# Persistent Extracellular Signal-Regulated Kinase Activation by the Histamine H4 Receptor in Spinal Neurons Underlies Chronic Itch

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Transient extracellular signal-regulated kinase (ERK) activation in the spinal cord triggers histamine-induced acute itch. However, whether persistent ERK activation plays an important role in chronic itch development remains unclear. This study investigated the role of spinal ERK activation in chronic itch. The results showed that repetitive DNFB painting on the nape of mice evoked not only initial scratching but also sustained, spontaneous scratching. In addition, DNFB induced itching rather than nociception, as demonstrated using a cheek model. Furthermore, ERK was persistently activated in the spinal cord of DNFB-treated mice, and the intrathecal inhibition of phosphorylation of ERK suppressed both spontaneous itching and ERK activation. ERK activation was observed in neurons but not in glia cells during chronic itch development. Finally, DNFB-induced spontaneous itching behavior and ERK activation were largely inhibited by the histamine H4 receptor antagonist JNJ7777120 but not by the H1 receptor antagonist chlorpheniramine. Our results indicate that persistent ERK activation via the histamine H4 receptor in spinal neurons underlies DNFB-induced chronic itch.

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## INTRODUCTION

Inflammatory skin diseases, such as allergic dermatitis and atopic dermatitis, are the most common chronic itch conditions seen in clinics and significantly affect patients' lives (Weisshaar and Dalgard, 2009). However, the mechanisms by which chronic itch is induced by these inflammatory skin diseases remain poorly understood. Because chronic itch is usually resistant to clinical antihistamine treatments (Dunford et al., 2007; Thurmond et al., 2008; Twycross et al., 2003), elucidating the cellular and molecular mechanisms underlying such itch conditions is urgently needed to develop new antipruritic treatment.

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*Abbreviations: ERK, extracellular signal-regulated kinase; PBS, phosphate buffered saline; pERK, phosphorylation of extracellular signal-regulated kinase*

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Extracellular signal-regulated kinase (ERK) transduces extracellular stimuli into intracellular signals through various transcriptional and post-translational mechanisms (Lee et al., 2014). Moreover, the activation of ERK in the spinal cord is important for synaptic plasticity and central sensitization, which are pivotal to the development of chronic pain and itch (Carstens and Akiyama, 2016; Ji, 2003, 2015; Stander and Schmelz, 2006). We previously reported that ERK activation in spinal neurons, which serve as a component of local neuronal circuits, contributes selectively to histamine-dependent acute itch (Jiang et al., 2015; Zhang et al., 2014). However, whether ERK is important in the development of chronic itch remains unclear.

Histamine is one of the best known pruritogens responsible for pruritic and allergic inflammatory conditions in humans and animals (Thurmond et al., 2015). Histamine mediates its effect via the G-protein–coupled receptors H1R, H2R, H3R, and H4R. The function of histamine in pruritus has long been thought to be mediated by H1R and, for this reason, clinically available antihistamines are referred to as antihistamine H1R treatments. Although anti-H1R compounds have been shown to effectively treat acute itch, these compounds are largely ineffective against chronic conditions (Kido-Nakahara et al., 2017). H4R is a newly identified histamine receptor that has received considerable attention for its role in mediating chronic inflammation (Dunford et al., 2007; Thurmond et al., 2008). However, the mechanisms by which H4R mediates chronic itch have not been elucidated to date.

In the present study, we demonstrated that DNFB elicited neuronal activation and itching but not painful behavior.

Repeated painting of DNFB induced sustained ERK activation as well as prolonged, spontaneous itch. Moreover, the histamine receptor H4R was found to be required for the ERK activation and spontaneous itch induced by DNFB.

## RESULTS

### Repeated painting with DNFB induced prolonged and spontaneous scratching behavior

DNFB models were produced by repetitively painting the nape of mice 4 times in 2 weeks (Figure 1a). Consistent with our previous report (Zhang et al., 2014), DNFB induced vigorous scratching immediately after each painting (Figure 1b). Moreover, the DNFB mice also exhibited robust and sustained spontaneous scratching after the cessation of DNFB administration, and this spontaneous scratching behavior lasted for at least 2 weeks (Figure 1b).

We also assessed the latency to initiate scratching following the first DNFB treatment. The first bout of scratching was always initiated within minutes, and the latency was much shorter in DNFB-treated mice compared to that of the control mice (Figure 1c). Furthermore, ERK was also activated in the spinal cord within minutes after DNFB treatment (Figure 1d). To assess whether the ERK activation in the spinal cord was induced by mechanical stimulation, mice received an Elizabeth collar, which can prevent scratching behavior. The expression of phosphorylation of ERK induced by DNFB was comparable between the mice with Elizabeth collar and the control mice (Figure 1e–g), indicating that ERK activation in the spinal cord was not due to scratching behavior.

### DNFB induced itching but not nociception in mice

Although DNFB produces robust scratching, whether this behavior is a manifestation of itching remains debated because algogens, such as capsaicin, also induce scratching when injected into the nape of mice. The cheek model can discriminate pain from itching behavior because painful stimuli always elicit wiping with the forelimb, whereas itch stimuli induce scratching with the hind paws (Shimada and LaMotte, 2008). DNFB was then painted on the cheek skin of mice to distinguish between the two behaviors. The results showed that DNFB induced substantial increases in scratching compared with controls (Figure 2b), whereas the number of wiping behaviors was minimal and not significantly different between the two groups (Figure 2a). These data suggest that DNFB is an itch stimulus, that is, it induces pruritic behavior rather than nociceptive behavior.

### Inhibition of ERK phosphorylation attenuated spontaneous scratching behavior and ERK activation in DNFB mice

We previously reported that the mitogen-activated protein kinase/ERK inhibitor U0126 decreases both ERK activation and histamine-induced itching behavior. To determine whether ERK is persistently activated in the spinal cord and to understand its role in chronic itch development, U0126 was intrathecally injected into the spinal cord 1, 3, 7, and 14 days after the final DNFB painting. The results showed that U0126 invariably inhibited the spontaneous scratching behavior of DNFB mice (Figure 3a). Moreover, prolonged activation of ERK in the spinal cord was inhibited by the spinal injection of U0126 (Figure 3b–3e), implying a sustained role of pERK in mediating DNFB-induced chronic itch.

### Cellular localization of ERK activation during DNFB-induced development of chronic itch

ERK is activated sequentially in neurons, microglia, and astrocytes in the spinal cord and contributes to the mechanical allodynia of neuropathic pain (Zhuang et al., 2005). To determine whether ERK activation induced by itch shares the same mechanisms, we co-immunostained spinal cord sections taken 3, 7, and 14 days after the cessation of DNFB application with pERK and NeuN, GFAP, or IBa1, which are markers of neurons, astrocytes and microglia, respectively. The results showed that pERK always co-labeled with NeuN but not with GFAP or IBa1 (Figure 4a–4l). These data suggest that ERK is activated in neurons, but not in astrocytes or microglia, in the spinal cord during chronic itch development.

### DNFB induced ERK activation and scratching behavior through the histamine H4R but not H1R

To investigate whether DNFB-related chronic itch is mediated by histamine receptors, H1R and H4R antagonists were intrathecally injected into mice. The spontaneous itch behavior was unaffected by H1R antagonist chlorpheniramine 3 days after the cessation of the DNFB application. However, both the chronic itch behavior and ERK activation were largely inhibited by JNJ7777120, the H4R antagonist (Figure 5a–5c). However, neither chlorpheniramine nor JNJ7777120 affected the initial scratching behavior immediately after the first application of DNFB (Figure 5d, 5e). By contrast, chlorpheniramine and JNJ7777120 substantially inhibited histamine-induced itch (Figure 5g, 5h). Furthermore, using real-time quantitative PCR, we found that the mRNA of H4R but not H1R significantly increased after DNFB treatment (Figure 5f). Our results suggest that histamine-induced acute itch and DNFB-induced chronic itch are mediated via different receptors.

### Incubation with DNFB and histamine induced ERK activation in spinal cord through different histamine receptors

To determine whether DNFB or histamine is sufficient to induce ERK activation in spinal cord neurons, we incubated spinal slices with histamine and DNFB. The ERK in the spinal cord was dramatically activated after the slices were incubated in Krebs solution containing either DNFB or histamine. Interestingly, an H4R antagonist but not an H1R antagonist inhibited DNFB-induced ERK activation (Figure 6b), while both H1R and H4R antagonists attenuated histamine-induced ERK activation (Figure 6a). Moreover, H4R agonist 4-methylhistamine induced ERK activation in the spinal cord (Figure 6c). Intrathecal injection of 4-methylhistamine also produced robust scratching behavior in mice (Figure 6d).

## DISCUSSION

We previously reported that ERK activation in the spinal cord is involved in histamine-induced acute itch and in the initial period of DNFB-induced itch (Zhang et al., 2014). In this study, we provide the evidence showing that ERK is persistently activated in the spinal cord and plays a role in chronic

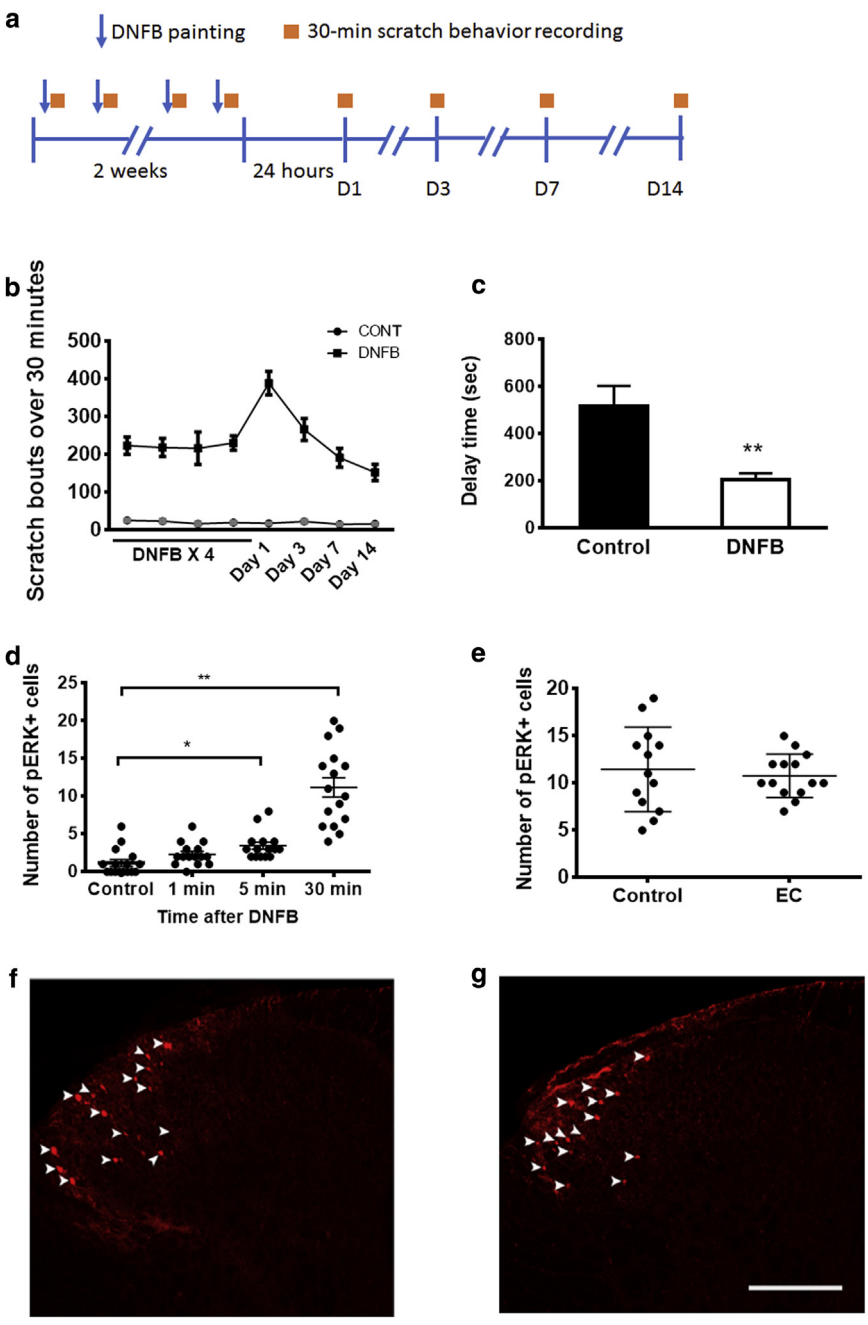


**Figure 1. Repetitive DNFB painting of the neck generates both initial scratching and prolonged spontaneous scratching behaviors in mice.** (**a**) The timeline of DNFB application and itch behavior observation. (**b**) DNFB application four times generates initial and spontaneous scratching behavior in mice (n = 12). (**c**) The latency to the first bout of scratching following DNFB treatment was shorter compared with that of the control treatment (n = 10). (**d**) Spinal cord ERK was activated in vivo within minutes after treatment with DNFB (n = 6). (**e**–**g**) ERK activation in the spinal cord 30 minutes after DNFB treatment was comparable in EC and the control mice (n = 6). (**f, g**) Showing the example of pERK expression in the spinal dorsal horn induced by DNFB in the EC (**g**) and control mice (**f**) (n = 5). $^{*}P < 0.05$; $^{**}P < 0.01$. EC, Elizabeth collar; ERK, extracellular signal-regulated kinase; pERK, phosphorylation of extracellular signal-regulated kinase. Scale bars = 100 μm.

itch development. H4R but not H1R contributes to the ERK activation and chronic itch induced by DNFB.

Allergic dermatitis and atopic dermatitis are common chronic skin diseases characterized by intense pruritus and have a significant negative impact on an individual's quality of life. Although they have different etiologies and distributions, both diseases are accompanied by chronic itch. DNFB is widely used for sensitization and challenging to induce allergic dermatitis in mouse models (Jin et al., 2009; Kim et al., 2015; Lee et al., 2014, 2015). In addition, repetitive challenging induces human atopic dermatitis–like pathogenesis in mice (Miyamoto et al., 2002). However, the scratching behavior induced by DNFB is usually observed during the initial period after a challenge. Few studies have investigated the late phase of spontaneous itching behavior, which is considered a hallmark of chronic itch. Our results showed that DNFB induced not only initial scratching but also long-lasting spontaneous scratching that lasted at least 2 weeks after the cessation of the DNFB application. Notably, DNFB, as a hapten, is usually sensitized and then challenged to induce an immune response (Kim et al., 2015; Lee et al., 2015). In our study, mice showed quick scratching responses and spinal cord ERK activation within minutes after DNFB administration, indicating that DNFB may directly or indirectly act on neuronal fibers in the skin to generate neuron-based responses that are independent of the immune activity. This result is consistent with a recent study showing that DNFB induces mast cell degranulation in the absence of

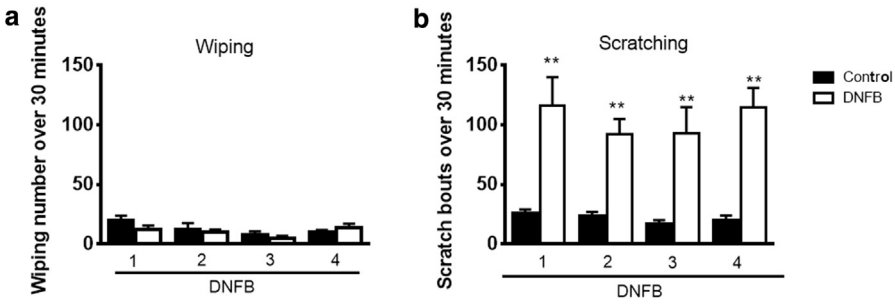


**Figure 2. Repetitive DNFB painting on the cheek leads to increased scratching but not wiping behavior in mice.** (**a**) DNFB application four times on the mouse cheek induced minimal wiping behavior, and there was no significant difference between the DNFB-treated mice and the control mice. (**b**) DNFB application induced robust scratching behavior, which was significantly different compared to that in the control mice (n = 6). $^{**}P < 0.01$.

serum proteins and IgE (Manabe et al., 2017). We also used an in vitro immunohistochemistry method to further demonstrate that DNFB, histamine, and H4R agonist 4-methylhistamine are all sufficient to induce ERK activation in spinal cord neurons. However, how DNFB mediates the activation of sensory neurons is unknown and needs further investigation.

Capsaicin injections produce nociceptive sensations. However, they also generate scratching behavior as pruritic agents do when injected into the skin of the nape (Shimada and LaMotte, 2008). In this study, we found that DNFB almost always increased scratching, whereas there was no difference in wiping behavior between the experimental mice and the control mice. Our results demonstrated that DNFB induces itching but not nociceptive behavior. Together with the sustained spontaneous behavioral data, it can be concluded that repeated painting with DNFB induced a chronic itching response not a nociceptive response.

Although several studies have implied that central modulation may play an important role in the development and maintenance of chronic itch (Mochizuki et al., 2017), little attention has been paid to the role of the central nervous system in the processing of itch. ERK signaling in the spinal cord is involved in regulating neuronal plasticity and contributes to the development of pathologic pain. Different durations of ERK activation have also been reported to induce different cell responses (Marshall, 1995). Transient ERK activation in the spinal cord has been shown to be involved in histamine-dependent itch (Zhang et al., 2014; Zhou et al., 2017). In the present study, persistent ERK activation was found in DNFB-induced chronic spontaneous itch. Moreover, ERK activation was always found in neurons but not in microglia and astrocytes during 2 weeks of DNFB-related spontaneous itch, implying different neuronal mechanisms of chronic itch and chronic pain secondary to ERK activation.

Four types of histamine receptors, named H1R, H2R, H3R, and H4R, have been reported to date (Seifert et al., 2013; Strasser et al., 2013). H1R and H4R appear to be key players in the induction of itch (Rossbach et al., 2011). H1R was the first identified histamine receptor. Clinical antihistamine treatment, referred to as anti-H1R, is commonly used to relieve itch and other symptoms associated with acute dermal allergic responses (Dunford et al., 2007). However, the effect of antihistamine treatment is limited in chronic situations. H4R is a recently discovered histamine receptor, and its function has not been fully elucidated yet. The mRNA of H4R has been detected in peripheral bone marrow eosinophils and mast cells (Leurs et al., 2009; Strakhova et al., 2009) as well as in sensory neurons (Rossbach et al., 2011; Strakhova et al., 2009) and

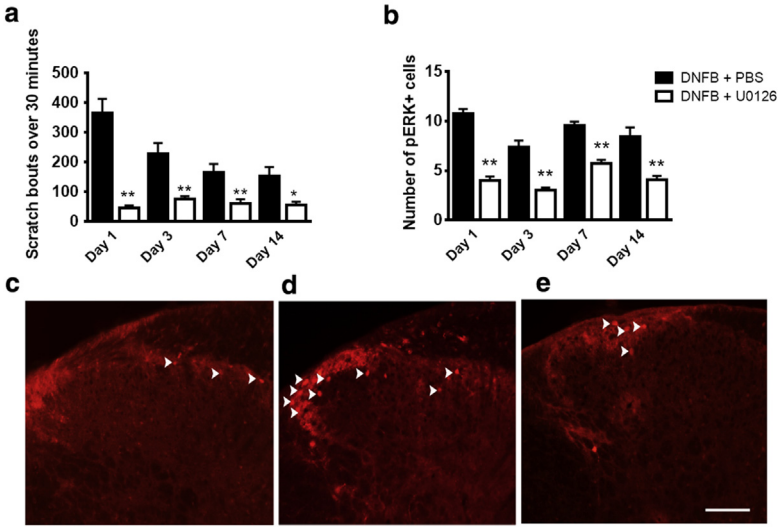


**Figure 3. Persistent activation of ERK in the spinal cord is required for chronic itch.** (**a**) Spinal injections of U0126 (1 μg) 1, 3, 7, and 14 days after the final application of DNFB significantly inhibited the spontaneous scratching behavior (n = 8). (**b**) Spinal injections of U0126 (1 μg) inhibited ERK activation in the spinal cord 1, 3, 7, and 14 days after the final DNFB application (n = 8). (**c**–**e**) Images show pERK expression in the spinal dorsal horn in control (**c**), day 7 of final DNFB application (**d**) and the day 7 DNFB mice with U0126 treated (**e**). $^{*}P < 0.05$, $^{**}P < 0.01$. ERK, extracellular signal-regulated kinase. Scale bars = 100 μm.

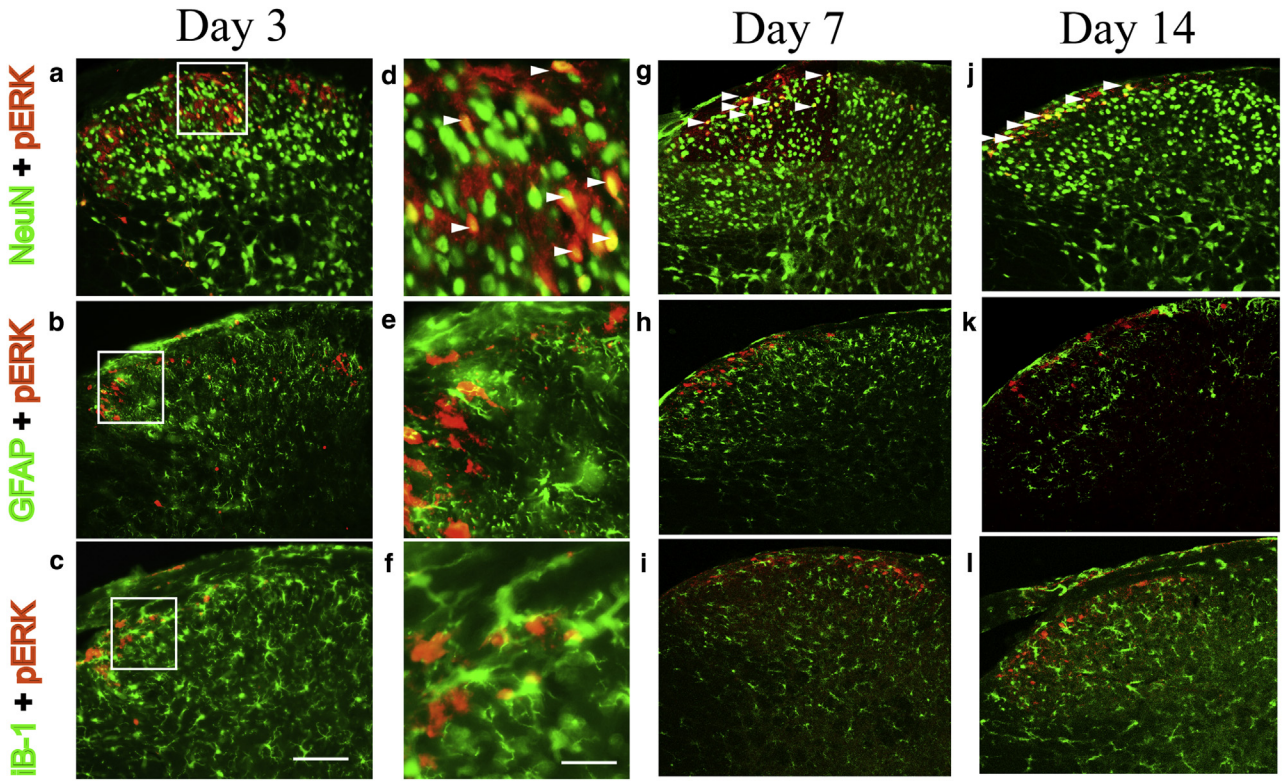


**Figure 4. Specific pERK expression in superficial spinal neurons in the DNFB chronic itch mice.** pERK expression was co-localized with NeuN (arrowheads in [**d**], [**g**], and [**j**]) but not with GFAP (**b**, **e**) or IBa1 (**c**, **f**) at 3 days (**a**–**f**), 7 days (**g**–**i**) and 14 days (**j**–**l**) after the final DNFB painting. The figures of (**d**), (**e**), and (**f**) are high magnification images of the boxed areas in (**a**), (**b**), and (**c**). Scale bars = 100 μm (**a**–**c, g-l**) and 40 μm (**d**–**f**) (n = 5). pERK, phosphorylation of extracellular signal-regulated kinase.

the spinal cord (Strakhova et al., 2009). H4R agonists activate H4R in the neurons of dorsal root ganglia by opening TRPV1 after stimulating the PLC pathway (Jian et al., 2016). A recent behavioral study showed that H4R modulates various neuronal functions of the central nervous system (Sanna et al., 2017). However, the mechanisms by which H4R is involved in chronic itch are poorly known. Our results showed that an H4R antagonist inhibited DNFB-induced spontaneous itch and ERK activation, indicating that DNFB induced persistent ERK activation and prolonged itch behavior via H4R. In contrast, both H1R and H4R antagonists largely inhibited histamine-induced itch behavior and ERK activation. Our data indicated that acute itch induced transient and chronic itch induced prolonged ERK activation in the spinal cord through different histamine receptors, which are in line with the recent study that both H1R and H4R enhance the intracellular concentrations of calcium and regulate mitogen-activated protein kinase activity by distinct signaling pathways (Beermann et al., 2015, 2014). Given that H1R may not be the only receptor involved in itch induction, one should exercise caution when defining histamine-independent itch when only considering the role of H1R. Other histamine receptors, such as H4R, should also be considered.

Our study shows that persistent ERK activation in the spinal cord underlies DNFB-induced chronic itch via H4R. Taken together with our previous study (Zhang et al., 2014), these findings suggest that different types of itch may have different molecular mechanisms. Nevertheless, targeting the ERK pathway promptly in spinal cord neurons may offer effective treatment for chronic itch.

## MATERIALS AND METHODS

### Animals

Adult male ICR mice (6–8 weeks old) were purchased from SLAC Laboratory Animal Company (Shanghai, China). The animals were housed with food and water available ad libitum and under controlled temperature (22° ± 2°C) and humidity (60%–80%) conditions in a 12-hour/12-hour light/dark cycle. All experiments were performed in accordance with the guidelines for animals used in biological studies of Tongji University and were approved by the Animal Study Committee at Tongji University School of Medicine, Shanghai, China.

### Behavioral tests

A DNFB model and a histamine model were established as described previously (Zhang et al., 2014). For the DNFB model, the mice received repeated skin painting with 150 μl 0.15% DNFB (Sigma-Aldrich, St Louis, MO) in acetone at the shaved nape or cheek, four times in 2 weeks. The control mice were painted using acetone in the same manner. To study acute itch behavior induced by DNFB, the number of scratches toward the painted sites was determined immediately after each application of DNFB or acetone. Chronic behavior was observed 1, 3, 7, and 14 days after the final DNFB administration. The scratch behavior 1 day after DNFB was observed 24 hours after the final DNFB painting. The scratch behaviors on days 3, 7, and 14 were assessed at that same time of day. For the histamine-induced itch model, 500 μg histamine was dissolved in 50 μl 0.01 M phosphate buffered saline (PBS; pH 7.4) and injected intradermally

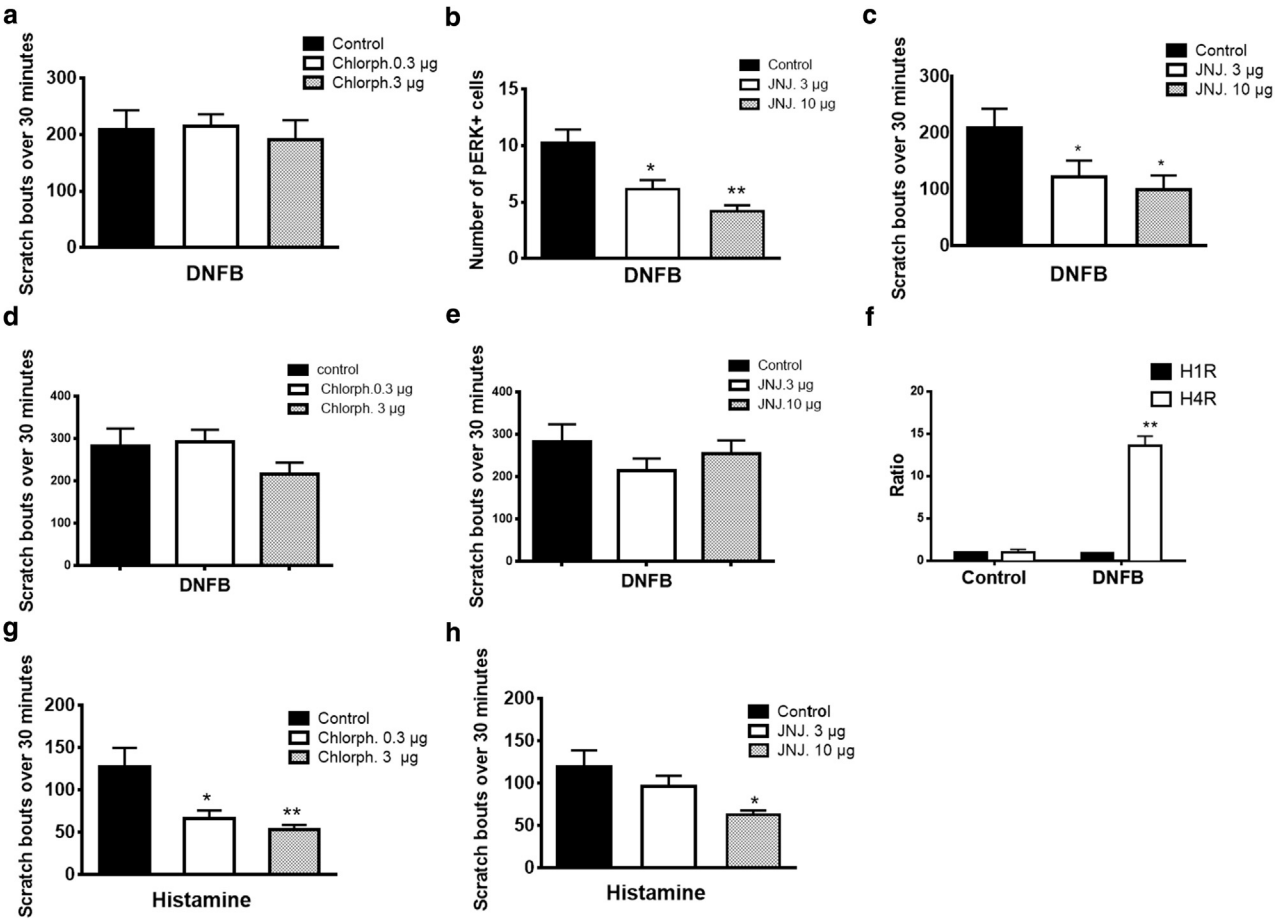


**Figure 5. H4R is required for DNFB-induced prolonged ERK activation and spontaneous scratching behavior.** (**a**) Spinal injection of the H1R receptor antagonist chlorpheniramine (0.3 μg or 3 μg) exerted no effect on spontaneous itching at day 3 after final DNFB application. (**b, c**) Spinal injection of the H4R antagonist JNJ7777120 (3 μg or 10 μg) inhibited DNFB-induced ERK activation and spontaneous itching behavior at day 3 after final DNFB application (n = 5–7). (**d, e**) Neither chlorpheniramine (0.3 μg or 3 μg) nor JNJ7777120 (3 μg or 10 μg) inhibited the initial scratching induced by DNFB (n = 9). (**f**) The mRNA of H4R but not H1R was increased after DNFB treatment (n = 3). (**g, h**) Both the H4R (**h**) and H1R (**g**) antagonists injected spinally inhibited histamine-induced acute itch (n = 5–7). $^{*}P < 0.05$, $^{**}P < 0.01$. ERK, extracellular signal-regulated kinase.

into the nape to generate acute histamine-dependent itch responses (Sun and Chen, 2007, Zhang et al., 2014).

All scratch behaviors were recorded using a video camera (SONY HDR-CX240; Sony, Tokyo, Japan) and calculated in 30-minute segments, and the counting and analysis were performed with the experimenter blinded to the treatment condition.

### Drug administration

The mitogen-activated protein kinase/ERK inhibitor U0126 (Sigma-Aldrich) was dissolved in dimethyl sulfoxide to prepare a 100× stock solution, which was stored at −80°C. The solution was diluted in PBS before use. Using a 30-gauge needle, U0126 (1 μg in 10 μl PBS) was administered intrathecally at the lumbar level (L4 and L5) 1, 3, 7, and 14 days after the final DNFB application. The H1R antagonist chlorpheniramine (Sigma-Aldrich) and H4R antagonist JNJ7777120 (Sigma-Aldrich) were dissolved in PBS and injected intrathecally. The control mice received an equal volume of PBS alone.

### In vivo immunohistochemistry

Mice were anesthetized using sodium pentobarbital perfused through the ascending aorta with PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the cervical cord segments of the mice were removed and post-fixed overnight. Samples were cut transversely into 30-μm-thick frozen sections on a cryostat. The cervical segment of the spinal cord was cut continuously into six sets. Every sixth section was collected as one set and then processed for immunofluorescence. The following primary antibodies were used: rabbit anti-pERK1/2 antibody (1:500; Cell Signaling Technology, Danvers, MA); mouse anti-NeuN antibody (1:1000; Millipore, Temecula, CA); mouse anti-GFAP antibody (1:1000; Millipore); and goat anti-Iba1 antibody (1:1000; Abcam, Cambridge, UK). The sections were incubated with the primary antibodies overnight at 4°C followed by biotin-conjugated horse anti-rabbit or horse anti-mouse (1:500; Vector Laboratories, Burlingame, CA) for 2 hours and, finally, with Cy3-conjugated streptavidin (1:500; Jackson Immuno-Research, West Grove, PA) for 1 hour at room temperature. For double immunofluorescence, sections were incubated with a mixture of rabbit polyclonal and mouse monoclonal primary antibodies followed by a mixture of Alexa Fluor 488- and biotin-conjugated horse anti-rabbit IgG and, finally, with Cy3-conjugated streptavidin.

### In vitro immunohistochemistry

As we reported previously (Gao et al., 2009; Kawasaki et al., 2008), a portion of the lumbar spinal cord (L4–L5) was removed from the

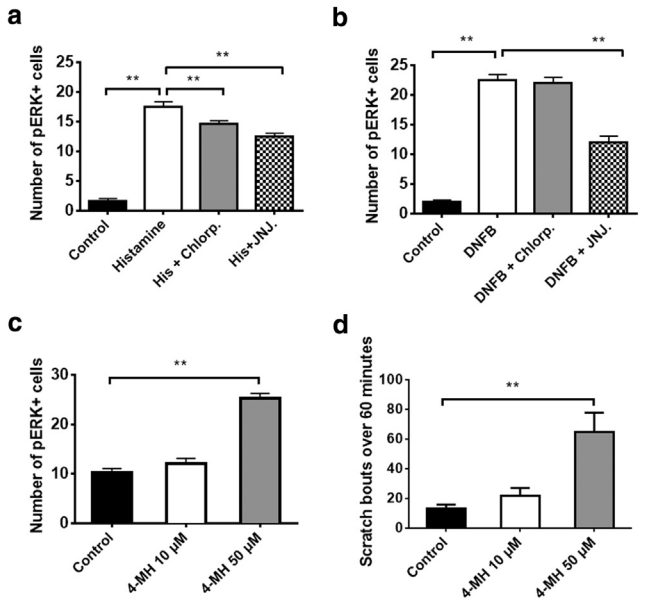


**Figure 6. ERK activation in the spinal cord induced by histamine and DNFB incubation via different histamine receptors.** (**a, b**) Both the incubation of histamine (10 mg/ml) and 0.15% DNFB induced pERK expression in the spinal dorsal horn. Chlorpheniramine (300 μg/ml) and JNJ7777120 (100 μg/ml) significantly inhibited histamine-induced ERK activation (**a**); JNJ7777120 but not chlorpheniramine inhibited DNFB-induced ERK activation (**b**). (n = 3–6). (**c**) 4-MH (10 μM, 50 μM) induced ERK activation in spinal cord. (**d**) Intrathecal injection of 4-MH (10 μM, 50 μM) evoked scratching behavior (n = 5–7), $^{**}P < 0.01$. 4-MH, 4-methylhistamine. ERK, extracellular signal-regulated kinase; pERK, phosphorylation of extracellular signal-regulated kinase.

mice (4–6 weeks old) under urethane anesthesia (2.5 g/kg, intraperitoneal) and kept in pre-oxygenated ice-cold Krebs solution. Spinal segments were placed in a shallow groove formed in an agar block glued to the bottom of the microslicer stage. Transverse slices (600 μm) were cut on a vibrating microslicer. The slices were incubated with Krebs solution saturated with 95% $O_2$ and 5% $CO_2$ at 35°C for at least 2 hours before experimentation. The Krebs solution contained the following (in mM): 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $NaH_2PO_4$, 25 $NaHCO_3$, and 11 glucose. The slices were stimulated with DNFB, histamine, or DNFB and histamine combined with chlorpheniramine or JNJ7777120 for 10 minutes and then fixed with 4% paraformaldehyde overnight and 10% sucrose for 2.5 hours. Thin spinal cord sections (15 μm) were cut on a cryostat and processed for immunohistochemistry using pERK antibodies (see Materials and Methods). The stained sections were observed under a Nikon fluorescence microscope equipped with a Nikon Coolpix digital camera (DS-Ri1; Nikon, Tokyo, Japan) or a laser confocal microscope (TCS SP5 II; Leica, Wetzlar, Germany). All images were made into figures using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA), with only minor adjustments to the contrast and brightness settings if necessary.

### Real-time quantitative reverse transcriptase PCR

Animals were decapitated, and the lumbar enlargement of the spinal cord was extracted by sterilized instruments 30 minutes after DNFB or acetone painting. The total RNA was extracted by an RNA Extraction Kit (Takara, Kyota, Japan). The isolated RNA was reverse-transcribed to synthesize the first-strand cDNA using a cDNA synthesis kit (Thermo Scientific, Waltham, MA). Sequences for all forward and reverse primers were as follows: forward and reverse primers of H1R were GGGTCCCTCCCTACCTTTTTA and GCGTGTGACTGTTTCCCTTTC; and forward and reverse primers of H4R were GGAAGCTAGCCAGGTCACTG and TTCCGTTCTGGGGTAAGTTG. An ABI 7500 Real-Time PCR System and SYBR Green I (Tiangen, Beijing, China) were used in the PCR. Real-time PCR liquid was prepared and reaction conditions were set following kit instructions. Glyceraldehyde-3-phosphate dehydrogenase served as an internal control. The melting curve was used to evaluate the reliability of the PCR results. Threshold cycle (CT) values (the inflection point of the amplification curve) were determined, and the relative expression of target genes was calculated using the $2^{-\Delta\Delta}$Ct method.

### Quantification and statistics

To count the pERK-positive cells in the cervical sections, all immunostained sections in one set were included. A cell was considered pERK-positive when the signal to noise ratio (intensity of immunofluorescence in the cell body vs. background) was >2. The values were averaged for each animal. The data are expressed as the mean ± SEM. Differences between two groups were compared using 2-tailed Student *t* tests. One-way analysis of variance followed by Fisher's post-hoc tests were conducted to evaluate differences among multiple groups. Differences with $P < 0.05$ were considered statistically significant.

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### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### AUTHOR CONTRIBUTIONS

L.Z and Q-X. W designed the research and wrote the paper. K.H, D-D.H, and D.B carried out immune staining, behavior testing, and data analysis. Z-Y.W and Y-Y.C did real-time quantitative PCR experiment and immune staining work; Y-J.Z and X.L prepared the sample. All authors read and approved the final manuscript.

### REFERENCES

Beermann S, Bernhardt G, Seifert R, Buschauer A, Neumann D. Histamine H(1)- and H(4)-receptor signaling cooperatively regulate MAPK activation. Biochem Pharmacol 2015;98:432–9.

Beermann S, Vauth M, Hein R, Seifert R, Neumann D. Distinct signalling pathways of murine histamine H1- and H4-receptors expressed at comparable levels in HEK293 cells. PLoS One 2014;9:e107481.

Carstens E, Akiyama T. Central mechanisms of itch. Curr Probl Dermatol 2016;50:11–7.

Dunford PJ, Williams KN, Desai PJ, Karlsson L, McQueen D, Thurmond RL. Histamine H4 receptor antagonists are superior to traditional antihistamines in the attenuation of experimental pruritus. J Allergy Clin Immunol 2007;119:176–83.

Gao YJ, Zhang L, Samad OA, Suter MR, Yasuhiko K, Xu ZZ, et al. JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. J Neurosci 2009;29:4096–108.

Ji RR. Neuroimmune interactions in itch: do chronic itch, chronic pain, and chronic cough share similar mechanisms? Pulm Pharmacol Ther 2015;35:81–6.

Ji RR, Kohno T, Moore KA, Woolf CJ. Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci 2003;26:696–705.

Jian T, Yang N, Yang Y, Zhu C, Yuan X, Yu G, et al. TRPV1 and PLC participate in histamine H4 receptor-induced itch. Neural Plast 2016;2016:1682972.

Jiang GY, Dai MH, Huang K, Chai GD, Chen JY, Chen L, et al. Neurochemical characterization of pERK-expressing spinal neurons in histamine-induced itch. Sci Rep 2015;5:12787.

Jin H, He R, Oyoshi M, Geha RS. Animal models of atopic dermatitis. J Invest Dermatol 2009;129:31–40.

Kawasaki Y, Zhang L, Cheng JK, Ji RR. Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. J Neurosci 2008;28:5189–94.

Kido-Nakahara M, Furue M, Ulzii D, Nakahara T. Itch in atopic dermatitis. Immunol Allergy Clin North Am 2017;37:113–22.

Kim GD, Park YS, Ahn HJ, Cho JJ, Park CS. Aspartame attenuates 2, 4-dinitrofluorobenzene-induced atopic dermatitis-like clinical symptoms in NC/Nga mice. J Invest Dermatol 2015;135:2705–13.

Lee JH, Lee YS, Lee EJ, Lee JH, Kim TY. Capsiate inhibits DNFB-induced atopic dermatitis in NC/Nga mice through mast cell and CD4+ T-cell inactivation. J Invest Dermatol 2015;135:1977–85.

Lee JH, Park CK, Chen G, Han Q, Xie RG, Liu T, et al. A monoclonal antibody that targets a NaV1.7 channel voltage sensor for pain and itch relief. Cell 2014;157:1393–404.

Leurs R, Chazot PL, Shenton FC, Lim HD, de Esch IJ. Molecular and biochemical pharmacology of the histamine H4 receptor. Br J Pharmacol 2009;157:14–23.

Manabe Y, Yoshimura M, Sakamaki K, Inoue A, Kakinoki A, Hokari S, et al. 1-Fluoro-2,4-dinitrobenzene and its derivatives act as secretagogues on rodent mast cells. Eur J Immunol 2017;47:60–7.

Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179–85.

Miyamoto T, Nojima H, Shinkado T, Nakahashi T, Kuraishi Y. Itch-associated response induced by experimental dry skin in mice. Jpn J Pharmacol 2002;88:285–92.

Mochizuki H, Schut C, Nattkemper LA, Yosipovitch G. Brain mechanism of itch in atopic dermatitis and its possible alteration through non-invasive treatments. Allergol Int 2017;66:14–21.

Rossbach K, Nassenstein C, Gschwandtner M, Schnell D, Sander K, Seifert R, et al. Histamine H1, H3 and H4 receptors are involved in pruritus. Neuroscience 2011;190:89–102.

Sanna MD, Ghelardini C, Thurmond RL, Masini E, Galeotti N. Behavioural phenotype of histamine H4 receptor knockout mice: focus on central neuronal functions. Neuropharmacology 2017;114:48–57.

Seifert R, Strasser A, Schneider EH, Neumann D, Dove S, Buschauer A. Molecular and cellular analysis of human histamine receptor subtypes. Trends Pharmacol Sci 2013;34:33–58.

Shimada SG, LaMotte RH. Behavioral differentiation between itch and pain in mouse. Pain 2008;139:681–7.

Stander S, Schmelz M. Chronic itch and pain—similarities and differences. Eur J Pain 2006;10:473–8.

Strakhova MI, Nikkel AL, Manelli AM, Hsieh GC, Esbenshade TA, Brioni JD, et al. Localization of histamine H4 receptors in the central nervous system of human and rat. Brain Res 2009;1250:41–8.

Strasser A, Wittmann HJ, Buschauer A, Schneider EH, Seifert R. Species-dependent activities of G-protein-coupled receptor ligands: lessons from histamine receptor orthologs. Trends Pharmacol Sci 2013;34:13–32.

Sun YG, Chen ZF. A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature 2007;448(7154):700–3.

Thurmond RL, Gelfand EW, Dunford PJ. The role of histamine H1 and H4 receptors in allergic inflammation: the search for new antihistamines. Nat Rev Drug Discov 2008;7:41–53.

Thurmond RL, Kazerouni K, Chaplan SR, Greenspan AJ. Antihistamines and itch. Handb Exp Pharmacol 2015;226:257–90.

Twycross R, Greaves MW, Handwerker H, Jones EA, Libretto SE, Szepietowski JC, et al. Itch: scratching more than the surface. QJM 2003;96:7–26.

Weisshaar E, Dalgard F. Epidemiology of itch: adding to the burden of skin morbidity. Acta Derm Venereol 2009;89:339–50.

Zhang L, Jiang GY, Song NJ, Huang Y, Chen JY, Wang QX, et al. Extracellular signal-regulated kinase (ERK) activation is required for itch sensation in the spinal cord. Mol Brain 2014;7:25.

Zhou FM, Cheng RX, Wang S, Huang Y, Gao YJ, Zhou Y, et al. Antioxidants attenuate acute and chronic itch: peripheral and central mechanisms of oxidative stress in pruritus. Neurosci Bull 2017;33:423–35.

Zhuang ZY, Gerner P, Woolf CJ, Ji RR. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. Pain 2005;114(1–2):149–59.