GRPR/Extracellular Signal–Regulated Kinase and NPRA/Extracellular Signal–Regulated Kinase Signaling Pathways Play a Critical Role in Spinal Transmission of Chronic Itch

Xueting Liu[1], Yanmei Wang[1], Tianyu Tao[1], Liping Zeng[1], De Wang[1], Yuhuan Wen[1], Yangyang Li[1], Zhongqiu Zhao[2,3] and Ailin Tao[1]

Intractable or recurrent chronic itch greatly reduces the patients' QOL and impairs their daily activities. In this study, we investigated whether there are certain key signaling molecules downstream of the recently identified peptides mediating itch in the spinal cord. RNA sequencing analysis of mouse spinal cord in chronic itch models induced by squaric acid dibutylester and imiquimod showed that extracellular signal–regulated kinase (ERK) 1/2 cascade is the most significantly upregulated gene cluster in both models. In four different mouse models of chronic itch, sustained ERK phosphorylation was detected mainly in spinal neurons, and MAPK/ERK kinase inhibitors significantly inhibited chronic itch in these models. Phosphorylated ERK was observed in the interneurons expressing the receptors of different neuropeptides for itch, including gastrin-releasing peptide receptor, natriuretic peptide receptor A, neuromedin B receptor, and sst2A. Blocking gastrin-releasing peptide receptor and natriuretic peptide receptor A by genetic approaches or toxins in mice significantly attenuated or ablated spinal phosphorylated ERK. When human embryonic kidney 293T cells transfected with these receptors were exposed to their respective agonists, ERK was the most significantly activated intracellular signaling molecule. Together, our work showed that phosphorylated ERK is a unique marker for itch signal transmission in the spinal cord and an attractive target for the treatment of chronic itch.

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INTRODUCTION

Chronic itch is a cardinal manifestation of dermatologic diseases (e.g., allergic dermatitis, atopic dermatitis, psoriasis) and a variety of nondermatologic conditions. The development and maintenance of chronic itch can result from peripheral (primary sensory neuron) sensitization or central (spinal cord, brain) sensitization (Dong and Dong, 2018; Nattkemper et al., 2018; Yosipovitch and Bernhard, 2013). Over the past decade, research has elucidated that many neurotransmitters, neuropeptides, cytokines, chemokines, and receptors present in either peripheral or central tissues are involved in the transduction, transmission, and modulation of itch signals. In particular, recent studies identified that neuropeptides such as gastrin-releasing peptide (GRP) (Sun and Chen, 2007; Sun et al., 2009), natriuretic polypeptide b (also known as brain-derived natriuretic peptide [BNP]) (Mishra and Hoon, 2013), somatostatin (SST) (Huang et al., 2018a), prodynorphin (Huang et al., 2018a; Kardon et al., 2014), neuromedin B (NMB) (Zhao et al., 2014), neurokinin B (Sakata et al., 2019), neuropeptide Y (Pan et al., 2019), and galanin (Liu et al., 2019) act as key mediators and work through their receptors and neural circuits for itch signaling from the periphery to the brain. However, the intracellular events and downstream signaling pathways activated by these neuropeptides in the spinal cord are still not well-understood.

In chronic pain settings, central sensitization represents significant neural plasticity of the central neurons and circuits and is considered responsible for long-lasting and persistent pain hypersensitivity, even when peripheral inflammation or nerve injuries no longer exist (Basbaum et al., 2009; Ji et al., 2016). Dozens of protein kinases, in particular, MAPKs such as extracellular signal–regulated kinase (ERK), p38, and c-Jun N-terminal kinase, are activated in both primary sensory and dorsal horn neurons where they can participate in the generation and maintenance of chronic pain (Ji et al., 2009; Zhuang et al., 2005). In chronic itch, it is unclear whether central sensitization occurs, and some recent studies have presented conflicting results. For example, genetically induced, sustained ERK phosphorylation (phosphorylated

[1]*The Second Affiliated Hospital, The State Key Laboratory of Respiratory Disease, Guangdong Provincial Key Laboratory of Allergy & Clinical Immunology, Center for Immunology, Inflammation, & Immune-Mediated Disease, Guangzhou Medical University, Guangzhou, China;* [2]*Center for the Study of Itch, Department of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri, USA; and* [3]*Respiratory Care Services, Barnes-Jewish Hospital, St. Louis, Missouri, USA*

Correspondence: Ailin Tao, The Second Affiliated Hospital, Guangzhou Medical University, Changgangdong 250, Guangzhou 510260, China. E-mail: taoailin@gzhmu.edu.cn

Abbreviations: BNP, brain-derived natriuretic peptide; ERK, extracellular signal–regulated kinase; GRP, gastrin-releasing peptide; GRPR, GRP receptor; HEK, human embryonic kidney; IMQ, imiquimod; MEK, MAPK/extracellular signal–regulated kinase kinase; NMB, neuromedin B; NMBR, NMB receptor; NPRA, natriuretic peptide receptor A; p-ERK, phosphorylated extracellular signal–regulated kinase; RNA-seq, RNA sequencing; SADBE, squaric acid dibutylester; SST, somatostatin

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ERK [p-ERK]) in sensory neurons could initiate and maintain long-lasting itch (Zhao et al., 2013). Meanwhile, in chronic itch models chemically induced with DNFB or in dry skin, only peripheral (in dorsal root ganglion) but not spinal ERK activation was observed (Zhao et al., 2013). Another study reported that the downstream of GRP receptor (GRPR) works through the phosphoinositide 3-kinases γ/protein kinase B but not through the p38 or ERK1/2 pathways in the spinal cord (Pereira et al., 2015). In contrast, two studies (Huang et al., 2018b; Zhang et al., 2014) showed that in the spinal cord, transient ERK activation contributes to histamine-dependent but not histamine-independent acute itch (Zhang et al., 2014), and persistent neuronal ERK is activated by histamine H4 receptor for DNFB-induced chronic itch (Huang et al., 2018b).

Recently, we reported that mice with allergic contact dermatitis caused by squaric acid dibutylester (SADBE) showed prolonged spontaneous scratching (Liu et al., 2020). SADBE-induced prolonged itch includes both histaminergic and nonhistaminergic components, which are relayed by natriuretic peptide receptor A (NPRA) and GRPR in the spinal cord, respectively (Liu et al., 2020). To further investigate the central mechanisms of chronic itch, we employed several different chronic itch models and performed RNA sequencing (RNA-seq) analysis. Because the ERK pathway was one of the most significantly enriched upregulated gene clusters, we investigated the role of ERK in chronic itch and the relationship between p-ERK and neural circuits in the spinal cord for chronic itch.

RESULTS

ERK1/2 cascade is highly enriched in the spinal cord of mice with SADBE- and imiquimod-induced chronic itch

To identify the signaling pathways relevant to chronic itch in mouse models, RNA-seq analysis was conducted with the spinal cord tissues of the SADBE-induced allergic contact dermatitis model and the imiquimod (IMQ)-induced psoriasis model (Sakai et al., 2016) (Figure 1a–c). As shown in the volcano plot, 1,573 (811 downregulated genes, 762 upregulated genes) and 2,933 (1,187 downregulated genes, 1,746 upregulated genes) genes were differentially expressed 3 days after the last SADBE challenge (Figure 1d) and 2 days after the IMQ painting (Figure 1e), respectively, compared with those of their controls. Meanwhile, the result of DESeq2 (version 1.16.1) differential expression analysis demonstrated that the differentially expressed genes in the spinal cords between chronic itch mice and their controls were hierarchically clustered dependent on the gene enrichment features (Figure 1f and g). To further investigate the molecular mechanisms underlying chronic itch, we carried out the Gene Ontology enrichment analysis of differentially expressed genes with a special focus on intracellular cell signaling pathways. Results obtained from the Gene Ontology analysis indicated that ERK1/2 cascade (Gene Ontology: 0070371) is the most significantly enriched upregulated gene cluster in both SADBE and IMQ groups (Figure 1h and i). A further analysis showed that 17 and 63 genes in the ERK1/2 cascade were upregulated in the mice treated with SADBE (Figure 1j) and IMQ (Figure 1k), respectively, some of which were confirmed by quantitative real-time RT-PCR assay (Figure 1l and m).

Chronic itch is associated with sustained ERK activation

Because our recent work showed that SADBE induced a prolonged chronic spontaneous itch (Liu et al., 2020), we next investigated whether p-ERK is responsible for chronic itch similar to the central role of p-ERK in chronic pain (Gao and Ji, 2009; Ji et al., 2018; Obata and Noguchi, 2004). We established a series of chronic itch models on mice before using their spinal cord tissues for sampling (Figure 2a–d). To see whether there is a correlation between the itch behavior and ERK activation, we examined the ERK activation kinetics in the cervical dorsal horn at 3, 7, 21, and 35 days after the last SADBE challenge. We found that SADBE induced a rapid onset and persistent ERK activation up to day 35 (Figure 2e and i). Significantly increased induction of p-ERK in the spinal cord was also found in mice with IMQ-induced psoriasis model (Figure 2f and i); MC903 (calcipotriol)-induced atopic dermatitis model (Oetjen et al., 2017) (Figure 2g and i); and acetone-, ether-, and water-induced dry skin model (Figure 2h and i). To determine the role of p-ERK in chronic itch, two specific MAPK/ERK kinase (MEK) inhibitors U0126 (Favata et al., 1998; Liu et al., 2017; Zhao et al., 2013) and PD0325901 (Alessi et al., 1995; Barrett et al., 2008; Liu et al., 2017) were intrathecally administered, which showed significant inhibition to chronic pain (Komatsu et al., 2014) or itch (Zhao et al., 2013) and very little unspecific central side effects. As expected, SADBE-; IMQ-; MC903-; and acetone, ether, and water–induced chronic itch was significantly alleviated by injection of either U0126 (10 nmol per mouse, intrathecally) (Figure 2j) or PD0325901 (25 nmol per mouse, intrathecally) (Figure 2k).

Double staining of p-ERK with traditional spinal neurochemical markers in the cervical spinal cord of SADBE mice showed that most of the p-ERK$^+$ cells were located in the superficial laminae (Figure 3a and b), and p-ERK was mainly found in neurons 3 (Figure 3c–e) and at 21 days (Supplementary Figure S1a–c) after the last SADBE painting. We double stained the p-ERK$^+$ cells with *Vglut2* (a glutamatergic neuronal marker) (Figure 3f) and *Vgat* (an inhibitory neuronal marker) in SADBE mice (Figure 3g) or with anti-GFP in GAD67-eGFP mice (Figure 3h) after SADBE treatment. The p-ERK$^+$ cell population included both inhibitory and excitatory neurons; about half (43.3%, 88 of 203) of the p-ERK$^+$ cells were excitatory (Figure 3i), and half of the p-ERK$^+$ cells were *Vgat*$^+$ (51.5%, 34 of 66 cells) (Figure 3j) or GAD67-eGFP$^+$ (47.2%, 241 of 511 cells) (Figure 3k).

ERK is a major intracellular signaling pathway downstream of GRPR

GRPR has long been regarded as the key itch receptor in the spinal cord (Dong and Dong, 2018; Huang et al., 2018a; Mishra and Hoon, 2013; Sun and Chen, 2007; Sun et al., 2009; Zhao et al., 2013), and recently, we elucidated that GRPR and NPRA are receptors that relay the prolonged itch in the spinal cord (Liu et al., 2020). Therefore, we next investigated whether p-ERK is the downstream signaling mediator for GRPR and NPRA. Double staining of p-ERK with

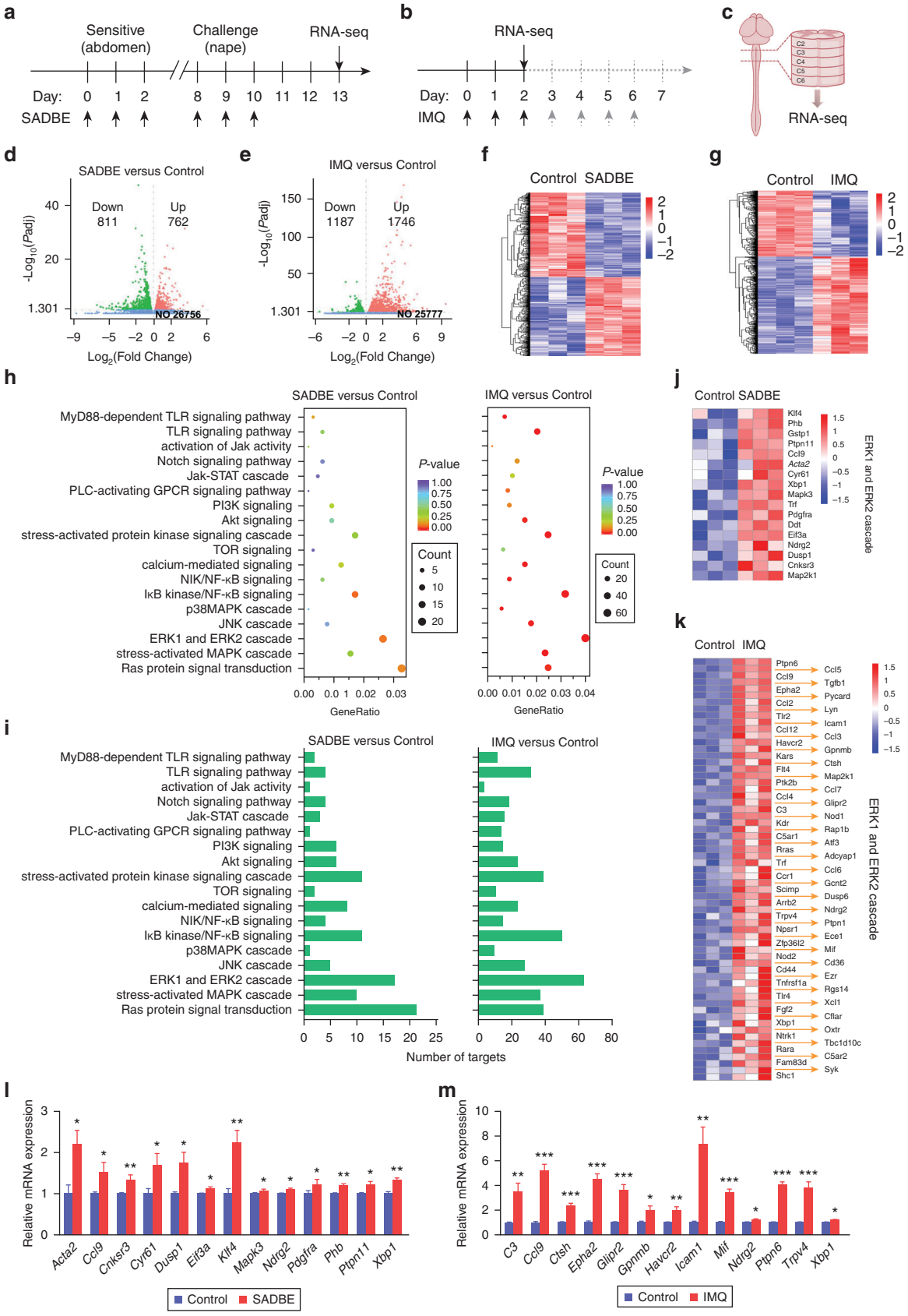

Figure 1. RNA-seq analysis of differential gene expression and enriched signaling pathways in the spinal cord of SADBE- and IMQ-treated mice. (**a**, **b**) Schematic schedule of RNA-seq analysis of cervical spinal cord tissues of mice after being treated with (**a**) SADBE or (**b**) IMQ. (**c**) Schematic drawings of c2−c6 spinal cord used for RNA-seq. (**d**, **e**) Volcano plots illustrating the expression of DEGs in the cervical spinal cord of mice between (**d**) SADBE or (**e**) IMQ groups and their respective controls. Values are presented as the Log_2 of tag counts. *P*adj < 0.05. (**f, g**) Heatmaps showing the hierarchical clustering of changed

Grpr$^+$ cells in C57BL/6J (referred to as C57) mice (Figure 4a and b) or with GFP staining in GRPR-eGFP mice (Figure 4c and d) 7 days after the last SADBE painting showed that a small subset of *Grpr*$^+$ cells (22.4%, 39 of 174 cells) or GRPR-eGFP$^+$ cells (21.2%, 14 of 66 cells) were p-ERK$^+$ (Figure 4b and d).

We next assessed the phosphorylation of several key cell-signaling molecules important in chronic pain or inflammation (Jensen et al., 2008; Liu et al., 2017) in human embryonic kidney (HEK)293T cells transiently expressing GRPR (GRPR-expressing HEK). GRP (20–2,000 nM) activated ERK in a concentration-dependent manner after 10 and 30 minutes (Supplementary Figure S2a and b). In contrast, other markers such as p38, c-Jun N-terminal kinase, protein kinase B, and p65 were only weakly activated (Supplementary Figure S2a and b). Over a wider period of time, the levels of p-ERK, phosphorylated c-Jun N-terminal kinase, phosphorylated p38, phosphorylated protein kinase B, and phosphorylated p65 in GRPR-expressing HEK cells after GRP treatment (2 μM) at 0, 2, 10, 30 minutes or 1, 3, 6, 24 hours were measured. Among all the markers examined, ERK was the only one strikingly activated by GRP with a peak time at 10 minutes and remained at a high level for 24 hours as the longest time tested (Figure 4e and f). The MEK inhibitor U0126 (0.4–50 μM) significantly inhibited GRP-induced ERK activation in a concentration-dependent manner (Figure 4g and h).

ERK is also an important intracellular signaling pathway downstream of NPRA, sst2A, and NMB receptor

In the spinal cord, NPRA/SST/sst2A (receptor of SST)/dynorphine-expressing cells form an SST-mediated itch microcircuit for modulating itch responses (Huang et al., 2018a; Kardon et al., 2014). Our RNAscope (Advanced Cell Diagnostics, Hayward, CA) data revealed that 29.5% (74 of 251 cells) of *Npr1*$^+$ cells doubled stained with p-ERK (Figure 5a and b). Similarly, p-ERK was partially coexpressed with *Sstr2*$^+$ (Supplementary Figure S3a) and *Prodyn* (Supplementary Figure S3b). In NPRA-expressing HEK cells, ERK was activated by BNP (0.1–50 μM) (Supplementary Figure S2c and d) with the peak time of 10 minutes (Supplementary Figure S2e and f). Among all the kinases tested in the NPRA- or sst2A-expressing HEK cells, ERK was the most highly activated by their agonists BNP (Figure 5c and d) and octreotide (Supplementary Figure S3c–f), respectively, and peaked at 10 minutes. The MEK inhibitor U0126, in a concentration-dependent manner, prevented ERK activation in NPRA-expressing HEK cells (Figure 5e and f) and sst2A-expressing HEK cells in response to BNP and octreotide (Supplementary Figure S3g and h), respectively.

NMB receptor (NMBR), another member of the mammalian bombesin receptor family, is expressed in the superficial spinal cord and responsible for histaminergic itch (Zhao et al., 2014). Double staining of p-ERK with GFP in NMBR-eGFP mice (Supplementary Figure S4a and b) or with *Nmbr*$^+$ cells in C57 mice (Supplementary Figure S4c and d) 7 days after the last SADBE painting showed that a proportion of NMBR-eGFP$^+$ cells or *Nmbr*$^+$ cells were p-ERK$^+$. Among all the kinases we tested, p-ERK was significantly activated by NMB and peaked at 10 minutes (Supplementary Figure S4e–h), and the activation was reversed by U0126 in a concentration-dependent manner (Supplementary Figure S4i and j). These results together support the hypothesis that ERK acts as the primary signaling pathway downstream of NPRA (Figure 5), sst2A (Supplementary Figure S3), and NMBR (Supplementary Figure S4).

Neurochemical heterogeneity of p-ERK$^+$ cells and dissection of spinal circuits for p-ERK in chronic itch

In the spinal cord, *Nmbr*, *Grpr*, and *Sstr2* were shown to be expressed in different populations (Supplementary Figure S5a and b) (Kiguchi et al., 2016; Liu et al., 2020; Mishra and Hoon, 2013; Zhao et al., 2014). Most *Prodyn*$^+$ cells were also *Sstr2*$^+$ (Supplementary Figure S5c) and partially *Nmbr*$^+$ (Supplementary Figure S5d) but typically not *Grpr*$^+$ (Supplementary Figure S5e). Nonetheless, p-ERK was positive in some *Grpr*$^+$ (Figure 4a and b), GRPR-eGFP (Figure 4c and d), *Npr1*$^+$ (Figure 5a and b), *Sstr2*$^+$ (Supplementary Figure S3a), *Prodyn*$^+$ (Supplementary Figure S3b), NMBR-eGFP (Supplementary FigS4a and b), and *Nmbr*$^+$ (Supplementary Figure S4c and d) cells in the cervical spinal cord 7 days after the last SADBE treatment, demonstrating the neurochemical heterogeneity of dorsal horn cells expressing p-ERK. Itch neuropeptides such as GRP, BNP, NMB, and SST were also upregulated to different extents in the dorsal root ganglions of the SADBE mice (Liu et al., 2020). Most of the *Nppb*$^+$ cells coexpressed *Sst* (Supplementary Figure S6a) (Huang et al., 2018a), but they rarely coexpressed *Grp* (Supplementary Figure S6b) (Liu et al., 2020) or *Mrgpra3* (Supplementary Figure S6c and Figure 6g). In addition, *Nmb*$^+$ cells did not coexpress *Grp* (Figure 6h) but greatly expressed *Hrh1* (Figure 6i), *Trpv1* (Supplementary Figure S6d), and *Sst* (Supplementary Figure S6e), supporting the idea that the NMB/NMBR pathway is dispensable for histaminergic itch (Zhao et al., 2014).

To further test the possibility that GRP and BNP directly activate ERK signaling, we intrathecally injected GRP and BNP into mice, and as predicted, both of them directly evoked ERK activation in the superficial spinal cord (Figure 6a and b). The examination of bombesin-saporin mice (Sun et al., 2009) after treatment with SADBE (Figure 6c and d) and IMQ (Supplementary Figure S6f) showed that bombesin-saporin treatment completely abolished ERK activation. In contrast, p-ERK staining in *Grpr*-knockout mice showed that *Grpr* inactivation significantly reduced ERK

DEGs; *P*adj < 0.05. (**h**, **i**) The GO analysis of upregulated enriched targets for cell signaling pathways. (**j**, **k**) Heatmaps of upregulated ERK1 and ERK2 cascade–related genes in the spinal cord of SADBE- and IMQ-treated mice. (**l**, **m**) A total of 13 representative DEG expressions determined by QRT-PCR. n = 3 per group for **d**–**k** and 5 per group for **l** and **m**. Akt, protein kinase B; DEG, differentially expressed gene; ERK, extracellular signal–regulated kinase; GO, Gene Ontology; GPCR, G-protein–coupled receptor; IMQ, imiquimod; JNK, c-Jun N-terminal kinase; Log, logarithm; NIK, NF-kB–inducing kinase; *P*adj, adjusted *P*-value; PI3K, phosphatidylinositol-3 kinase; PLC, phospholipase C; QRT-PCR, quantitative real-time RT-PCR; RNA-seq, RNA sequencing; SADBE, squaric acid dibutylester; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; TOR, target of rapamycin.

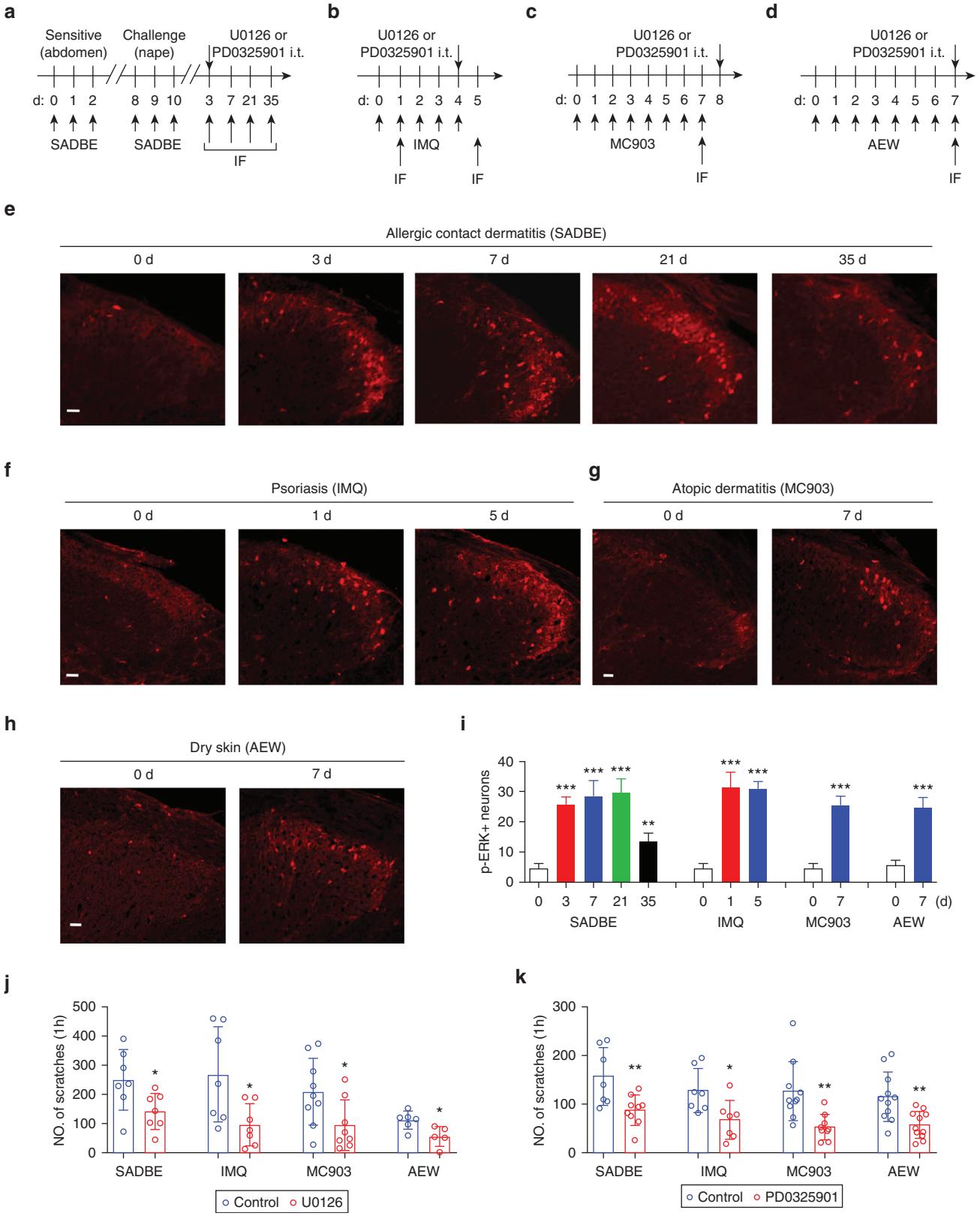

Figure 2. ERK activation is observed in the spinal cord and contributes to chronic itch. (**a**–**d**) Schematic experimental protocols of four chronic itch mouse models and the time points of drug administration or tissue sampling for IF staining. (**e**–**i**) Representative (**e**–**h**) immunostaining images and the (**i**) quantification graphs showing p-ERK immunoreactivity in transverse sections of cervical spinal dorsal horn at 3, 7, 21, and 35 d after SADBE painting; 1 and 5 d after the last IMQ painting; or d 7 of the MC903 and AEW model. n = 3 per group in **i**. (**j**–**k**) SADBE-, IMQ-, MC903-, and AEW-induced chronic itch was significantly attenuated by i.t. injection of U0126 (10 nmol per mouse for **j**) or PD0325901 (25 nmol per mouse for **k**) at 10 minutes before recording. Bars = 25 μm for **e**–**h**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$, compared to the control using one-way ANOVA followed by Bonferroni's test. AEW, acetone, ether, and water; d, day; ERK, extracellular signal–regulated kinase; h, hour; i.t., intrathecal; IF, immunofluorescent; IMQ, imiquimod; NO., number; p-ERK, phosphorylated extracellular signal–regulated kinase; SADBE, squaric acid dibutylester.

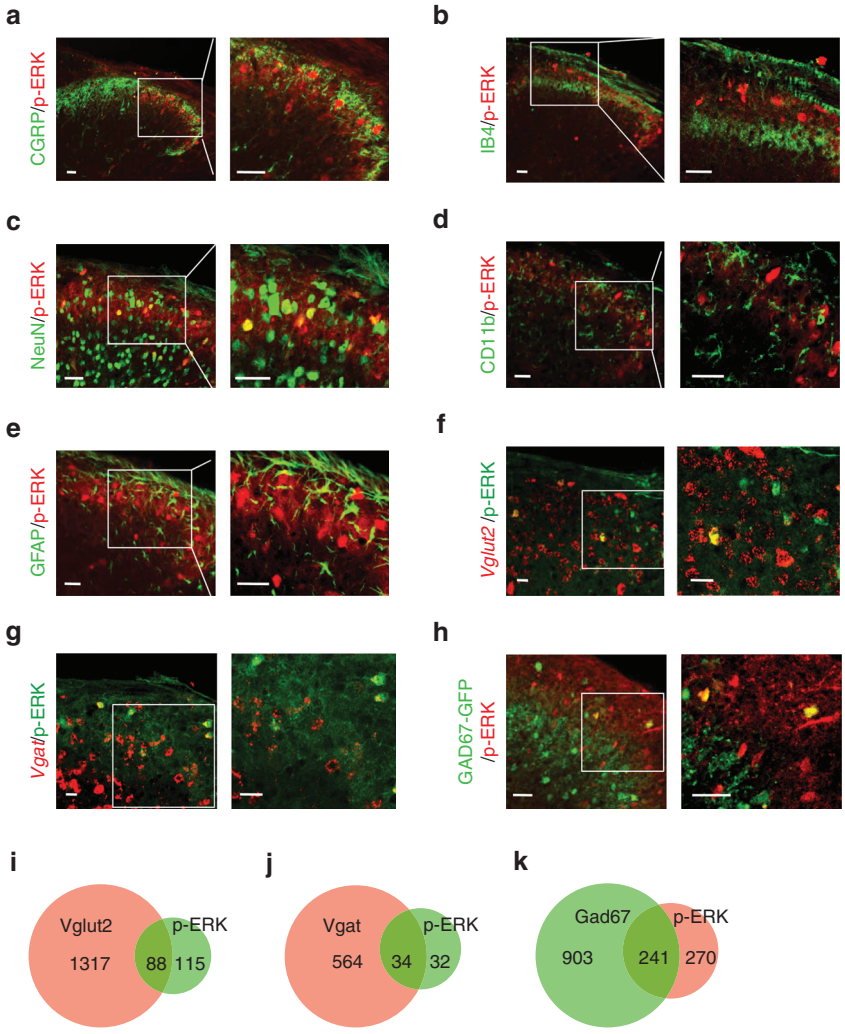

Figure. 3. Characterization of spinal ERK activation in mice with SADBE-induced prolonged itch. (**a**–**e**) Double staining of p-ERK with (**a**) CGRP, (**b**) IB4, and (**c**) markers of neurons (NeuN), (**d**) microglia (CD11b), and (**e**) astrocytes (GFAP) on sections in c4–c6 spinal dorsal horn of mice at 3 days after SADBE painting. (**f**–**h**) Cross sections of cervical spinal cords from mice with SADBE-induced–prolonged itch were immunostained for p-ERK and RNAscope ISH stained for (**f**) *Vglut2* (red), (**g**) *Vgat* (red), or (**h**) GAD67-GFP (green). **(i–k)** Venn diagrams for the quantification of p-ERK overlaps with the indicated markers. $n = 3–4$ mice per group. Bars = 25 μm. ERK, extracellular signal–regulated kinase; ISH, in situ hybridization; p-ERK, phosphorylated ERK; SADBE, squaric acid dibutylester.

activation, which was further reduced by the coinjection of H1R antagonist olopatadine and H4R antagonist JNJ7777120 (Figure 6c and d). In contrast, *Npr1* inactivation (Supplementary Figure S6g) or the BNP-saporin (Mishra and Hoon, 2013) (Supplementary Figure S6h) only partially reduced p-ERK in the cervical spinal cords. Thus, whereas the distribution of peptides and/or receptors subpopulations in the spinal cord is relatively independent, to varying degrees, they activate a common intracellular pathway, p-ERK, in the case of SADBE-induced prolonged itch as well as in psoriatic itch induced by IMQ, atopic dermatitis–like itch induced by MC903, or dry skin–induced itch by acetone, ether, and water, and in turn, the p-ERK is instrumental for these circuits to transmit the chronic itch (Figure 6j).

DISCUSSION

In this study, we have delineated the important role of spinal p-ERK for neural signaling in the mouse chronic itch models. By using RNA-seq analysis, we demonstrated that ERK1/2 cascade is one of the most significantly upregulated gene clusters. By in vitro analysis, we validated from a number of signal transduction pathways that ERK is the primary signaling pathway downstream of GRPR, NPRA, sst2A, and NMBR. By combining neurochemical, pharmacologic, genetic, and neurotoxin methods, we identified the micro-circuits formed by GRPR, NPRA, sst2A, NMBR, and dynorphin-expressing cells that express p-ERK as a key downstream signal molecule in the signaling of chronic itch in the spinal cord. Taken together, our study reveals that p-ERK is a reliable spinal marker for the central sensitization of itch and explains how it regulates spinal itching.

People think that chronic itch and chronic pain share some similarities such as peripheral sensitization, central sensitization, glial regulation, and immune regulation (Ji, 2015). Phosphorylation of ERK in dorsal horn neurons occurs in response to peripheral inflammation or nerve injury, which had been proposed as nociceptive specific and used as a marker of central sensitization (Gao and Ji, 2009; Ji et al., 2018; Obata and Noguchi, 2004). However, there are some differences in ERK activation in the spinal cord between chronic pain and itch. For example, spinal p-ERK in chronic pain is short or transient (within minutes or hours) (Gao and Ji, 2009; Ji et al., 2018; Obata and Noguchi, 2004), but in DNFB-induced chronic itch (Huang et al., 2018b) and our mouse models, p-ERK in the spinal cord was sustained. As we had shown, in the periphery of mice with chronic itch, various cytokines, chemokines, and

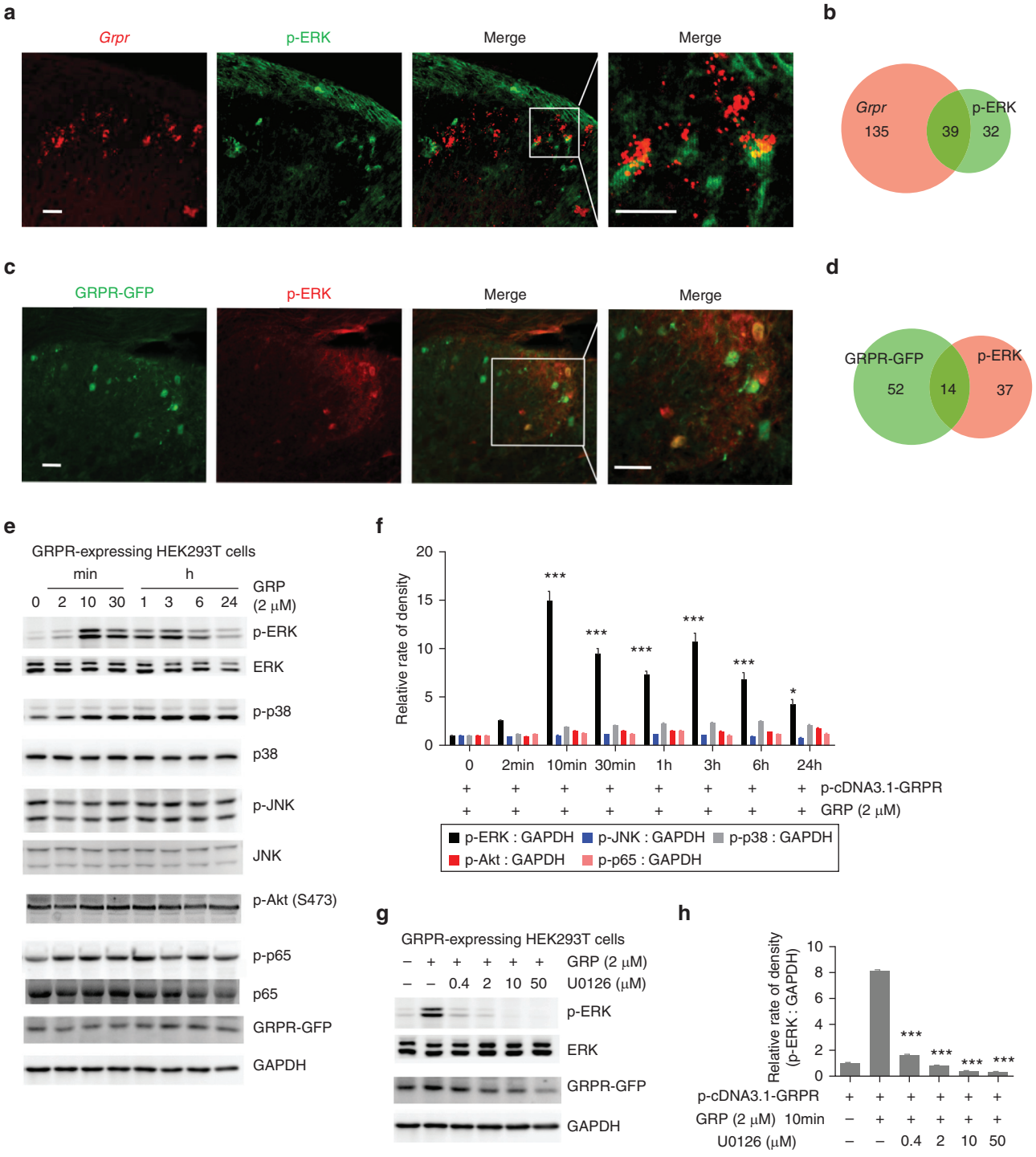

Figure 4. GRPR signals through the ERK pathway. (**a**–**d**) Double staining of p-ERK with (**a**) *Grpr* mRNA in C57 mice or (**c**) GFP antibody staining in GRPR-eGFP mice at 7 days after the last SADBE painting. Bars = 25 μm. (**e**–**h**) Representative (**e**) western blots and (**f**) quantification results of p-ERK, p-JNK, p-p38, p-Akt, and p-p65 versus GAPDH in GRPR-expressing HEK cells and treated with GRP (2 μM) or the representative (**g**) western blots and (**h**) quantification results in GRPR-expressing HEK cells treated with GRP (2 μM) for 10 mins after incubation with the MEK inhibitor U0126 (0.4, 2, 10, and 50 μM). In **f** and **h**, data are expressed as mean ± SEM of three independent experiments. One-way ANOVA followed by Tukey test; $*P < 0.05$, $***P < 0.001$, compared with vehicle (1 × PBS). ERK, extracellular signal–regulated kinase; GRP, gastrin-releasing peptide; GRPR, GRP receptor; h, hour; HEK, human embryonic kidney; JNK, c-Jun N-terminal kinase; MEK, MAPK/extracellular signal–regulated kinase kinase; min, minute; p-Akt, phosphorylated protein kinase B; p-cDNA, phosphorylated cDNA; p-ERK, phosphorylated extracellular signal–regulated kinase; p-JNK, phosphorylated c-Jun N-terminal kinase; p-p38, phosphorylated p38; p-p65, phosphorylated p65; SADBE, squaric acid dibutylester.

peptides, including IL-33, TSLP, GRP, and BNP, displayed significant upregulation for weeks (Liu et al., 2020), which may lead to sustained ERK activation. Other inflammatory agents such as IL-31 (Cevikbas et al., 2014) and B1R agonist (Chen et al., 2016) would activate ERK signaling to cause prolonged itch as well.

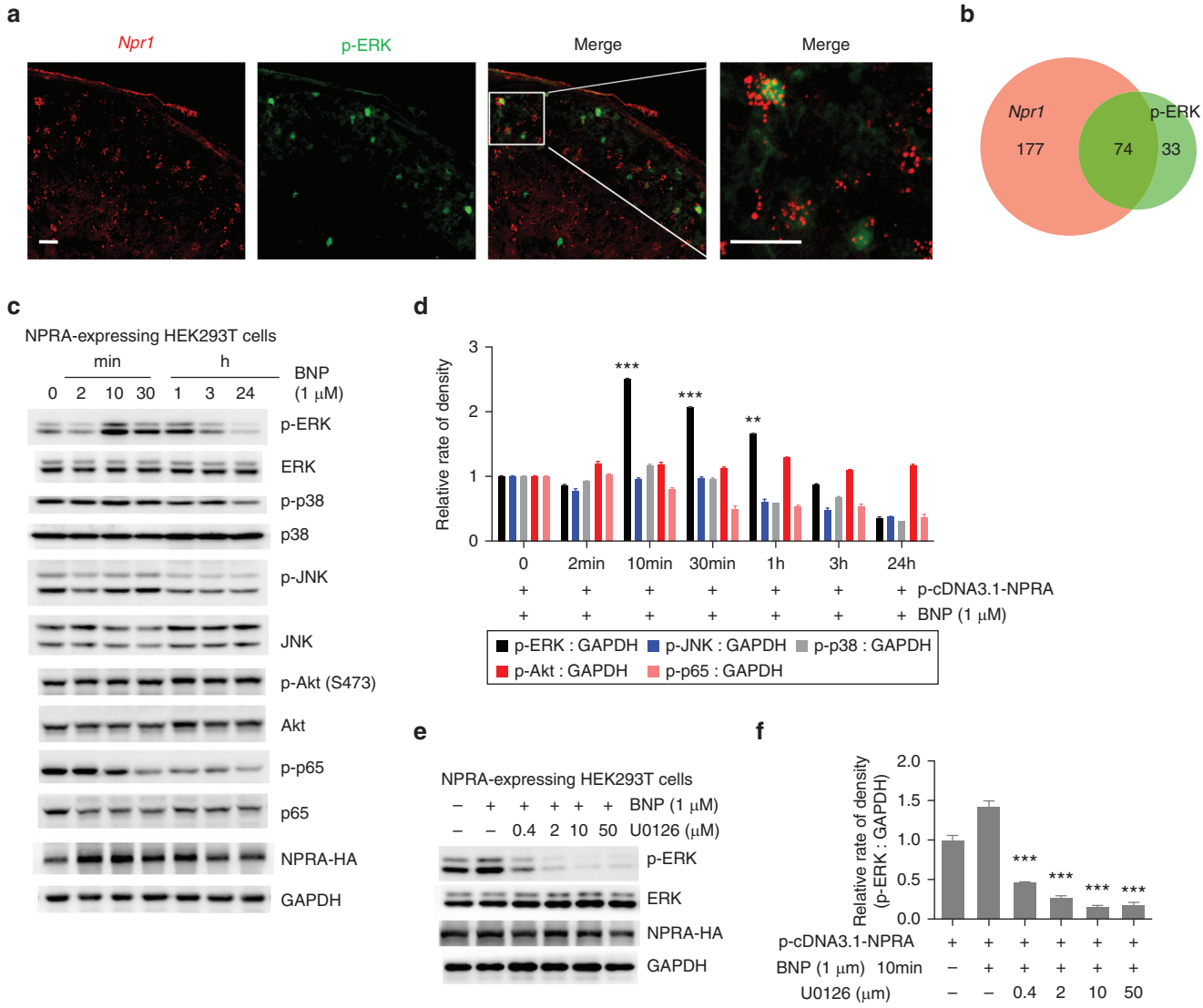

Figure 5. ERK is the intracellular signaling pathway downstream of NPRA. (**a**, **b**) *Npr1* (red) and p-ERK (green) double staining of *Npr1* and p-ERK in the dorsal horn of SADBE mice at 7 days after the last SADBE treatment. Bars = 25 μm. (**c**, **d**) Representative (**c**) western blot and (**d**) quantification results of p-ERK, p-JNK, p-p38, p-Akt, and p-p65 versus GAPDH in NPRA-expressing HEK cells treated with BNP (1 μM). (**e**, **f**) Representative (**e**) western blots of p-ERK, total ERK, NPRA-HA, and quantification results of p-ERK versus (**f**) GAPDH in NPRA-expressing HEK cells treated with BNP (1 μM) for 10 min after incubation with MEK inhibitor U0126 (0.4, 2, 10, and 50 μM). In **d** and **f**, $^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA followed by Tukey test compared with the vehicle (1 × PBS). Akt, protein kinase B; BNP, brain-derived natriuretic peptide; ERK, extracellular signal–regulated kinase; h, hour; HEK, human embryonic kidney; JNK, c-Jun N-terminal kinase; MEK, MAPK/ extracellular signal–regulated kinase kinase; min, minute; NPRA, natriuretic peptide receptor A; p-Akt, phosphorylated protein kinase B; p-cDNA, phosphorylated cDNA; p-ERK, phosphorylated extracellular signal–regulated kinase; p-JNK, phosphorylated c-Jun N-terminal kinase; p-p38, phosphorylated p38; p-p65, phosphorylated p65; SADBE, squaric acid dibutylester.

SADBE-induced p-ERK could be significantly attenuated by GRPR or NPRA blockade, antihistamines, and others. In other words, p-ERK responded to a considerable number of known antipruritic reagents. Our in vitro study data showed that respective agonists significantly activate ERK signaling in HEK293T cells that transiently express GRPR, NPRA, sst2A, and NMBR. Therefore, p-ERK significantly serves as a key factor for prolonged itch. Previously, the lack of spinal p-ERK detected in DNFB or dry skin chronic itch models (Zhao et al., 2013) might be due to the observation time being too early (~24 hours). What will ERK signaling trigger to further promote chronic itch? Next, the identification of ERK-dependent transcriptional events will be a logical step in elucidating how sustained p-ERK encodes chronic behavioral output. We did some preliminary explorations and found that *Ccl9*, *Xbp1*, *Ndrg2*, and others might be the possible common molecular targets downstream of p-ERK for chronic itch (Figure 1l–m), which certainly need to be confirmed by further experiments.

Our study also provides more information for proposing a spinal circuit for p-ERK in chronic itch. Firstly, chronic itch had been believed to be mostly histamine independent, and antihistamines are ineffective in treating it, but our recent work (Liu et al., 2020) suggests that this view is incorrect at least because of prolonged itch associated with allergic contact dermatitis. Grp^+ with Nmb^+ (Figure 6g), *Sst*+ (Supplementary Figure S6a), and $Nppb^+$ (Liu et al., 2020)

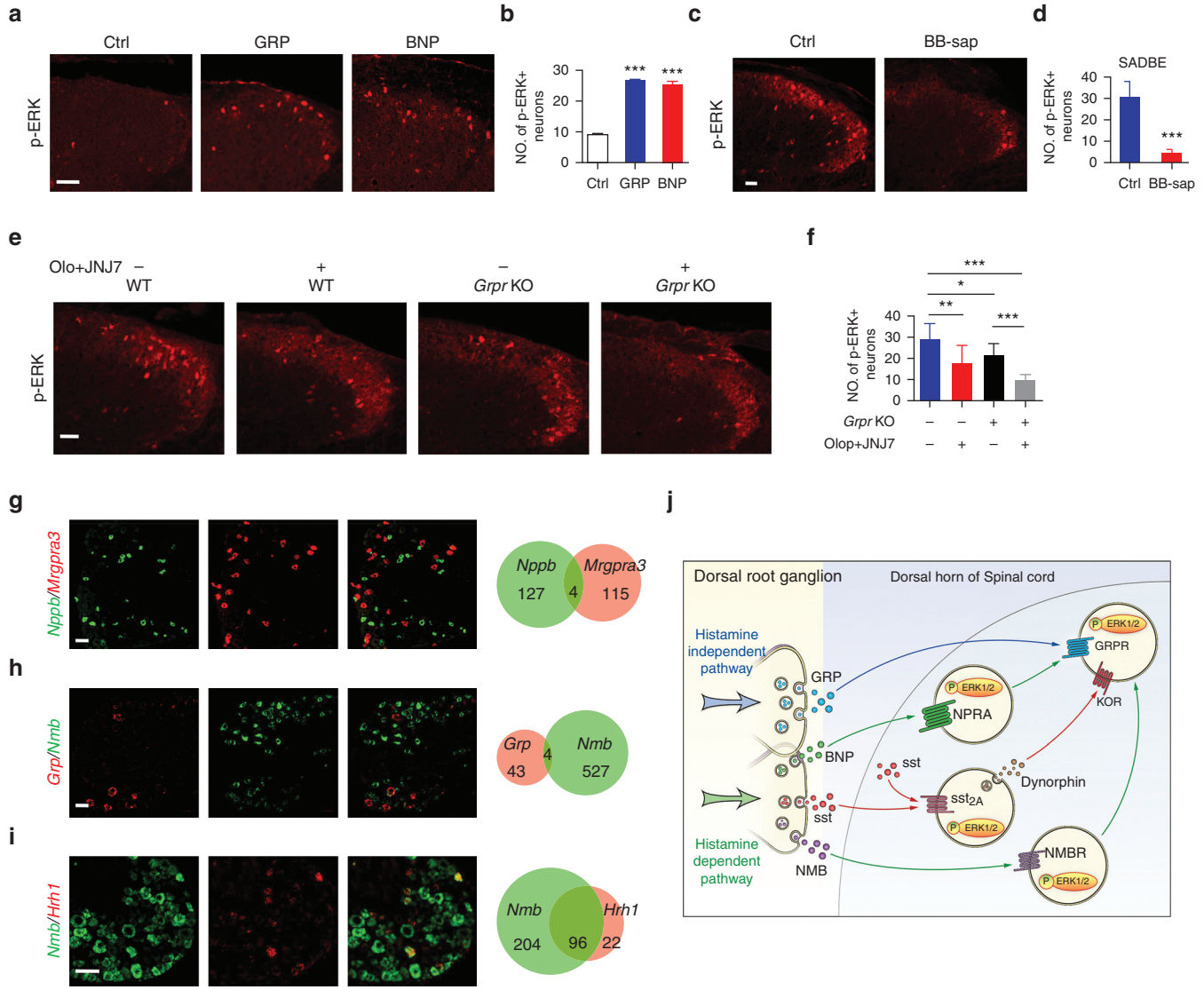

Figure 6. Intrathecal GRP and BNP evoke spinal p-ERK, and genetic and pharmacologic blockade of chronic itch eliminates p-ERK. (**a**, **b**) p-ERK+ cells were significantly increased in the cervical (c2−c7) superficial spinal cord of in C57 mice 10 minutes after intrathecal injection of GRP (0.1 nmol) and BNP (5 μg). (**c**, **d**) p-ERK+ cells were ablated in C57 mice at 7 days after the last SADBE painting (21 days after treatment with BB-sap). (**e**, **f**) *Grpr*-KO mice showed impaired SADBE-induced p-ERK+ that was further reduced by the coinjection of H1R antagonist olopatadine and H4R antagonist JNJ7777120. (**g**–**i**) Double RNAscope ISH images and quantifications reveal the overlap between itch markers in DRG. For all quantifications, n = 4 mice. $*P < 0.05$, $**P < 0.01$, $**P < 0.001$, compared to the control or that showed in the figure. Bars = 25 μm. (**j**) Schematic diagram of proposed spinal neuronal circuits for p-ERK in chronic itch. Repeated exposure of SADBE/IMQ/MC903/AEW results in the release of a variety of itch-related neuropeptides such as GRP, BNP, SST, and NMB, which originate from DRG neurons and probably also from the spinal cord and bind to their specific receptors on the membrane of interneurons in the dorsal horn. Dorsal horn interneurons, to a large extent, do not coexpress these receptors but form a specific neural circuit. p-ERK is a common intracellular signaling molecule for the activation of GRPR+, NPRA+, sst2A+, and NMBR+ cells in itch. After activation of NPRA+, sst2A+, and NMBR+ neurons, itch information is transmitted by GRPR+ neurons through the excitatory synapse to the spinal projection neurons and then transmitted to the brain. AEW, acetone, ether, and water; BB-sap, bombesin-saporin; BNP, brain-derived natriuretic peptide; ctrl, control; DRG, dorsal root ganglion; ERK, extracellular signal−regulated kinase; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; IMQ, imiquimod; ISH, in situ hybridization; KO, knockout; NMB, neuromedin B; NMBR, neuromedin B receptor; NO., number; NPRA, natriuretic peptide receptor A; p-ERK, phosphorylated extracellular signal−regulated kinase; SADBE, squaric acid dibutylester; SST, somatostatin; WT, wild type.

overlap little in the dorsal root ganglion, and similarly, *Grpr*$^+$ overlaps little with *Npr1*$^+$ (or *Sstr2*+) in the spinal cord (Huang et al., 2018a; Liu et al., 2020; Mishra and Hoon, 2013). Secondly, most of the *Grpr*$^+$ (Aresh et al., 2017; Liu et al., 2020), *Nmbr*$^+$ (Zhao et al., 2014), and *Npr1*$^+$ (Liu et al., 2020) cells are excitatory, and most of the sst2A$^+$ cells are inhibitory (Kardon et al., 2014). However, ERK is activated in all these subsets of cells to different extents. Because GRPR$^+$ cells act as a downstream switch (Huang et al., 2018a; Liu et al., 2020; Mishra and Hoon, 2013; Sun et al., 2009; Zhao et al., 2013), the ablation of GRPR$^+$ cells with bombesin-saporin resulted in a blockade of signaling from NPRA, sst2A, NMBR, and others. Consequently, ERK was unable to be activated in these subsets and thus led to itch abolishment. Together, not only BNP/NPRA and GRP/GRPR are activated but also the cells expressing these itch peptides and their receptors may form a circuit (Huang et al., 2018a; Liu et al., 2020; Mishra and Hoon,

2013; Sun et al., 2009) using p-ERK as a hub in the spinal cord for chronic itch (Figure 6j).

Current chronic itch treatment includes topically and/or systemically administering corticosteroids, immunosuppressants, antihistamines, phototherapy, and others. (Yosipovitch et al., 2018). Our findings support that both peripheral and central sensitizations occur in chronic itch settings. There is a need for a therapeutic strategy that targets peripheral and central sensitizations of refractory pruritus in particularly targeting the ERK pathway. Recently, a small synthetic ERK inhibitor was found to be alleviating IMQ-induced mice skin lesions (Huang et al., 2019). Inspired by the finding that the combined treatment of BRAF inhibitors and MEK inhibitors has become an excellent strategy for treating melanoma and avoiding resistance to BRAF inhibitors (Savoia et al., 2019), our work supports the development of small-molecule signal transduction inhibitors targeting both peripheral (Zhao et al., 2013) and central p-ERK for the treatment of chronic itch.

MATERIALS AND METHODS

Mice and reagents

Adult male C57BL/6J mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). First breeders of *Grpr*-knockout mice (Zhao et al., 2013), *Npr*- knockout mice (Oliver et al., 1997), GRPR-eGFP mice (Zhao et al., 2014), and NMBR-eGFP mice (Zhao et al., 2014) were provided by Dr Zhoufeng Chen at Washington University (St. Louis, MO), and GAD67-eGFP mice were from Dr Hui Li at the Fourth Military Medical University (Xi'an, China). Genomic DNA was isolated from tail biopsies, and the presence of targeting transgenes was confirmed by PCR-based genotyping (Supplementary Figure S7). All animal experiments were performed under the protocol number A2019-001 approved by the Animal Studies Committee of Guangzhou Medical University (Guangzhou, China). All drugs and reagents used for behavioral tests are listed in the Supplementary Materials.

Behavioral studies

The contact sensitizer SADBE (Sigma-Aldrich, St. Louis, MO) was used to induce contact allergic dermatitis in mice (Liu et al., 2020). A topical application of 5% IMQ cream (3M Pharmaceuticals, Maplewood, MN) was used to induce a psoriasis mouse model (Sakai et al., 2016). To induce atopic dermatitis–like inflammation and chronic itching in mice, MC903 (calcitriol, Tocris Bioscience, Bristol, United Kingdom) was used on the nape of neck skin (Oetjen et al., 2017). A mouse model of dry skin–evoked chronic itch was induced by acetone, ether, and water (Akiyama et al., 2010; Zhao et al., 2013). Scratching behaviors were performed as described in previous work (Sun et al., 2009; Zhao et al., 2013). Details for behavioral tests and tissue sampling are described in the Supplementary Materials.

RNA-seq analysis, immunohistochemistry, and RNAscope in situ hybridization assay

RNA-seq experiments were performed by Novogene (Beijing, China) following the manufacturer's instructions by using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). Sequencing libraries were generated, and index codes were added to attribute sequences to each sample. Immunohistochemical staining and RNAscope in situ hybridization were performed similarly to our recent work (Liu et al., 2020). For detailed experimental procedures and antibody information, see the Supplementary Materials.

HEK293T cell transfection and western blot analysis

cDNAs for mouse *Grpr*, *Npr1*, *sst2a*, and *Nmbr* were cloned into the plasmid vector pcDNA3.1 and transiently transfected to HEK293T cells for 48 hours using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfected HEK293T cells were incubated with respective agonists followed by western blot analysis to study their effects on the activation of different signaling pathways. More details are in the Supplementary Materials.

Statistical analyses

All quantitative values are reported as mean ± SEM. Unless otherwise specified, Prism 6 (version 6.0e; GraphPad, San Diego, CA) was used for statistical analysis. To compare the difference between the two groups, an unpaired two-tailed *t*-test was used. To compare more than two groups, one-way ANOVA was used, followed by Tukey's post hoc analysis. $P < 0.05$ was considered statistically significant.

Data availability statement

The RNA sequencing datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152637 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152646, hosted at National Center for Biotechnology and Information Gene Expression Omnibus (series accession numbers GSE152637 and GSE152646).

ORCIDs

Xueting Liu: http://orcid.org/0000-0001-5419-198X
Yanmei Wang: http://orcid.org/0000-0002-8623-075X
Tianyu Tao: http://orcid.org/0000-0002-5803-5009
Liping Zeng: http://orcid.org/0000-0001-7115-6836
De Wang: http://orcid.org/0000-0001-8586-4593
Yuhuan Wen: http://orcid.org/0000-0002-6425-7281
Yangyang Li: http://orcid.org/0000-0003-2838-8206
Zhongqiu Zhao: http://orcid.org/0000-0002-6048-345X
Ailin Tao: http://orcid.org/0000-0002-0584-921X

CONFLICT OF INTEREST

The authors state no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: XL, ZZ, AT; Funding Acquisition: AT, XL; Investigation: XL, DW, YW, TT, LZ, YL; Methodology: XL, YW, DW, LZ, TT; Supervision: AT; Writing - Original Draft Preparation: XL, ZZ, AT; Writing - Review and Editing: XL, ZZ, AT

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.09.008.

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