 in immune cell signaling (Idzko et al., 2004), which may contribute to 5-HT release in the skin; in the AD model, we found that 5-HT levels in eczematous skin isolated from *Htr7*<sup>-/-</sup> and *Trpa1*<sup>-/-</sup> mice were indistinguishable from those of wild-type mice (Fig-

ure 6C, red). Thus, HTR7 and TRPA1 are not required for the release of 5-HT in atopic dermatitis skin. Taken together, our data identify HTR7 and TRPA1 as key molecular determinants of atopic dermatitis (Figure 7).

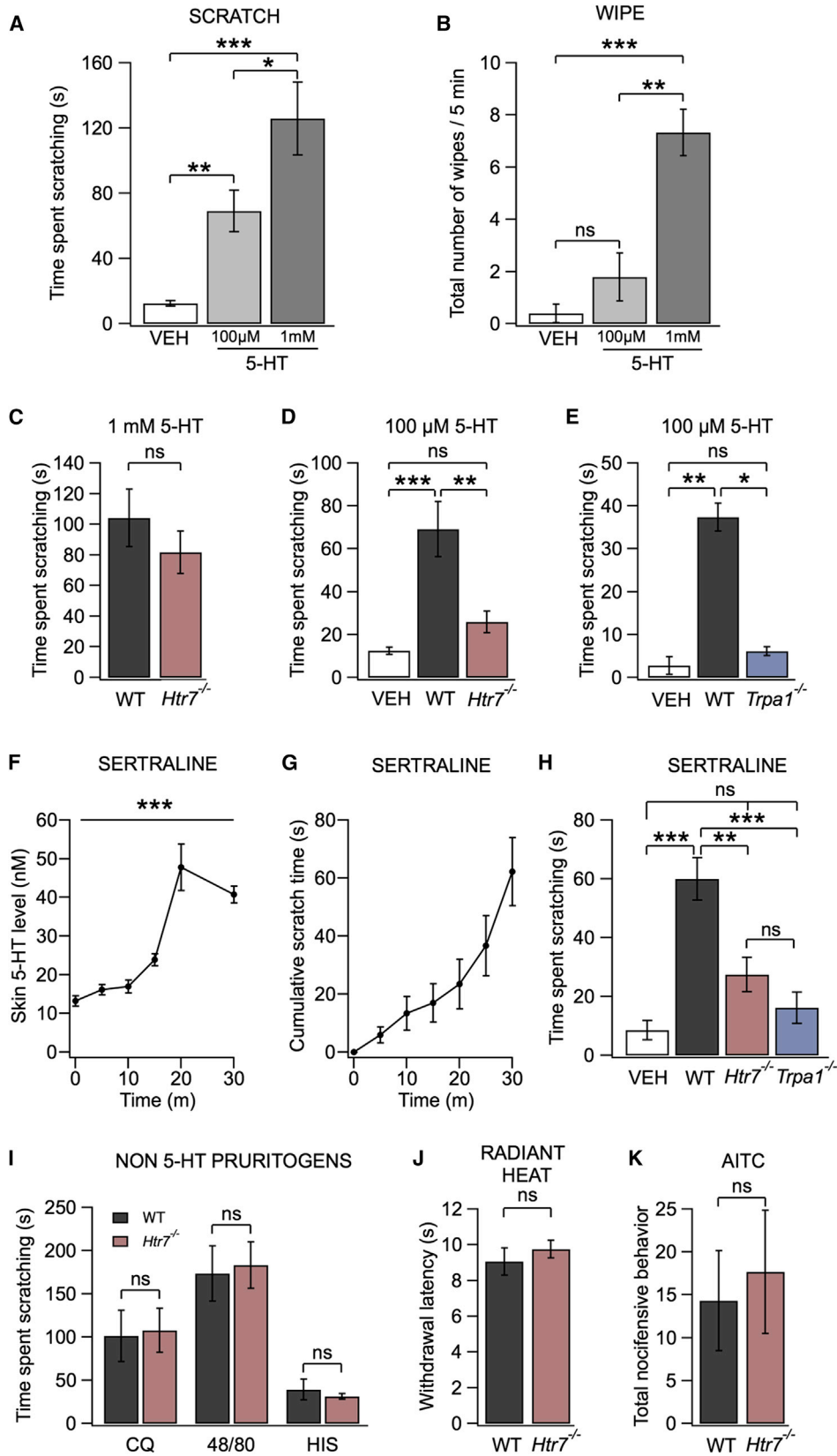
## DISCUSSION

In this work, we have taken advantage of natural variation in itch behavior in mice and gene expression to identify novel molecular determinants of itch in primary afferent sensory neurons. Many of the genes that we have found to be co-regulated with itch behavior have already been implicated in somatosensation, including *Htr1d* (Ahn and Basbaum, 2006; Tepper et al., 2002), *Scn8a* (Xie et al., 2013), and *Ano1* (Cho et al., 2012). However, a number of novel genes were also identified, many of which may represent new candidate itch transducers. We focused on the 5-HT receptor, HTR7, and established its role as a key transducer of serotonergic acute and chronic itch.

In the periphery, 5-HT can act as an algogen to trigger acute pain or pain hypersensitivity (Bardin, 2011; Julius and Basbaum, 2001; Kayser et al., 2007; Zeitz et al., 2002), a pruritogen to trigger itch-evoked scratching, rash, or wheal (Imamachi et al., 2009; Kalz and Fekete, 1960; Nojima and Carstens, 2003), or in some cases, both itch and pain (Akiyama et al., 2010; Moser and Giesler, 2014). A variety of 5-HT receptor subtypes are expressed in primary afferent sensory neurons, with HTR1–3 being the best studied. Pharmacological and genetic ablation studies have shown that HTR1a, HTR1b, HTR2a, HTR2b, and HTR3a mediate some forms of acute and inflammatory pain transmission (Abbott et al., 1996; Kayser et al., 2007; Lin et al., 2011; Nojima and Carstens, 2003; Van Steenwinckel et al., 2008; Zeitz et al., 2002). Pharmacological studies have also implicated peripheral HTR2a, HTR2b, and HTR3 receptors, and central HTR1a receptors in itch (Bonnet et al., 2008; Imamachi et al., 2009; Kyriakides et al., 1999; Liu et al., 2013; Zhao et al., 2014), but to date there has been no genetic evidence for any 5-HT receptor in transducing serotonergic itch signals in primary afferent neurons. We have used genetic and pharmacological tools to show that HTR7 plays a selective role in acute serotonergic and atopic dermatitis itch, but not pain. Our results augment the previous characterization of HTR7 in the CNS in circadian rhythm function (Glass et al., 2003; Lovenberg et al., 1993), central thermoregulation (Hedlund et al., 2003), neuroendocrine function (Jørgensen, 2007; Kim et al., 2013), migraine (Hedlund, 2009), and antinociception (Bardin, 2011; Santello and Nevia, 2015).

### HTR7 Mediates Acute Serotonergic Itch

Our data implicate HTR7 as a key component of the 5-HT itch pathway. In designing methods to assay 5-HT-evoked itch, we found that different concentrations of 5-HT evoked distinct behaviors. Micromolar 5-HT concentrations triggered itch but not pain behaviors, while millimolar concentrations triggered both itch and pain. Most experimental analyses of 5-HT-evoked behavior have used millimolar concentrations (1–94 mM; Akiyama et al., 2010; Kuraishi et al., 2000; Nojima and Carstens, 2003; Zeitz et al., 2002), which are significantly higher than the 5-HT levels measured in chronic itch conditions



(legend on next page)

(Kerr et al., 1992; Liu et al., 2013; Lundberg et al., 1999; Soga et al., 2007) and thus may not target physiologically relevant sites and 5-HT receptor subtypes. Our results make clear that HTR7 is required for itch behaviors evoked by 5-HT at 100  $\mu$ M, but not for itch and pain at 1 mM concentrations. These findings suggest that HTR7 may be preferentially activated by low 5-HT levels, which dovetail with studies showing that HTR7 has the highest affinity for 5-HT among HTR family members (Vanhoenacker et al., 2000). A role for HTR7 in only a subset of 5-HT-evoked behaviors under particular conditions, rather than all 5-HT responses, is not surprising given that many 5-HT receptors are expressed in sensory neurons (Bardin, 2011; Manteniotis et al., 2013) and that HTR7 is expressed in only ~35% of the 5-HT-positive neuronal population. The emerging picture is thus one of specific physiological responses to different doses of 5-HT that are mediated by distinct molecular and cellular mechanisms, with HTR7 mediating itch at 5-HT levels comparable to those measured in chronic itch skin.

### HTR7 Mediates Acute SSRI-Evoked Itch

In analyzing the role of HTR7 in serotonergic itch, we also examined itch behaviors in response to injection of the SSRI sertraline in the cheek model of itch. SSRI induced an increase in local 5-HT levels and a concomitant increase in scratching behaviors in mice; HTR7 was required for SSRI-evoked itch behaviors. An estimated 2%–4% of human patients report itch, rash, and other related skin conditions as a side effect of taking SSRIs (Cederberg et al., 2004; Sannicandro et al., 2002; FDA, 2014). As HTR7 is also expressed in human primary afferent neurons (Figure 2B), it is tempting to speculate that antagonists may attenuate SSRI-evoked itch in humans. Paradoxically, SSRIs can also partially alleviate some forms of chronic itch (Anjaneyulu and Chopra, 2004; Browning et al., 2003; Zylicz et al., 2003). The mechanisms underlying this dual role for SSRIs in alleviating and causing itch in humans are unknown; it will be interesting to determine whether they involve different 5-HT signaling molecules between normal and chronic itch patients, or differential targeting of peripheral versus central targets (Browning et al., 2003).

### HTR7 Couples to the Irritant Receptor TRPA1

Previous studies showed that serotonergic itch is mediated by a subset of TRPV1-expressing neurons, though functional TRPV1

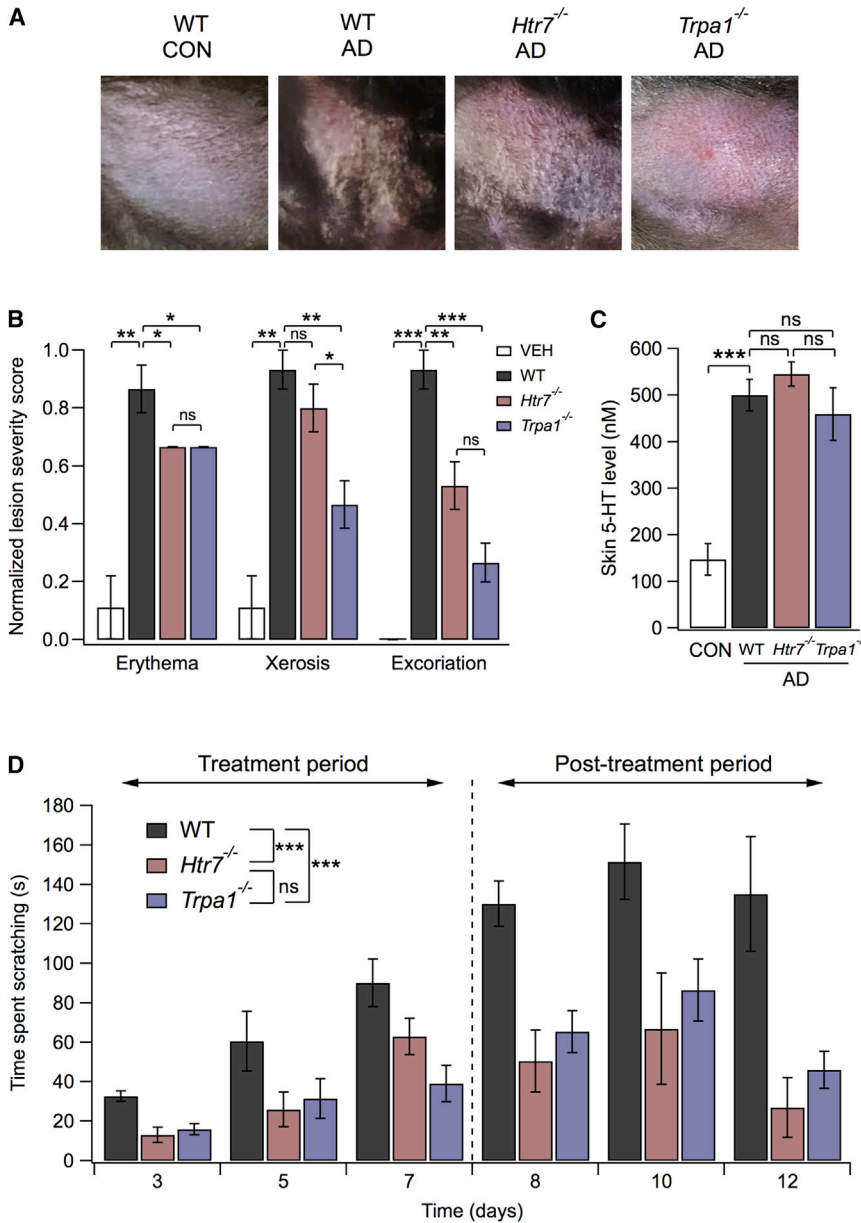
ion channels were dispensable for itch behaviors (Imamachi et al., 2009). Thus, the receptors and downstream signaling pathways responsible for 5-HT itch transduction were unknown. Our results establish TRPA1 as the transduction channel required for HTR7-mediated acute serotonergic and SSRI-evoked itch behaviors, echoing the known role for TRPA1 in other forms of non-histaminergic itch (Cevikbas et al., 2014; Liu et al., 2013; Oh et al., 2013; Wilson et al., 2011, 2013a, 2013b). Furthermore, we found that HTR7 activation of TRPA1 required functional adenylate cyclase (Figure 3), consistent with previous studies showing that HTR7 is a  $G\alpha_s$ -coupled GPCR that stimulates adenylate cyclase and cAMP production and that TRPA1 activity is regulated by AC and cAMP in sensory neurons (Schmidt et al., 2009). Interestingly, TRPA1 does not couple to all HTRs, as activation of HTR2 by  $\alpha$ -methyl 5-HT triggers itch and pain behaviors independent of TRPA1 (Liu et al., 2013; Schmidt et al., 2009; Wilson et al., 2011). Overall, our data further highlight the importance of TRPA1 in mediating non-histaminergic itch.

### Do HTR7 and TRPA1 Receptors Act in Neurons to Mediate Itch Behaviors?

HTR7 and TRPA1 expression have been reported in a variety of neurons in the CNS and non-neuronal cells in the skin (Bayer et al., 2007; Idzko et al., 2004; Kwan et al., 2009). Our study provides several lines of evidence that HTR7 in somatosensory neurons mediates itch behaviors. First, we found that HTR7 activation in mast cell-deficient mice evokes a normal itch response. Second, primary mouse and human keratinocytes do not express functional HTR7 receptors. Third, *K14-Cre;Trpa1<sup>fl/fl</sup>* mice and *Trpa1<sup>fl/fl</sup>* mice displayed similar HTR7-evoked itch responses. Fourth, neuronal ablation of TRPV1-expressing neurons resulted in a dramatic decrease in itch response evoked by HTR7 activation. Finally, HTR7 functions in the subset of the MrgprA3 population of neurons (Figure 2) that have proven to be indispensable in itch but not pain (Han et al., 2013). However, 5-HT is well known to play important roles in modulating itch and pain signal transmission at multiple levels in the CNS. Indeed, pharmacological activation of HTR7 in the forebrain has been recently shown to reduce neuropathic mechanical pain (Santello and Nevia, 2015). Also, 5-HT acts on a variety of immune and other non-neuronal cells to modulate itch and inflammation (Idzko et al., 2004; Soga et al., 2007). Thus, our findings using

### Figure 5. HTR7 Is Required for the Detection of 5-HT-Dependent Acute Itch

- (A) 5-HT (100  $\mu$ M and 1 mM) but not vehicle injection (VEH; 20  $\mu$ l, PBS) causes robust scratching in the cheek model of itch.  
 (B) Injection of 1 mM but not 100  $\mu$ M 5-HT evokes wiping behaviors.  
 (C) Scratching behaviors evoked by 1 mM 5-HT are similar between *Htr7<sup>+/+</sup>* (WT) and *Htr7<sup>-/-</sup>* mice.  
 (D) Scratching behaviors evoked by 100  $\mu$ M 5-HT are significantly attenuated in *Htr7<sup>-/-</sup>* versus *Htr7<sup>+/+</sup>* (WT) mice. VEH; 20  $\mu$ l, PBS.  
 (E) Scratching behaviors evoked by 100  $\mu$ M 5-HT are reduced in *Trpa1<sup>-/-</sup>* compared to *Trpa1<sup>+/+</sup>* (WT) mice.  
 (F) Skin 5-HT levels increase after injection with the selective serotonin reuptake inhibitor (SSRI), sertraline (100  $\mu$ M). One-way ANOVA, \*\*\* $p$  < 0.001.  
 (G) Time course of cumulative scratching behavior to sertraline (100  $\mu$ M) follows the time-dependent increase in skin 5-HT levels following sertraline injection, as shown in (F).  
 (H) *Htr7<sup>-/-</sup>* and *Trpa1<sup>-/-</sup>* mice show significant decreases in scratching behaviors compared to wild-type (WT) mice following sertraline injection (100  $\mu$ M).  
 (I) Chloroquine (CQ, 40 mM), compound 48/80 (48/80, 4 mM), and histamine (HIS, 27 mM) injection evokes similar itch behaviors in both *Htr7<sup>+/+</sup>* (WT) and *Htr7<sup>-/-</sup>* mice.  
 (J) There is no difference in acute heat sensitivity between *Htr7<sup>+/+</sup>* (WT) and *Htr7<sup>-/-</sup>* mice.  
 (K) AITC application (10% in mineral oil) evokes similar nocifensive behaviors in *Htr7<sup>+/+</sup>* (WT) and *Htr7<sup>-/-</sup>* mice. One-way ANOVA, Tukey-Kramer post hoc test; ns,  $p$   $\geq$  0.05; \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001;  $n$  = 5–9 mice/genotype/behavior. Error bars represent SEM.



**Figure 6. HTR7 Is Required for the Development of Chronic Itch in a Model of Atopic Dermatitis**

(A) Representative images of skin isolated from wild-type (WT), *Htr7*<sup>-/-</sup>, and *Trpa1*<sup>-/-</sup> mice treated topically with vehicle control (CON; 20 μl 100% ethanol) or the vitamin D analog, MC903 for the induction of atopic dermatitis (AD; 200 μM). (B) Atopic dermatitis lesion severity scores in mice treated with MC903 or vehicle (as described in A). (C) The AD model triggers an increase in the concentration of 5-HT-treated skin isolated from WT, *Htr7*<sup>-/-</sup>, and *Trpa1*<sup>-/-</sup> mice. One-way ANOVA, Tukey-Kramer post hoc test; ns, p ≥ 0.05; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; n = 5/genotype/treatment. (D) Scratching behavior during and after VITD treatment is attenuated in *Htr7*<sup>-/-</sup> and *Trpa1*<sup>-/-</sup> mice. ANOVA for multivariate linear models; \*\*\*p < 0.001; n = 10/genotype. Error bars represent SEM.

mouse model is the first in which spontaneous itch behaviors manifest in the absence of a daily skin treatment: we observed itch behaviors that persisted 5 days after the last skin treatment, establishing this paradigm as a model of chronic rather than acute itch. We found that *HTR7*- and *TRPA1*-deficient mice both displayed less severe skin lesions and scratched significantly less than wild-type mice (Figure 6D). However, these mutant animals displayed elevated levels of 5-HT in atopic dermatitis skin, similar to levels observed in wild-type (Figure 6C). Thus, *HTR7* and *TRPA1* are required for the detection, rather than the release of 5-HT under atopic dermatitis-like conditions.

When considered in light of previous studies, our data support a dual role for *TRPA1* in atopic dermatitis. First, when co-expressed with the TSLP receptor, *TRPA1* acts as a transducer of TSLP-

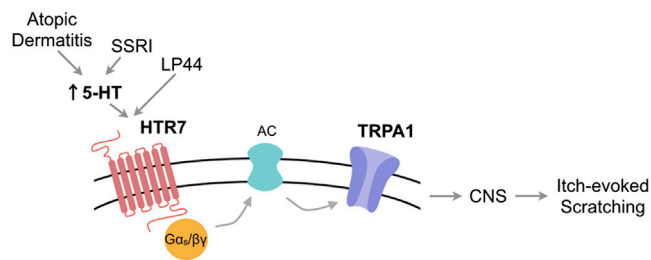
the global *HTR7* knockout do not exclude a role for *HTR7* outside primary afferent neurons. Future studies using tissue-specific knockout mice will determine the contributions of sensory neurons and other cell types to acute and chronic itch.

**HTR7 and TRPA1 Are Both Required for Development of Atopic Dermatitis**

In addition to our study of acute serotonergic itch, we also examined the role of *HTR7* and *TRPA1* in a mouse model of atopic dermatitis, motivated by the well-known links between 5-HT and itch in this condition (Huang et al., 2004; Soga et al., 2007). Our results revealed for first time that the MC903-induced mouse model of atopic dermatitis recapitulates the dysregulation of 5-HT in the skin, and the itch behaviors, characteristic of the human disorder (Huang et al., 2004). In addition, our

evoked itch and inflammation (Wilson et al., 2013b). Second, when co-expressed with *HTR7*, *TRPA1* acts as a transducer of 5-HT-evoked itch. We also highlight the importance of 5-HT signaling in the pathology of atopic dermatitis. Patients with severe atopic dermatitis eventually progress to develop asthma and allergic rhinitis, in a process known as the “atopic march” (Spergel and Paller, 2003). It is notable that elevated 5-HT levels are also linked to asthma (Bayer et al., 2007), and future studies will elucidate how *TRPA1*-expressing neuronal subtypes orchestrates the progression of atopic diseases.

Animals lacking *Htr7* and *Trpa1* still exhibited a residual degree of eczematous skin lesions and itch behaviors in the atopic dermatitis model. Thus, an important question is the identity of the other molecular players required for the complete development and/or maintenance of atopic dermatitis. Such



**Figure 7. Schematic Diagram Depicting the Putative Roles of HTR7 and TRPA1 Signaling in Acute and Chronic Itch**

Model by which increased 5-HT levels in the skin, triggered by direct injection of 5-HT, SSRI administration, or atopic dermatitis activates the 5-HT receptor, HTR7. HTR7 in turn signals to adenylylate cyclase via  $G\alpha$  and  $G\beta\gamma$ , to open TRPA1 ion channels, promote neuronal depolarization and action potential firing, and ultimately trigger itch-evoked scratching.

determinants may include TSLP, given its upregulation in the human disease and the atopic dermatitis mouse model (Li et al., 2006), and its activity in sensory neurons that promotes itch behaviors (Wilson et al., 2013b). Interestingly, another 5-HT receptor co-regulated with itch, *Htr1d* (Figure 1B), has been previously implicated in inflammatory pain (Ahn and Basbaum, 2006; Tepper et al., 2002) and is also a compelling candidate mediator of atopic dermatitis and itch. Moreover, numerous inflammatory mediators are released in atopic dermatitis in addition to 5-HT and TSLP (Brandt and Sivaprasad, 2011), whose mechanisms have yet to be revealed.

Despite these complexities, our data clearly show that HTR7 and TRPA1 are key mediators of serotonergic acute and chronic itch. Aside from AD, a variety of human chronic itch disorders are linked to 5-HT. It is likely, therefore, that HTR7 antagonists may prove to be useful for the selective attenuation of itch that results from these pathological conditions.

## EXPERIMENTAL PROCEDURES

### RNA-Seq Analysis

We mapped RNA-seq reads from the DRG of each BXD strain to the DBA/2J and C57BL/6J using Tophat. HTSeq was used to sum the total read counts per gene. Read counts were normalized with the EDASeq package in R using the upper-quartile method. Gene ontology enrichment analysis was performed using the topGO package in R. Information regarding analyses packages is available in the Supplemental Experimental Procedures. All expression data have been deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>).

### Histology

Histology on skin and in situ hybridization on DRG was carried out as previously described (Gerhold et al., 2013).

### Cell Culture

Cell culture and transfection was carried out as previously described (Wilson et al., 2011). Cholera toxin subunit B, Alexa Fluor 594 conjugate (Life Technologies) was used for retrograde labeling of cutaneous afferent neurons.

### Electrophysiology

Electrophysiology was performed as previously described (Wilson et al., 2013b). Whole-cell recordings in transfected cells were performed using the Port-a-patch system (Brueggemann et al., 2004) (Nanion Technologies).

### Calcium Imaging

$Ca^{2+}$  imaging experiments were carried out as previously described (Wilson et al., 2011). Image analysis and statistics were performed using Igor Pro (WaveMetrics).

### Behavioral Studies

*Htr7*<sup>-/-</sup> mice were obtained from Jackson Laboratory, *Trpv1*<sup>-/-</sup> and *Trpa1*<sup>-/-</sup> mice were described previously (Bautista et al., 2006; Caterina et al., 2000), and *K14-Cre;Trpa1*<sup>fl/fl</sup> mice were provided by Cheryl Stucky. Behavioral experiments were performed as previously described (Wilson et al., 2011). All experiments were performed under the policies and recommendations of the International Association for the Study of Pain and approved by the University of California, Berkeley Animal Care and Use Committee.

### Atopic Dermatitis Model

MC903 (R&D Systems) was applied to the mouse cheek once per day, for 7 days. Character of lesion scoring was adapted from previous studies (Hamp-ton et al., 2012; Liu et al., 2013; Yun et al., 2011).

### Statistical analysis

The following statistical tests were used where appropriate: Student's t test, one-way ANOVA, and ANOVA for multivariate linear models.

### ACCESSION NUMBERS

The accession number for the expression profiling of dorsal root ganglia in BXD mouse strains is GEO: GSE69374.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one figure, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2015.05.044>.

### AUTHOR CONTRIBUTIONS

T.M. performed and analyzed gene expression experiments. S.P.M., L.M.B., J.F.W., and C.L.S. designed and implemented the behavioral studies. S.P.M. and L.M.B. designed the histological studies. T.M., S.P.M., M.P., S.R.W., and D.M.B. carried out the cellular imaging experiments. M.P., M.A.K., K.L., and A.S.B.O. performed electrophysiology experiments. T.M., S.P.M., L.M.B., R.B.B., and D.M.B. analyzed data. T.M., S.P.M., R.B.B., and D.M.B. wrote the manuscript. R.B.B. and D.M.B. provided advice and guidance.

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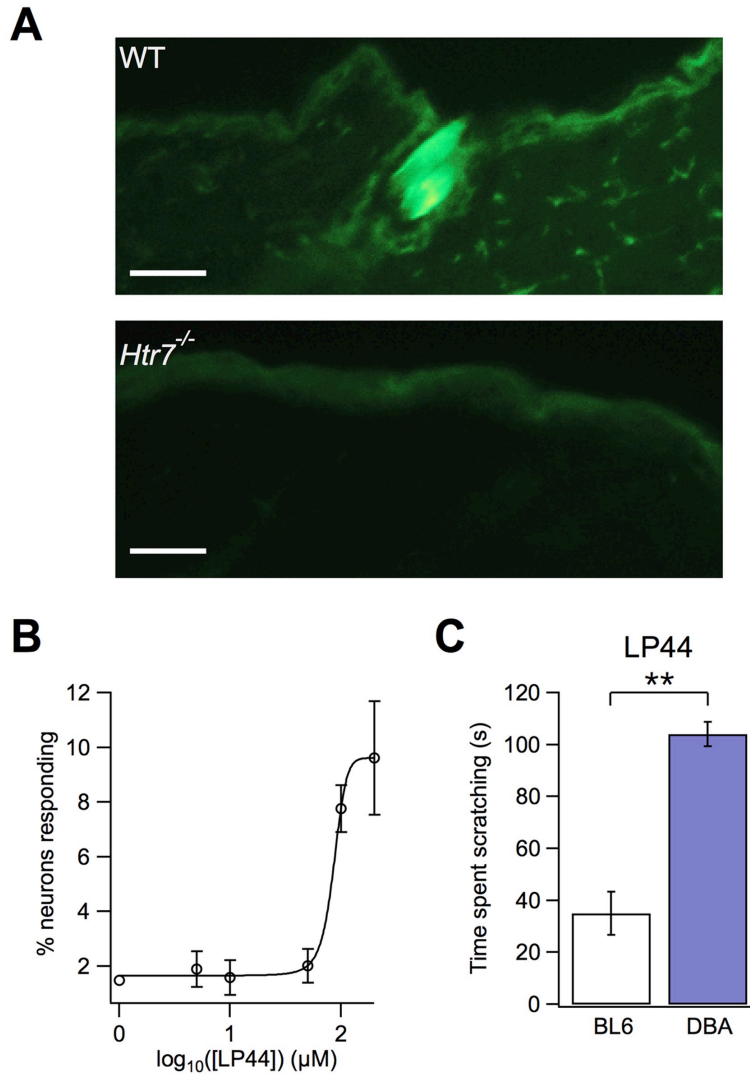


**Neuron**

**Supplemental Information**

## **HTR7 Mediates Serotonergic Acute and Chronic Itch**

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**Figure S1**

## SUPPLEMENTAL FIGURE LEGENDS

**Figure S1, Related to Figures 2 and 4. LP44 activates a subset of sensory neurons that express HTR7.**

(A) Validation of HTR7 antibody. Immunohistochemistry of hairy skin sections with an antibody against HTR7 shows neuronal staining in WT (top) but not in *Htr7<sup>-/-</sup>* (bottom). Scale bar, 50  $\mu$ m. (B) Dose-response curve of LP44 on primary DRG culture assayed by calcium imaging,  $n = 50-100$  cells/concentration. (C) LP44 evokes differential itch response between BL6 and DBA. Student's t-test,  $**p < 0.01$ ,  $n = 7$ /genotype. Error bars represent SEM.

## SUPPLEMENTAL TABLES

**Table S1, Related to Figure 1. Top candidate itch genes.**

List of candidate genes displaying strong correlation between DRG gene expression and CQ-evoked itch behavior ( $|r| > 0.55$ ).

Positive genes			Negative genes		
<i>r</i>	Gene symbol	<i>r</i>	Gene symbol	<i>r</i>	Gene symbol
0.704	<i>Ppig</i>	0.582	<i>Kcnc3</i>	-0.670	<i>Gm14399</i>
0.680	<i>Gm15535</i>	0.582	<i>Flt3</i>	-0.634	<i>4931406C07Rik</i>
0.674	<i>Spsb4</i>	0.581	<i>Endod1</i>	-0.630	<i>Rqcd1</i>
0.666	<i>Arl4c</i>	0.580	<i>Fam184b</i>	-0.617	<i>BC030867</i>
0.650	<i>Ppargc1a</i>	0.579	<i>Faim2</i>	-0.612	<i>Gm14326</i>
0.649	<i>Htr7</i>	0.578	<i>Ag1</i>	-0.610	<i>Atp1b3</i>
0.645	<i>Slc35d3</i>	0.578	<i>Scn8a</i>	-0.604	<i>Tmem176a</i>
0.638	<i>Inhbb</i>	0.577	<i>Mtap2</i>	-0.599	<i>BC048355</i>
0.638	<i>Phkb</i>	0.577	<i>Cbln2</i>	-0.598	<i>2210404O09Rik</i>
0.632	<i>Unc80</i>	0.576	<i>Ano1</i>	-0.593	<i>Ada</i>
0.629	<i>Zfp940</i>	0.573	<i>1700040D17Rik</i>	-0.588	<i>Med22</i>
0.626	<i>2610528B01Rik</i>	0.572	<i>Gm10676</i>	-0.583	<i>Tmem176b</i>
0.625	<i>Tmem47</i>	0.572	<i>Shh</i>	-0.581	<i>Pla2g16</i>
0.617	<i>Gm17638</i>	0.571	<i>2510049J12Rik</i>	-0.580	<i>Gm14325</i>
0.616	<i>Htr1d</i>	0.570	<i>Phospho1</i>	-0.578	<i>Sh3gl1</i>
0.615	<i>Hapln4</i>	0.569	<i>Rasa1</i>	-0.576	<i>Mrps6</i>
0.614	<i>Myh14</i>	0.568	<i>Atp2b2</i>	-0.575	<i>Gm14305</i>
0.613	<i>Onecut1</i>	0.565	<i>D630023F18Rik</i>	-0.572	<i>Cdkn1a</i>
0.611	<i>Cabp7</i>	0.564	<i>Kcnq4</i>	-0.572	<i>Api5</i>
0.611	<i>Cnnm1</i>	0.562	<i>Ppp1r12b</i>	-0.569	<i>Sertad1</i>
0.610	<i>Cpsf6</i>	0.559	<i>Cables2</i>	-0.569	<i>Casp1</i>
0.607	<i>Gm17111</i>	0.558	<i>Ogdhl</i>	-0.568	<i>Gm6543</i>
0.607	<i>Esrrg</i>	0.558	<i>Cnnm4</i>	-0.568	<i>Wtap</i>
0.604	<i>Wipf2</i>	0.557	<i>Kcnh7</i>	-0.565	<i>0610040B10Rik</i>
0.604	<i>Cntnap2</i>	0.556	<i>Epb4.111</i>	-0.565	<i>Rnh1</i>
0.602	<i>Rtbdn</i>	0.555	<i>Papd5</i>	-0.564	<i>Rxrg</i>
0.602	<i>Sh3rf3</i>	0.555	<i>Scrt2</i>	-0.564	<i>Bcl10</i>
0.601	<i>1700007I08Rik</i>	0.554	<i>Ube3b</i>	-0.563	<i>Cd9</i>
0.601	<i>Dynll2</i>	0.553	<i>Gm17443</i>	-0.563	<i>Setd6</i>
0.596	<i>Pcdh8</i>	0.553	<i>Cds1</i>	-0.561	<i>Ctnna1</i>
0.595	<i>Kcnc1</i>	0.553	<i>Scrt1</i>	-0.559	<i>Trp53i13</i>
0.591	<i>Heatr5a</i>	0.551	<i>A930033H14Rik</i>	-0.558	<i>Insc</i>
0.591	<i>9630001P10Rik</i>	0.551	<i>Hhatl</i>	-0.558	<i>Dok1</i>
0.590	<i>Chgb</i>	0.550	<i>Myrip</i>	-0.557	<i>Zfp119b</i>
0.589	<i>Clstn2</i>	0.550	<i>Gm17456</i>	-0.556	<i>Gm7325</i>
0.585	<i>Strbp</i>			-0.556	<i>Hmgcl</i>
0.583	<i>Bcl2</i>			-0.552	<i>Tssc4</i>

**Table S2, Related to Figure 1. Gene ontology (GO) enrichment of top candidate itch genes.**

List of GO terms that are enriched in top candidate genes displaying strong correlation between DRG gene expression and CQ-evoked itch behavior ( $r > 0.55$ ).

<b>GO ID</b>	<b>Term</b>	<b>p-value</b>
GO:0022843	voltage-gated cation channel activity	0.00011
GO:0005251	delayed rectifier potassium channel activity	0.00019
GO:0005249	voltage-gated potassium channel activity	0.0002
GO:0005516	calmodulin binding	0.00027
GO:0015267	channel activity	0.00035
GO:0022803	passive transmembrane transporter activity	0.00035
GO:0005244	voltage-gated ion channel activity	0.00041
GO:0022832	voltage-gated channel activity	0.00041
GO:0022836	gated channel activity	0.00046
GO:0022839	ion gated channel activity	0.00046
GO:0005267	potassium channel activity	0.00075
GO:0004993	serotonin receptor activity	0.00111
GO:0015079	potassium ion transmembrane transporter activity	0.00119
GO:0046873	metal ion transmembrane transporter activity	0.00144
GO:0005216	ion channel activity	0.00174
GO:0022838	substrate-specific channel activity	0.00182
GO:0005261	cation channel activity	0.00206
GO:0001948	glycoprotein binding	0.00276
GO:0015077	monovalent inorganic cation transmembr...	0.00292
GO:0022890	inorganic cation transmembrane transport...	0.0032

## **SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

### **RNA extraction and library preparation**

DRG neurons from one animal of each BXD progeny strain (see Figure 1B, bottom, for strains used) were removed and homogenized in Trizol (Life Technologies). Total RNA was extracted according to the manufacturer's protocol and genomic DNA was removed with Turbo DNase I (Life Technologies). Libraries were prepared using TruSeq RNA Sample Prep kit v2 (Illumina) and sequenced on Illumina HiSeq 2000 machines with 50bp single-end reads to obtain 30-40 million reads per sample.

### **RNA-seq analysis**

To map reads from DRG RNA-seq libraries from BXD mice, we first generated a DBA/2J reference genome by amending the C57BL/6J genome ([www.ensembl.org](http://www.ensembl.org), release NCBIM37) with DBA/2J SNPs and indels (<http://www.sanger.ac.uk>, release 'REL-1211-SNPs\_Indels'), using only those with a genotype quality Phred score of 99. We then mapped each RNA-seq read set to this DBA/2J reference and, separately, to the C57BL/6J reference, using Tophat (Trapnell et al., 2009) in each case. We retained for analysis only those reads in each mapping run that mapped uniquely with no mismatches. For a given read that met this criterion for each of the two references, we eliminated it from further analysis if the position to which it mapped was not orthologous between the two. HTSeq (Anders et al., 2015) was used to sum the total read counts per gene. Read counts were normalized with the EDASeq package in R

(Risso et al., 2011) using the upper-quartile method. Gene ontology enrichment analysis was performed using the topGO package in R (Alexa et al., 2006).

## **Histology**

For immunohistochemistry, caudal back skin tissue was harvested from mice and flash frozen in OCT (Tissue-Tek). Tissue was cryosectioned at 14 - 20  $\mu\text{m}$  and mounted on glass coverslips for staining. Histology was carried out as previously described (Gerhold et al., 2013). Antibodies: sheep anti-TRPA1 100  $\mu\text{g/ml}$  (Abcam), rabbit anti-HTR7 1:5000 (Abcam), Alexa 488 goat anti-rabbit 1:5000 (Life Technologies), and Alexa 568 donkey anti-sheep 1:5000 (Life Technologies). Skin sections were counterstained with DAPI (Life Technologies). For *in situ* hybridization, DRG were harvested and sectioned as described for immunohistochemistry. *Htr7* probes (Panomics) were used following the Quantigene protocol (Panomics). Images were taken using a Zeiss LSM710 confocal microscope at the Biological Imaging Facility, UC Berkeley.

## **Cell culture**

Preparation of neurons and ratiometric  $\text{Ca}^{2+}$  imaging were carried out as previously described (Wilson et al., 2011). Briefly, neurons from sensory ganglia were dissected and incubated for 10 min in 1.4  $\text{mg ml}^{-1}$  Collagenase P (Roche) in Hanks calcium-free balanced salt solution, followed by incubation in 0.25% standard trypsin (vol/vol) STV versene-EDTA solution for 3 min with gentle agitation. Cells were then triturated, plated onto glass coverslips and used within

20 h. (media: MEM Eagle's with Earle's BSS medium, supplemented with 10% horse serum (vol/vol), MEM vitamins, penicillin/streptomycin and L-glutamine). For retrograde labeling of cutaneous afferent neurons, cholera toxin subunit B, Alexa Fluor 594 conjugate (Life Technologies) was intradermally injected (4mg/ml in PBS, 10  $\mu$ l), and after 24 h, sensory neurons were dissected and cultured as described above. HEK293 cells (ATCC) were transfected with 500 ng of GFP tagged human HTR7 plasmid (Origene) either alone or with 50 ng of human TRPA1 using Lipofectamine 2000 (Life Technologies) per the manufacturer's instructions. Cells were plated on glass coverslips and used within 24 h for calcium imaging and whole cell recordings. Mouse primary keratinocytes (Yale Dermatology Cell Culture Facility) were plated on glass coverslips and used within 24 h for calcium imaging. All media and cell culture supplements were from the UCSF Cell Culture Facility.

### **Electrophysiology**

Whole cell recordings in transfected cells were performed using the Port-a-patch system (Brueggemann et al., 2004) (Nanion Technologies). All solutions used were provided by the manufacturer. Briefly, whole cell configuration was achieved with a high calcium external solution (in mM: 80 NaCl, 3 KCl, 10 MgCl<sub>2</sub>, 35 CaCl<sub>2</sub>, 10 HEPES, pH 7.4) which was replaced with a conventional external solution (in mM: 140 NaCl, 4 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 5 d-Glucose monohydrate, 10 HEPES, pH 7.4) prior to recording. The internal solution consisted of (in mM) 50 KCl, 10 NaCl, 60 KF, 20 EGTA, 10 HEPES, pH 7.2. Cells were held at -80 mV



and ramped every second from -100 mV to 100 mV over 300 ms. Inward currents were determined at -100 mV and plotted as a function of time. Sensory neuron recordings were collected at 5 kHz and filtered at 2 kHz using an Axopatch 200B and PClamp software. Electrode resistances were 2–6 M $\Omega$ . Stimulation protocol: 10 ms step to -80 mV, 150 ms ramp from -80 mV to +80 mV. Current clamp internal solution (in mM): 140 KCl, 5 EGTA, and 10 HEPES (pH 7.4 with KOH). Series resistance of all cells were <30 M $\Omega$ . Cells were perfused with external solution containing either 100  $\mu$ M LP-44 (Sigma Aldrich) or 100  $\mu$ M AITC (Sigma Aldrich). Cells were defined to be responsive to LP-44 if there was at least a 190% increase in current (chosen based on an average increase of 184% in the HTR7-TRPA1 co-transfected cells).

### **Calcium imaging**

Ca<sup>2+</sup> imaging experiments were carried out as previously described (Wilson et al., 2011). Cells were loaded for 30 min at room temperature with Fura-2AM, 10  $\mu$ M for neuronal culture, and 2  $\mu$ M for keratinocytes and HEK293 cells, supplemented with 0.01% Pluronic F-127 (wt/vol, Life Technologies), in a physiological Ringer's solution containing (in mM) 140 NaCl, 5 KCl, 10 HEPES, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub> and 10 D-(+)-glucose, pH 7.4. All chemicals were purchased from Sigma. Acquired images were displayed as the ratio of 340 nm to 380 nm. Cells were identified as neurons by eliciting depolarization with high potassium solution (75 mM) at the end of each experiment. Neurons were deemed to be sensitive to an agonist if the peak response was  $\geq$ 10% above baseline. Image analysis and

statistics were performed using custom routines in Igor Pro (WaveMetrics). All graphs displaying Fura-2 ratios were normalized to the baseline ratio F340/F380 = (Ratio)/(Ratio t = 0).

## PCR

cDNA was synthesized using SuperScript III reverse transcriptase (Life Technologies). Samples were diluted 1:10 and used as template for PCR experiments. The following primer pairs were used: mouse *Htr7* (forward, 5'-GCCATCTCCGCTCTCTCATC-3'; reverse, 5'-CCATAGTTGATCTGCTCCCCG-3'), mouse *Gapdh* (forward, 5'-CCGTAGACAAAATGGTGAAGGT-3'; reverse, 5'-GGGCTAAGCAGTTGGTGGT-3'), human *Htr7* (forward, 5'-CCCAGAGCAGTGTTTGTTC-3'; reverse, 5'-AGACCCTTCAGAGCACGAGA-3'), human *Gapdh* (forward, 5'-CCACTCCTCCACCTTTGAC-3'; reverse, 5'-ACCCTGTTGCTGTAGCC-3').

## Behavioral studies

*Htr7*<sup>-/-</sup> mice were obtained from Jackson Laboratory (Hedlund et al., 2003), *Trpv1*<sup>-/-</sup> and *Trpa1*<sup>-/-</sup> mice were described previously (Bautista et al., 2006; Caterina et al., 2000), and *K14-Cre;Trpa1*<sup>fl/fl</sup> mice were provided by Cheryl Stucky (Zappia et al., in preparation). Mice (20–35 g) were housed in 12 h light-dark cycle at 21°C. Itch behavioral measurements were performed as previously described (Wilson et al., 2011). Compounds injected: 2 mM LP44 (Santa Cruz), 100 µM or 1 mM 5-HT (Sigma), 40 mM chloroquine (Sigma), 3.5 mM BAM8-22 (Tocris), 4 mM

compound 48/80 (Sigma), 15 nM TSLP (R&D Systems), 100  $\mu$ M sertraline (Tocris), and 27 mM histamine (Tocris) in PBS. For all behavioral experiments, pruritogens were injected using both the neck model (50  $\mu$ l), and the cheek model (20  $\mu$ l) of itch, as previously described (Wilson et al., 2011); all differences observed between wild type and knockout animals did not vary by injection site. For the neuronal ablation experiment, resiniferatoxin (1  $\mu$ g/ml in 0.05% ascorbic acid and 7 % tween 80) was injected to the cheek two days prior to pruritogen injection. For AITC behavior, 5  $\mu$ l 10% AITC (Sigma) in mineral oil was applied to the right hind paw. Radiant heat paw withdrawal latencies, before and after injection with LP44 or 5-HT were performed as previously described (Tsunozaki et al., 2013). All mice were acclimated behavioral chambers on 2 subsequent days prior to treatment. Mice were injected with 20  $\mu$ l LP44 (2 mM) or 5-HT (10  $\mu$ M) into the hind paw, and their paw withdrawal latencies were measured 15 min pre- and 15 min and 30 min post-injection. Behavioral scoring was performed while blind to experimental condition and mouse genotype. All experiments were performed under the policies and recommendations of the International Association for the Study of Pain and approved by the University of California, Berkeley Animal Care and Use Committee.

### **Vitamin D model of Atopic Dermatitis**

Mice were singly housed and cheek hair was shaved one week prior to the start of the assay. 200  $\mu$ M (in 20  $\mu$ l EtOH) MC903 (R&D Systems) was applied to the cheek once per day, for 7 days. Behavior was recorded for 30 minutes on days 3,

5, 7 (ongoing treatment) and days 8, 10 and 12 (post-treatment) and the time spent scratching was quantified. Character of lesion scoring (COL) was adapted from (Hampton et al., 2012; Liu et al., 2013; Yun et al., 2011). Lesion severity was scored for redness (erythema), dryness (xerosis), and scabbing (excoriation) based on a 0-3 scale as follows: 0 = none, 1 = mild, 2 = moderate, 3 = severe. Behavioral and lesion scoring was performed while blind to genotype, treatment, and treatment duration.

### **Serotonin measurements**

Cheek skin was homogenized in 0.2 N HClO<sub>4</sub> (10 µl/mg of tissue) and neutralized with equal volume of 1 M borate buffer (pH 9.5). 5-HT levels were measured using the serotonin ELISA kit (Beckman Coulter).

### **Statistical analysis**

Values are reported as the mean ± s.e.m. For comparison between two groups, Student's *t*-test was used. For single-point comparison between >2 groups, a one-way ANOVA followed by a Tukey-Kramer *post hoc* test was used. For the time course comparison between >2 groups, ANOVA for multivariate linear models was used. Significance was labeled as: ns, not significant,  $p \geq 0.05$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

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