Astrocytic STAT3 activation and chronic itch require $IP_3R1/TRPC$-dependent Ca^{2+} signals in mice

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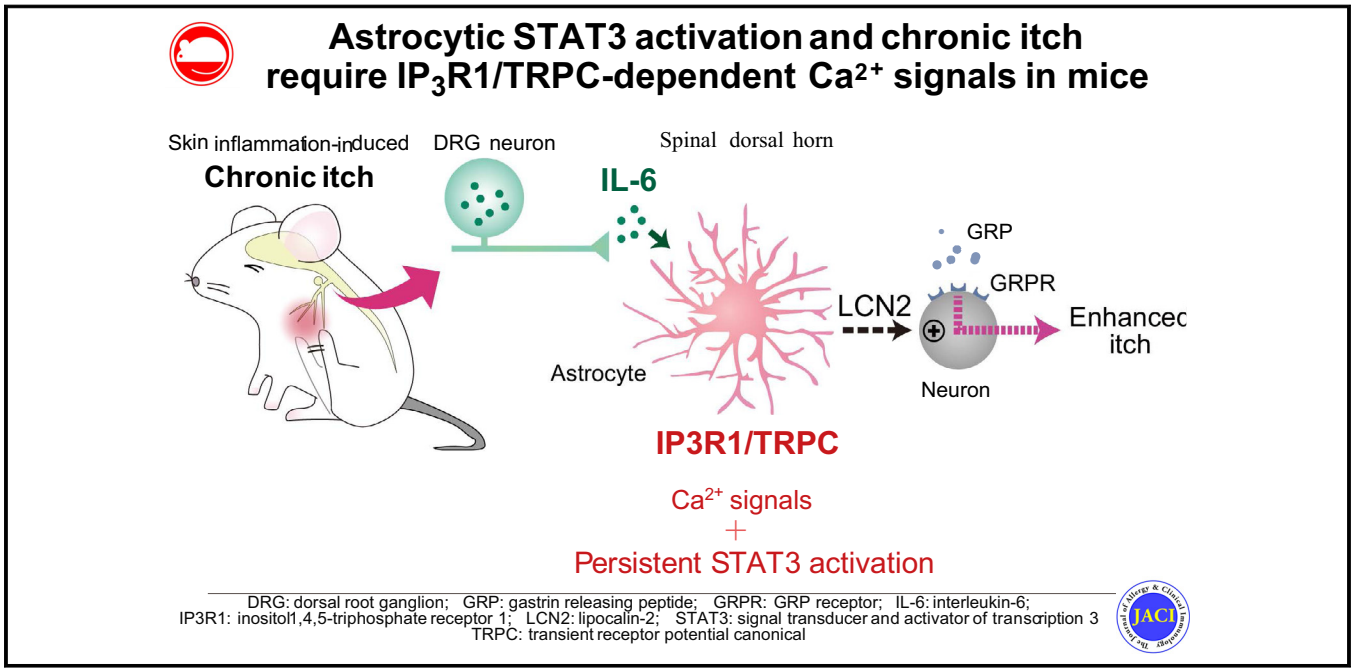
GRAPHICAL ABSTRACT



Background: Chronic itch is a debilitating symptom of inflammatory skin diseases, but the underlying mechanism is poorly understood. We have recently demonstrated that astrocytes in the spinal dorsal horn become reactive in models of atopic and contact dermatitis via activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) and critically contribute to chronic itch. In general, STAT3 is transiently activated; however, STAT3 activation in reactive astrocytes of chronic itch model mice persistently occurs via an unknown mechanism.

Objective: We aimed to determine the mechanisms of persistent activation of astrocytic STAT3 in chronic itch conditions.

Methods: To determine the factors that are required for persistent activation of astrocytic STAT3, Western blotting and calcium imaging with cultured astrocytes or spinal cord slices were performed. Thereafter, chronic itch model mice were used

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for genetic and behavioral experiments to confirm the role of the factors determined to mediate persistent STAT3 activation from *in vitro* and *ex vivo* experiments in chronic itch.

Results: IP_3 receptor type 1 (IP_3R1) knockdown in astrocytes suppressed IL-6–induced persistent STAT3 activation and expression of lipocalin-2 (LCN2), an astrocytic STAT3-dependent inflammatory factor that is required for chronic itch. IP_3R1-dependent astrocytic Ca^{2+} responses involved Ca^{2+} influx through the cation channel transient receptor potential canonical (TRPC), which was required for persistent STAT3 activation evoked by IL-6. IL-6 expression was upregulated in dorsal root ganglion neurons in a mouse model of chronic itch. Dorsal root ganglion neuron–specific IL-6 knockdown, spinal astrocyte–specific IP_3R1 knockdown, and pharmacologic spinal TRPC inhibition attenuated LCN2 expression and chronic itch.

Conclusion: Our findings suggest that IP_3R1/TRPC channel–mediated Ca^{2+} signals elicited by IL-6 in astrocytes are necessary for persistent STAT3 activation, LCN2 expression, and chronic itch, and they may also provide new targets for therapeutic intervention. (J Allergy Clin Immunol 2021;147:1341-53.)

Key words: *Contact dermatitis, chronic itch, astrocytes, STAT3, lipocalin-2, Ca^{2+} signal, IP_3R1, TRPC, IL-6, primary afferent sensory neuron*

Abbreviations used

AAV: Adeno-associated virus
2APB: 2-aminoethoxydiphenylborane
CNS: Central nervous system
DCP: Diphenylcyclopropenone
DRG: Dorsal root ganglion
ESYN: Enhanced synapsin
GRP: Gastrin-releasing peptide
IP_3R: Inositol 1,4,5-triphosphate receptor
IP_3R1: IP_3 receptor type 1
IP_3R2: IP_3 receptor type 2
JAK: Janus kinase
KO: Knockout
LCN2: Lipocalin-2
PYK2: Proline-rich tyrosine kinase 2
Pyr3: Pyrazole-3
SDH: Spinal dorsal horn
shRNA: Short hairpin RNA
siRNA: Small interfering RNA
STAT3: Signal transducer and activator of transcription 3
TRPC: Transient receptor potential canonical
WT: Wild-type

Itch is an unpleasant cutaneous sensation that elicits scratch reflex or a desire to scratch. Generally, itch and subsequent scratching serve as an alarm or a self-defense to harmful substances in the skin. However, in various pathologic settings such as inflammatory skin diseases, the itching becomes chronic, which causes unbearable unpleasant feelings and repetitive scratching-induced skin lesions. Chronic itch negatively affects the quality of life of millions of people worldwide; however, there are few effective treatment options.[1] As a result of discovery of neuronal pathways selective for itch transmission,[2-4] chronic itch is increasingly recognized as an expression of pathologic functioning of the nervous system.[5,6] However, the mechanism underlying chronic itch is poorly understood.

Astrocytes, an abundant cell type of glial cells, have emerged as important players in regulating neuronal excitability in the central nervous system (CNS).[7,8] Under pathologic conditions in the CNS, astrocytes become reactive with changes in gene expression and they produce inflammatory responses[9] that contribute to disease pathology.[8-10] We have recently demonstrated that astrocytes in the spinal dorsal horn (SDH) are activated in models of atopic and contact dermatitis and that reactive astrocytes and their released inflammatory factor lipocalin-2 (LCN2) are necessary for chronic itch.[11,12] The transcription factor signal transducer and activator of transcription 3 (STAT3) plays a pivotal role in the reactive process of astrocytes.[9] Indeed, pharmacologic and genetic inhibition of STAT3 suppresses reactive states of astrocytes in models of chronic itch.[11] STAT3 is transiently activated via phosphorylation by Janus kinases (JAK) following stimulation of cytokine receptors.[13] Astrocytic STAT3 has also been shown to be persistently activated under chronic itch[11] and other pathologic conditions of the nervous system, including spinal cord injury,[14] peripheral nerve injury,[15,16] and Parkinson disease.[17] However, the mechanism underlying persistent activation of STAT3 remains unknown.[9,18]

Astrocytes are nonexcitable cells that use intracellular Ca^{2+} to control their functions, including the release of gliotransmitters such as ATP and glutamate. In pathologic settings such as trauma, Alzheimer disease and epilepsy, reactive astrocytes have increased levels of intracellular Ca^{2+} or exhibit Ca^{2+} oscillations.[19-21] A major driver for intracellular Ca^{2+} mobilization could be inositol 1,4,5-triphosphate receptors (IP_3Rs).[22-24] Among three subtypes of IP_3Rs, type 2 IP_3Rs (IP_3R2s) have been reported to mediate Ca^{2+} signal–dependent gliotransmitter release from astrocytes,[25] and IP_3R2-knockout (IP_3R2-KO) mice have been shown to display a depression-like behavior,[26] reduction of extracellular K^+ uptake, and increases in injury-associated neuronal death.[20,27] However, several studies have also shown that IP_3R2-KO mice exhibit only a partial attenuation of behavioral deficits in a model of brain ischemia[28] or even display no effects on synaptic plasticity, neurovascular coupling, motor and sensory function, anxiety, learning, and memory.[29-31] Furthermore, Ca^{2+} responses in astrocytes lacking IP_3R2s are largely reduced, but subtle, long-lasting Ca^{2+} responses still remain.[32,33] Therefore, these findings suggest the presence of IP_3R2-independent Ca^{2+} signals in astrocytes; however, their mechanism and, importantly, their functional role in CNS health and disease are still uncertain.

In this study, we have shown that IP_3R type 1 (IP_3R1), but not IP_3R2, is critically involved in persistent STAT3 activation in astrocytes and expression of genes related to inflammatory reactive states of astrocytes. Furthermore, IP_3R1-dependent small and long-lasting Ca^{2+} increases in astrocytes were suppressed by inhibitors of transient receptor potential canonical (TRPC) channels, suggesting involvement of an influx of extracellular Ca^{2+} via TRPC channels. Moreover, astrocyte-specific knockdown of IP_3R1 in the SDH or a pharmacologic blockade of spinal TRPC channels in a model of contact dermatitis attenuated LCN2 expression in the SDH and chronic itch–related scratching behavior, a reaction that depends on astrocytic STAT3 and LCN2.[11] Thus, these findings indicate that

IP_3R1/TRPC-dependent Ca^{2+} signals play a crucial role in persistent activation of STAT3, astrocytic LCN2 expression, and chronic itch.

METHODS

Details of the methods used are presented in the Methods section of the Online Repository (in this article's Online Repository at www.jacionline.org).

Animals

C57BL/6J mice (CLEA, Tokyo, Japan and Charles River Japan, Kanagawa, Japan) and IP_3R2-KO mice (kindly provided by Katsuhiko Mikoshiba, MD, PhD) were used. All animal experiments were conducted according to relevant national and international guidelines contained in the Act on Welfare and Management of Animals (Ministry of Environment of Japan) and Regulation of Laboratory Animals (Kyushu University) and under the protocols approved by the Institutional Animal Care and Use Committee review panels at Kyushu University (A19-080/081-0).

Cell culture

Primary cultured astrocytes were prepared as described previously.[11]

Western blotting

As described previously,[11] protein lysates from primary cultured astrocytes and segments C3 to C5 of the SDH were used. Proteins were separated by using SDS-PAGE gel (Bio-Rad, Hercules, Calif). After the transfer, the blots were incubated with primary antibodies. Following incubation, these blots were incubated in horseradish peroxidase–conjugated secondary antibody (1:1000 [GE Healthcare, Madison, Wis]).

RT-PCR

As described previously,[11] total RNA extracts from primary cultured astrocytes and segments C3 to C5 of the dorsal root ganglion (DRG) or SDH were used.

Knockdown with siRNA

Cells were transfected with small interfering RNAs (siRNAs) by using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, Calif) according to the manufacturer's protocol.

Calcium imaging (*in vitro*)

Primary cultured astrocytes were transfected with Lck-GCaMP6m using adeno-associated virus (AAV) (AAV2/9-gfaABC1D-Lck-GCaMP6m-WPRE-polyA; 4×10^{10} viral genomic copies/well). Fluorescent signals were acquired with a confocal laser microscope (LSM 700, Carl Zeiss, Oberkochen, Germany).

Calcium imaging (*ex vivo*)

After microinjection of virus (AAV2/9-gfaABC1D-Lck-GCaMP6m-WPRE-polyA; 8.5×10^{12} viral genomic copies/mL) into the cervical SDH, cervical segments of the spinal cord were removed and used.

A chronic itch model

Diphenylcyclopropenone (DCP) (Wako, Osaka, Japan) was dissolved in acetone. To induce contact dermatitis, mice were shaved on the back and DCP was topically applied by painting 0.2 mL (1% or 2%) on the skin of their back. Seven days after the first painting, DCP was repainted on the same area of skin.

Measurement of IL-6 protein in the DRG

Lysates from segments C3 to C5 of the DRG were analyzed by using a Mouse IL-6 ELISA Kit (R&D Systems, Minneapolis, Minn) according to the manufacturer's protocol.

In vivo primary afferent sensory neuron or astrocyte-specific knockdown with AAV vector encoding shRNA

All recombinant AAV vectors were generated by using pZac2.1-ESYN or gfaABC1D-AcGFP–shRNAmir-WPRE including a targeting sequence for *Il-6* (GTCCTTCAGAGAGATACAGAA) or *Itpr1* (AGGCCTTGGTCT TCTTGGACT) as described previously.[11] For viral injection for primary afferent sensory neuron–specific IL-6 knockdown, 20 μL of virus (1×10^{13} viral genomic copies/mL) was subcutaneously injected into neonatal mice (postnatal day 0-1).[34] For viral injection for astrocyte-specific IP_3R1 knockdown, 0.5 μL of virus (1×10^{12} viral genomic copies/mL) per side was delivered bilaterally to the cervical SDH (near the C4-C6 level, ≲250 μm in depth).

Drug administration to the intrathecal space

Catheterization was performed by the day before the first DCP treatment as described previously.[11] DCP-treated mice were injected with Pyr3 (5 nmol/5 μL [Sigma, St Louis, Mo]) once (at day 10 after the first DCP treatment) or anti-mouse IL-6 antibody (50 ng/5 μL [R&D Systems]) once a day for 3 days (8-10 days after the first DCP treatment) under anesthesia. Recombinant IL-6 (100 ng/5 μL [R&D systems]) was intrathecally injected into intact C57BL/6J mice without anesthesia.

Behavioral studies

Scratching behavior was automatically detected and objectively evaluated by using MicroAct (Neuroscience, Tokyo, Japan) in accordance with a method described previously.[11] Movements of magnets (Neuroscience) implanted into the hind paws were recorded for 24 hours and analyzed by using MicroAct software.

Immunohistochemistry

The segments C3 to C5 of the spinal cord and DRG were fixed by using 4% paraformaldehyde. Spinal cord (30-μm) sections were incubated with primary antibodies. Following incubation, the tissue sections were washed and incubated in secondary antibody solution (Alexa Fluor 546, 1:1000 [ThermoFisher Scientific]). The tissue sections were coverslipped with Vectashield Hardmount (Vector Laboratories, Burlingame, Calif). DRG (15-μm) sections were coverslipped with Vectashield Hardmount. Fluorescent images were obtained with an LSM700 confocal laser microscope (Carl Zeiss).

RNAscope *in situ* hybridization

Segments C3 to C5 of the spinal cord were fixed by using 4% paraformaldehyde. Target mRNAs in spinal cord sections (12-μm) were detected with RNAscope assay (Advanced Cell Diagnostics, ACD, Hayward, Calif) according to the manufacturer's protocols using Advanced Cell Diagnostics probes. Fluorescent images were obtained with an LSM700 confocal laser microscope (Carl Zeiss).

Statistics

Statistical analyses of the results were performed by using the unpaired *t* test, the Mann-Whitney *U* test, 1-way ANOVA *post hoc* Tukey test, 2-way repeated measures ANOVA, and chi-square test (GraphPad Prism7 software, GraphPad Software Inc, San Diego, Calif). Values were considered significantly different at a P value less than .05.

RESULTS

We examined the temporal pattern of STAT3 phosphorylation at Tyr705, an active form of STAT3, in primary cultured astrocytes stimulated by IL-6, a proinflammatory cytokine widely used for STAT3 activation.[13] IL-6 induced strong phosphorylation of STAT3, which peaked at approximately 10

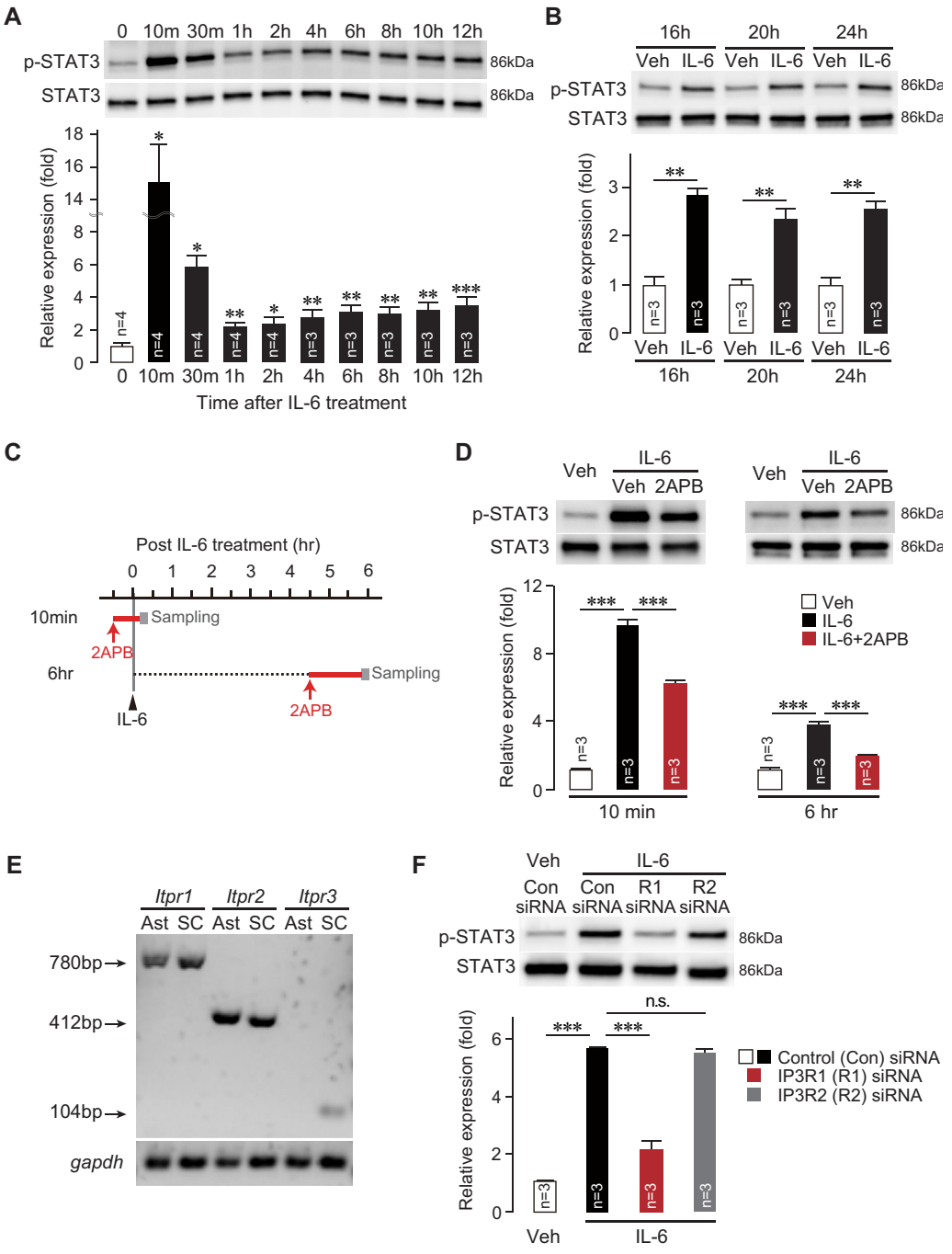

FIG 1. IL-6 induces persistent STAT3 activation via IP_3R1, but not via IP_3R2, in astrocytes. **A** and **B**, Time course of STAT3 phosphorylation after IL-6 treatment in primary cultured astrocytes: 0 minutes (m) to 12 hours (h [also hr]) (**A**) and 16 to 24 hours (n = 3 and 4); 2-tailed Mann-Whitney *U* test (0 and 30 minutes); 2-tailed unpaired *t* test (1-24 hours) (**B**). **C,** Schematic time line for 2APB treatment. **D,** Effects of 2APB (a nonspecific inhibitor for IP_3 receptors) on IL-6–induced STAT3 phosphorylation (n = 3; 1-way ANOVA *post hoc* Tukey test). **E,** PCR analysis of all IP_3R subtypes in primary cultured astrocytes (Ast) or the spinal cord (SC). **F,** Effect of subtype-specific siRNA for IP_3R1 or IP_3R2 on phosphorylation of STAT3 at 6 hours after IL-6 treatment (n = 3; 1-way ANOVA *post hoc* Tukey test). $*P < .05$; $**P < 01$; $***P < .001$. Data are means ± SEMs. *n.s.*, Not significant; *Veh*, vehicle.

minutes after treatment (Fig 1, *A*). STAT3 phosphorylation declined thereafter, but the significantly elevated phosphorylation levels were retained for a long period (Fig 1, *A*), continuing for at least 24 hours after treatment (Fig 1, *B*). To investigate whether Ca^{2+} signaling is necessary for persistent STAT3 activation, we treated cells with 2-aminoethoxydiphenylborane (2APB) (Fig 1, *C*), an inhibitor of IP_3Rs, and found that 2APB suppressed IL-6–induced STAT3 phosphorylation not

only in the early phase (at 10 minutes) but also in the late phase (at 6 hours) (Fig 1, *D*). These data suggest that in addition to transient STAT3 activation in the early phase, IL-6 induces IP_3R-dependent persistent STAT3 activation that lasts for a long time after stimulation.

We determined the IP_3R subtype that mediates IL-6–induced persistent STAT3 activation. In primary cultured astrocytes, mRNAs for IP_3R1 and IP_3R2, but not for IP_3R type 3, were highly expressed (Fig 1, *E*). A similar result was obtained in extracts from the mouse SDH (Fig 1, *E*). Using IP_3R subtype-specific siRNAs that selectively knocked down expression of each IP_3R type (see Fig E1 in this article's Online Repository at www.jacionline.org), we found that STAT3 phosphorylation at 6 hours after IL-6 stimulation was inhibited by IP_3R1 siRNA but not by IP_3R2 siRNA (Fig 1, *F*). IP_3R1 siRNA also inhibited STAT3 phosphorylation at 10 minutes after IL-6 treatment (see Fig E2 in this article's Online Repository at www.jacionline.org). These results suggest that IP_3R1 critically contributes to the persistent activation of STAT3 in IL-6–stimulated astrocytes.

Because STAT3 regulates expression of the inflammatory factor LCN2 that is crucial for chronic itch,[9,11] we examined whether IP_3R1-dependent persistent STAT3 activation influences astrocytic LCN2 expression. We found that IL-6 changed the levels of LCN2 mRNA at 24 hours after treatment and most of these changes were prevented by IP_3R1 siRNA (Fig 2, *A*). STAT3 has also been implicated in expression of other genes related to reactive astrocytes. The levels of mRNA of these genes (eg, *Steap4*, *S1pr3*, and *Timp1*)[35] in IL-6–stimulated astrocytes were also reduced by IP_3R1 siRNA treatment (Fig 2, *B-K*). Therefore, IP_3R1 substantially contributes to the regulation of expression of LCN2 and other genes associated with reactive states of astrocytes.

Previous studies using smooth muscle or immune cells have shown that the IP_3R1-dependent intracellular Ca^{2+} increase also involves an influx of extracellular Ca^{2+} through ion channels (such as TRPC or Orai1 channels).[36-38] To examine the role of these channels in persistent activation of STAT3 in IL-6–stimulated astrocytes, we tested the effect of inhibitors of TRPC (SKF96365 and pyrazole-3 [Pyr3]) and Orai1 (lanthanum) channels. The persistent phosphorylation of STAT3 was inhibited only by TRPC inhibitors (Fig 3, *A*). Because astrocytes have been reported to express several subtypes of TRPC channels (TRPC1, TRPC3, TRPC6, and TRPC 7),[39,40] we treated astrocytes with siRNA for each TRPC channel subtype and found significant suppression of IL-6–induced persistent STAT3 phosphorylation by either TRPC3 or TRPC7 siRNA (Fig 3, *B*). We also confirmed that extracellular Ca^{2+} was required for STAT3 phosphorylation in IL-6–stimulated astrocytes using 1,2-bis(*o*-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid, a chelator of extracellular Ca^{2+} (Fig 3, *C*). These results suggest that IL-6–induced persistent STAT3 activation in astrocytes involves Ca^{2+} signals via an IP_3R1-TRPC3/7 pathway.

To examine astrocytic Ca^{2+} responses via the IP_3R1-TRPC3/7 pathway, we performed Ca^{2+} imaging in primary cultured astrocytes. Because TRPCs are expressed on cell surfaces,[37] we monitored near-membrane Ca^{2+} events by using the membrane-tethered Ca^{2+} indicator Lck-GCaMP6m.[41] In addition, to omit a component of IP_3R2-dependent Ca^{2+} responses, we used primary cultured astrocytes prepared from IP_3R2-KO mice. IL-6 produced small and prolonged Ca^{2+} responses in IP_3R2-KO astrocytes (Fig 4, *A*) and increased the percentage of responding cells (Fig 4, *B*). However, the percentage of responding cells in wild-type (WT) astrocytes was indistinguishable between the IL-6– and vehicle-treated groups (see Fig E3, *A* and *B* in this article's Online Repository at www.jacionline.org), which is consistent with the previous findings.[32] Next, we examined the effects of 2APB and Pyr3 on the IL-6–induced Ca^{2+} responses in IP_3R2-KO astrocytes and found that the number of responsive cells induced by IL-6 was decreased by 2APB and Pyr3 (Fig 4, *C*). Moreover, siRNAs for IP_3R1, TRPC3, and TRPC7 also reduced the percentage of IL-6–induced responsive cells compared with the control siRNA (Fig 4, *D*). Furthermore, using spinal cord slices from adult IP_3R2-KO mice with astrocytes expressing Lck-GCaMP6m (see Fig E4 in this article's Online Repository at www.jacionline.org),[42] we also observed small and prolonged astrocytic Ca^{2+} responses after IL-6 treatment in the SDH (Fig 4, *E* and *F*), which were inhibited by 2APB and Pyr3 (Fig 4, *G* and *H*). These observations strongly suggest that persistent Ca^{2+} responses in IL-6–stimulated astrocytes involve the IP_3R1-TRPC3/7 pathway.

We have previously demonstrated that astrocytic STAT3 is persistently activated in the SDH of mouse models of dermatitis, which is required for LCN2 upregulation in SDH astrocytes and chronic itch.[11] Thus, to investigate the *in vivo* role of the IP_3R1-TRPC3/7 pathway under chronic itch, we used a mouse model of contact dermatitis developed by treatment with DCP (Fig 5, *A* and *B*). First, we examined the role of IL-6 and found that expression of IL-6 mRNA was elevated in the DRG but not in the SDH (Fig 5, *C*). The protein level of IL-6 was also increased in the DRG (Fig 5, *D*). Because the DRG contains the cell bodies of primary afferent sensory neurons and their neuronal axons project to the SDH, we predicted that DRG neuron–derived IL-6 participates in chronic itch. Intrathecal injections of a neutralizing antibody against IL-6 suppressed scratching behavior of DCP-treated mice (Fig 5, *E*). Furthermore, we generated mice with selective knockdown of IL-6 in primary afferent sensory neurons by systemic injection of an AAV vector that allows expression of short hairpin RNA (shRNA) targeting IL-6 under the control of the neuronal promoter enhanced synapsin (ESYN) into neonatal mice. In adult mice injected with AAV, AcGFP was expressed in the soma of DRG neurons (Fig 5, *F*) and their axon terminals in the SDH but not in SDH neurons (see Fig E5 in this article's Online Repository at www.jacionline.org)[34]; IL-6 level was reduced in the DRG (Fig 5, *G*). We found that these mice displayed suppression of the DCP-induced scratching behavior and expression of LCN2 in the SDH (Fig 5, *H* and *I*), indicating a pivotal role of primary afferent–derived IL-6 in astrocytic LCN2 expression and chronic itch.

Next, we examined expression of IP_3R1 in the SDH (Fig 6, *A*). IP_3R1 immunofluorescence in the SDH was primarily observed in cells positive for glial fibrillary acidic protein, a marker of astrocytes, whereas only a few IP_3R1-positive cells were double-stained by neuronal nuclei, a neuronal marker (Fig 6, *A*). To determine the role of IP_3R1, we knocked down IP_3R1 expression specifically in astrocytes in the SDH by microinjecting an AAV vector expressing an IP_3R1-shRNA under control of the astrocytic promoter gfaABC$_1$D into the SDH (Fig 6, *B* and *C*).[11] In these mice with SDH astrocyte–specific knockdown of IP_3R1, the DCP-induced scratching behavior, the numbers of SDH astrocytes with nuclear localized STAT3 (an index of STAT3 activation), and LCN2 expression in the SDH were inhibited (Fig 6,

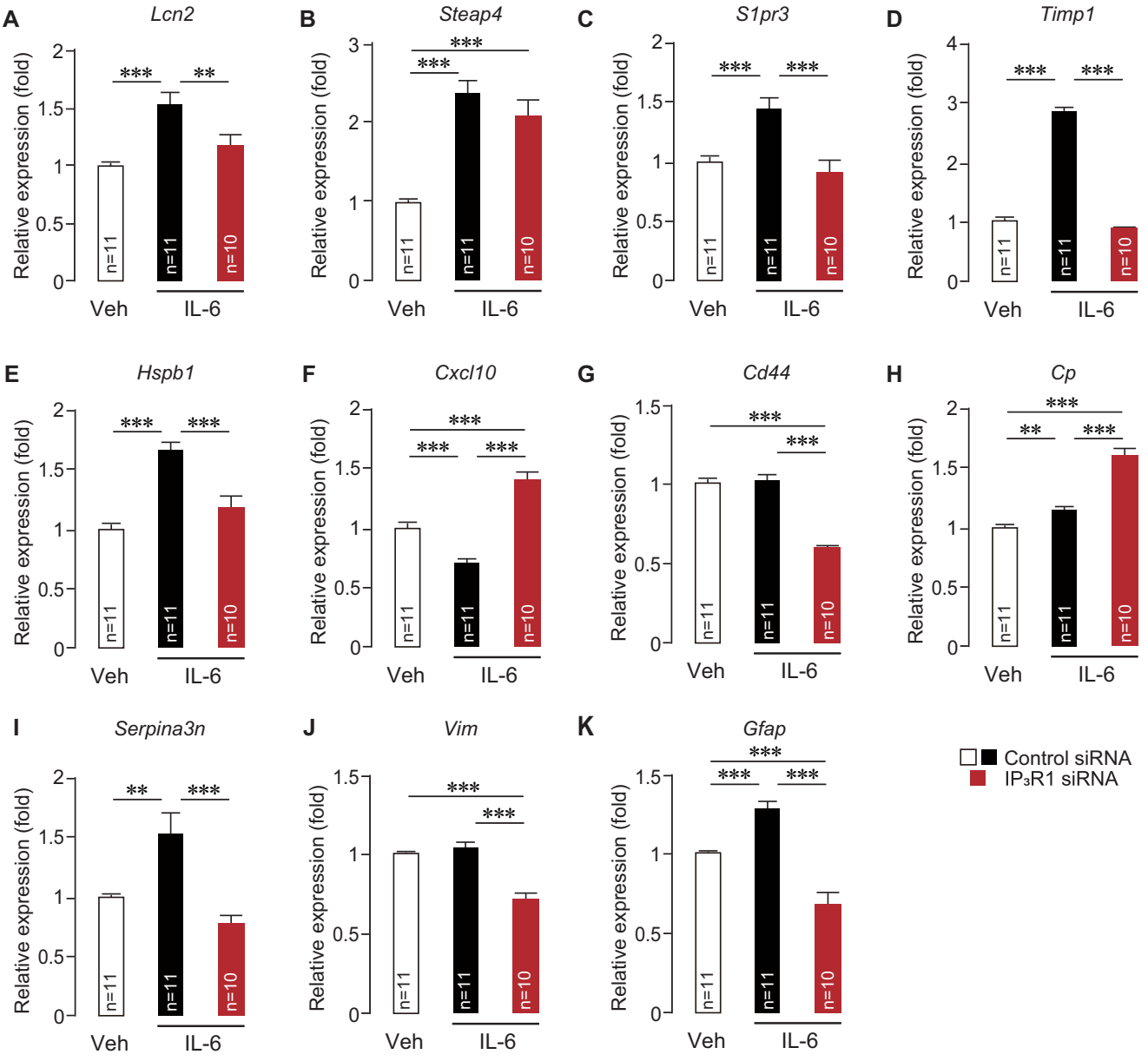

FIG 2. IP_3R1 is involved in expression of genes related to reactive states of astrocytes. **A-K,** Effect of IP_3R1 siRNA on IL-6–induced change in expression of mRNA of reactive astrocyte-related genes in primary cultured astrocytes (n = 10 and 11; 1-way ANOVA *post hoc* Tukey test). Total RNA extracted from primary cultured astrocytes 24 hours after IL-6 treatment was subjected to real-time PCR analysis. $^{**}P < .01$; $^{***}P < .001$. Data are means ± SEMs. *Veh*, Vehicle.

D-F). In contrast, the loss of IP_3R2 had no effect on DCP-induced scratching and LCN2 upregulation compared with in WT mice (see Fig E6 in this article's Online Repository at www.jacionline.org). We also found that a single intrathecal injection of the TRPC3 inhibitor Pyr3 suppressed both scratching behavior and expression of LCN2 in the SDH of DCP-treated mice (Fig 6, *G* and *H*). Together, these results suggest that astrocytic IP_3R1 and TRPC channels contribute to LCN2 upregulation in the SDH and chronic itch, both of which require IL-6 derived from DRG neurons and STAT3 activation in SDH astrocytes.[11]

DISCUSSION

Astrocytic Ca^{2+} signals have been shown to elicit release of gliotransmitters such as ATP and glutamate, which leads to regulation of neuronal excitability.[23,24,43] Because Ca^{2+} responses by activation of G_q protein–coupled receptors such as metabotropic ATP receptors and glutamate receptors were largely reduced in IP_3R2-KO astrocytes,[25,31] IP_3R2 has been considered to be a major determinant for astrocytic functions. However, recent studies have shown that Ca^{2+} events are still observed in astrocytes lacking IP_3R2, suggesting the presence of an IP_3R2-independent

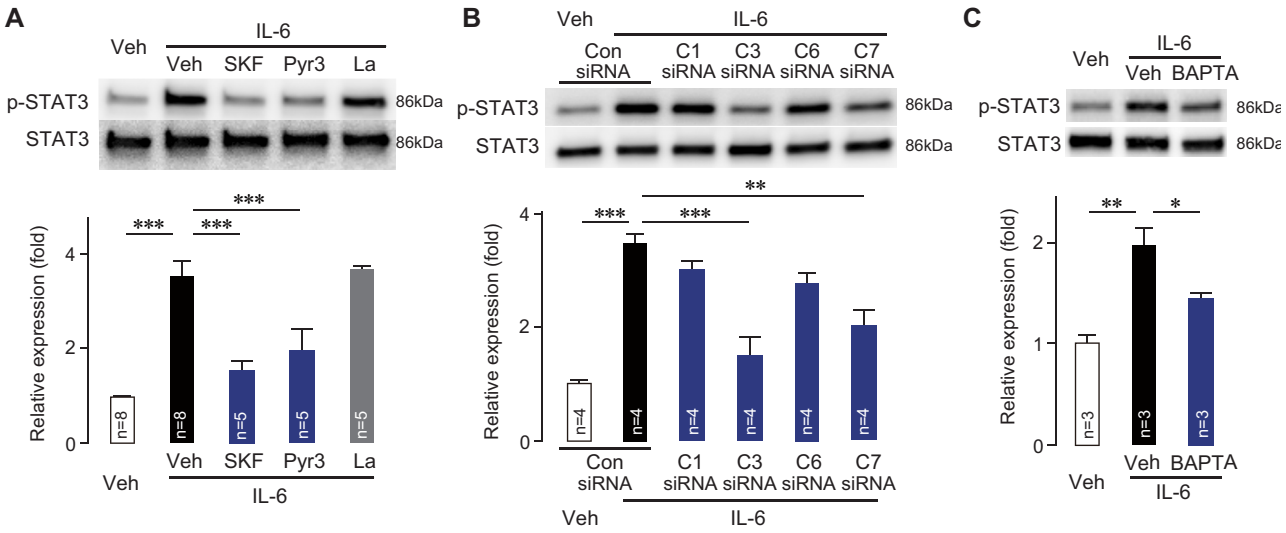

FIG 3. Ca^{2+} influx through TRPC channels is necessary for IP_3R1-dependent STAT3 activation. **A,** Effect of inhibitors for TRPC (SKF, Pyr3) or Orai (La [lanthanum]) channels on phosphorylation of STAT3 at 6 hours after IL-6 treatment (n = 5 and 8; 1-way ANOVA *post hoc* Tukey test). **B,** Effect of subtype-specific siRNA for TRPC channels (C1, C3, C6, and C7) on phosphorylation of STAT3 at 6 hours after IL-6 treatment (n = 3; 1-way ANOVA *post hoc* Tukey test). **C**, Effect of 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) (5 mM), an extracellular Ca^{2+} chelator, on phosphorylation of STAT3 at 6 hours after IL-6 treatment (n = 3; 1-way ANOVA *post hoc* Tukey test). $*P < 05$; $**P < .01$; $***P < .001$. Data are means ± SEMs. *Con*, Control; *Veh*, vehicle.

mechanism.[32,33,44,45] In this study, we demonstrated that IP_3R1 is crucial for both IL-6–induced persistent activation of STAT3 and expression of genes associated with reactive astrocytes. This is supported by our and previous findings showing the presence of IP_3R1s in astrocytes[46] and IP_3R1-dependent astrocytic Ca^{2+} responses. Although IP_3R1 knockdown also induced abnormal changes in some genes such as *Vim, Cxcl10* and *Cp*, it may involve other signals that are regulated by IP_3R1-dependent Ca^{2+} (eg, calcineurin and nuclear factor of activated T cells[47]). More importantly, we provide evidence indicating that astrocytic IP_3R1 critically contributes to behavioral and molecular alterations associated with chronic itch by demonstrating that astrocyte-specific IP_3R1 knockdown suppressed scratching behavior and LCN2 upregulation in a contact dermatitis model. Therefore, it is conceivable that IP_3R1-dependent Ca^{2+} signals induce astrocyte reactivity by regulating transcriptional activities via STAT3 activation, which in turn contributes to chronic itch. A pivotal role of IP_3R1 seems to be highlighted by our further findings showing no effect of IP_3R2 siRNA on IL-6–induced STAT3 activation and no alterations in chronic itch and LCN2 upregulation in IP_3R2-KO mice compared with in WT mice. Therefore, our results provide *in vivo* evidence for a distinct role of each IP_3R subtype in astrocytes. This is supported by recent studies showing that sleep is normal in IP_3R2-KO mice[26] but is disrupted by inhibition of IP_3R-induced astrocytic Ca^{2+} release by IP_3 5-phosphatase, an IP_3 hydrolyzing enzyme.[48]

IP_3R1- and IP_3R2-dependent intracellular Ca^{2+} dynamics were also different in astrocytes. Whereas IP_3R2-dependent Ca^{2+} signals are transient and strong,[32] IP_3R1-dependent signals were long-lasting, weak, and mostly masked by IP_3R2-dependent Ca^{2+} events in WT astrocytes (Fig 4, *A* and see Fig E3, *A*). The reason for this difference in Ca^{2+} dynamics remains unclear; however, the difference may be caused by an interaction between IP_3R1 and surface-localized channels such as TRPC channels. By using coimmunoprecipitation and immune fluorescence resonance energy transfer, previous studies have demonstrated that IP_3R1 physically and functionally interacts with TRPC3 in smooth muscle cells.[36,49] Functionally, activation of IP_3R1 induces Ca^{2+} influx via TRPC3, which has been implicated in the production of persistent constriction in cerebral arteries.[36,49] In addition, as supported by an ability of TRPC3 to form a complex with TRPC7,[50] this study showed that knockdown of either TRPC3 or TRPC7 had an inhibitory effect on IP_3R1-dependent Ca^{2+} responses. Therefore, it is possible that a putative interaction between IP_3R1 and TRPC3/7 may be critically involved in the persistent activation of STAT3 in IL-6-stimulated astrocytes. Furthermore, our *in vivo* data obtained from mice with a specific knockdown of IP_3R1 in SDH astrocytes and with intrathecal injection of Pyr3 emphasize that astrocytic IP_3R1 and TRPC channels contribute to STAT3 activation and LCN2 upregulation in SDH astrocytes and chronic itch. Although intrathecally injected Pyr3 can target TRPC3 not only in spinal cells but also in DRG cells, *Trpc3* mRNA expression in SDH astrocytes (see Fig E7 in this article's Online Repository at www.jacionline.org) and our *in vitro* data obtained by using primary cultured astrocytes (Fig 3), which included a marked suppression of IL-6–evoked LCN2 production by TRPC3 knockdown (see Fig E8 in this article's Online Repository at www.jacionline.org), strongly support our view. However, it should be noted that *Trpc3* mRNA was detected in microglia (see Fig E7) and DRG neurons.[51,52] Although activation of microglia is not observed in the SDH of chronic itch models,[11,53] the role of TRPC3 in SDH microglia and DRG neurons in astrocytic responses and chronic itch cannot be excluded. This will be clarified by further investigation using cell type–specific TRPC3-knockout mice.

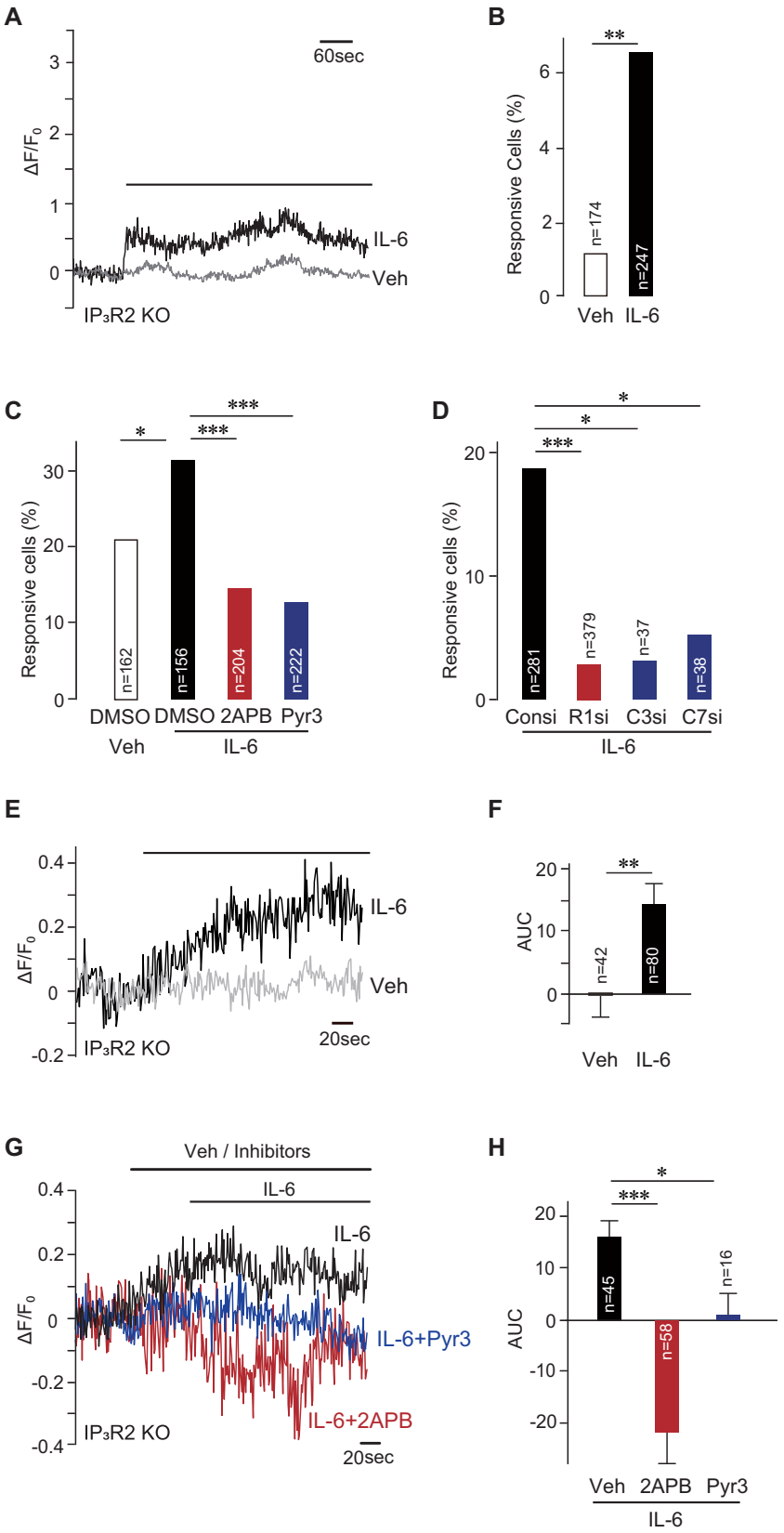

FIG 4. IL-6 evokes long-lasting and subtle Ca^{2+} signals via IP_3R1 in astrocytes. **A,** Representative Ca^{2+} responses to IL-6 or vehicle (Veh) in IP_3R2-KO primary cultured astrocytes transfected with Lck-GCaMP6m. **B,** Percentages of cells showing persistent Ca^{2+} responses after vehicle or IL-6 treatment (n = 174 and 247; 2-sided chi-square test). **C,** Effect of inhibitors of IP_3Rs (2APB) or TRPC3 (Pyr3) on the percentage of cells showing persistent Ca^{2+} responses after IL-6 treatment (n = 156, 162, 204, and 222; 2-sided chi-square test). **D,** Effect of siRNA targeting IP_3R1, TRPC3, or TRPC7 on the percentages of cells showing persistent Ca^{2+}

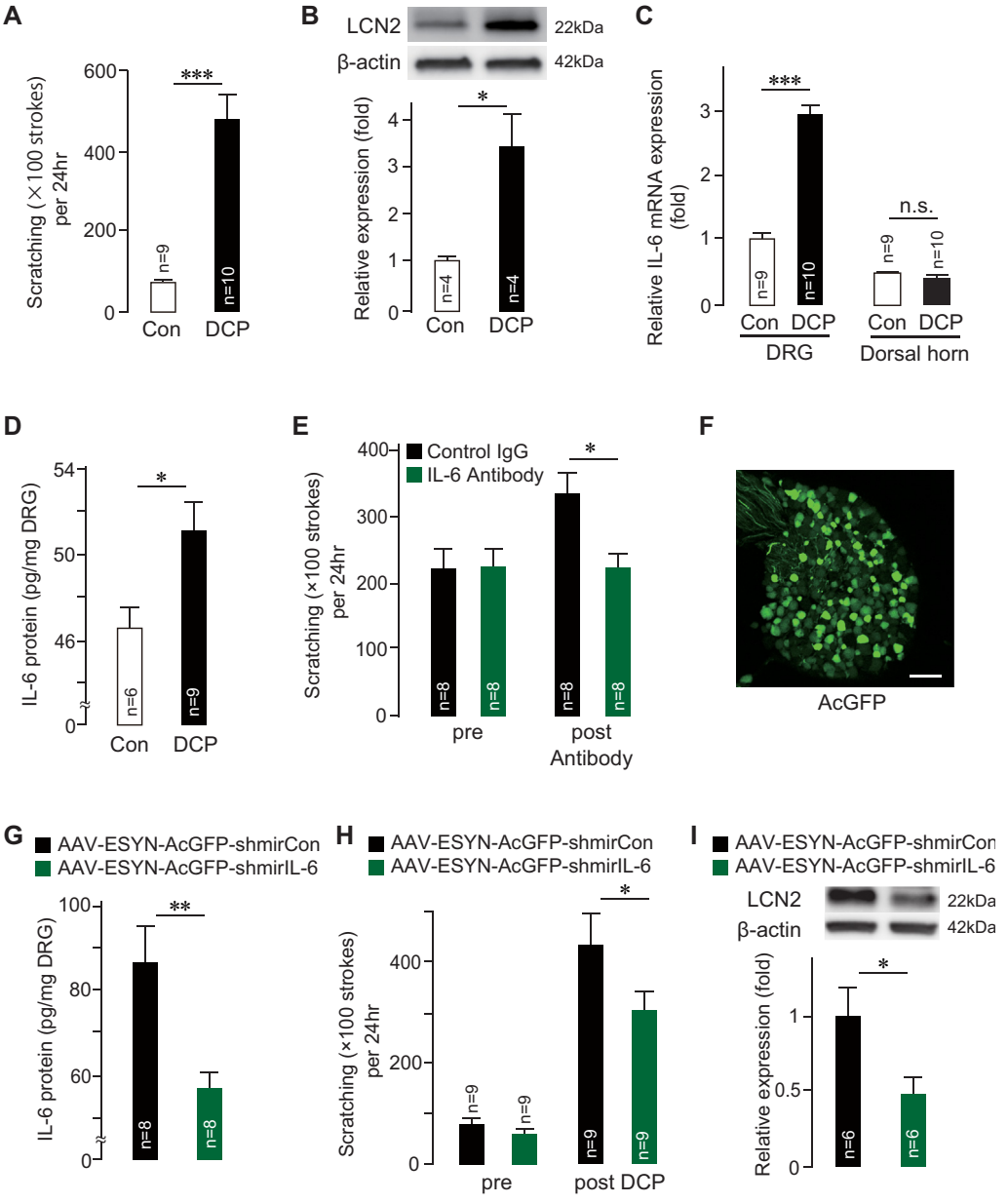

FIG 5. DRG neuron–derived IL-6 elicits upregulation of LCN2 in the SDH and aggravates chronic itch. **A,** Scratching during the course of 24 hours (hr) in DCP-treated mice (n = 9 and 10; 2-tailed Mann-Whitney *U* test). **B,** LCN2 protein, an astrocytic STAT3-dependent factor, in the cervical SDH of DCP-treated mice (n = 4; 2-tailed Mann-Whitney *U* test). **C,** *Il6* mRNA in the cervical DRG or spinal cord (n = 9 and 10). **D,** IL-6 protein in the cervical DRG (n = 6 and 9; 2-tailed unpaired *t* test). **E,** Effect of repeated intrathecal injection of IL-6–neutralizing antibody on DCP-induced scratching behaviors (before, day 7; after, day 10; n = 8; 2-way repeated measures ANOVA). **F,** Fluorescence of green fluorescent protein (GFP) in the DRG (*green*) of mice injected with AAV-ESYN-AcGFP-shmirControl (Con). Scale bar = 100 μm. **G,** IL-6 protein in the cervical DRG after injection of AAV-ESYN-AcGFP-shmirCon or IL-6 (n = 8; 2-tailed unpaired *t* test; $P = .0012$). **H,** Scratching in DCP-treated mice with knockdown of IL-6 in DRG neurons (n = 9; 2-way repeated measures ANOVA). **I,** LCN2 protein in the cervical SDH of DCP-treated mice with knockdown of IL-6 in DRG neurons (n = 6; 2-tailed unpaired *t* test). *$P < .05$; **$P < .01$; ***$P < .001$. Data are means ± SEMs. *Shmir*, miRNA-embedded shRNA.

responses after IL-6 treatment (n = 37, 38, 281, and 379; 2-sided chi-square test). **E,** Representative Ca^{2+} responses to IL-6 or vehicle in Lck-GCaMP6m–expressing astrocytes of IP_3R2-KO spinal cord slices. **F,** Area under the curve (AUC) of Ca^{2+} responses to IL-6 or vehicle in astrocytes of IP_3R2-KO spinal cord slices (n = 42 and 80; 2-tailed unpaired *t* test). **G** and **H,** Effect of IP_3R (2APB) or TRPC3 (Pyr3) inhibitors on IL-6–induced Ca^{2+} responses in Lck-GCaMP6m–expressing astrocytes of IP_3R2-KO spinal cord slices (representative Ca^{2+} responses [**G**]; AUC of Ca^{2+} responses [**H**]; n = 16, 45, and 56; 2-tailed Mann-Whitney *U* test). *$P <$ .05; **$P < .01$; ***$P < .001$. Data are means ± SEMs. *Con*, Control; *Consi*, control siRNA; *DMSO*, dimethyl sulfoxide; *sec*, second.

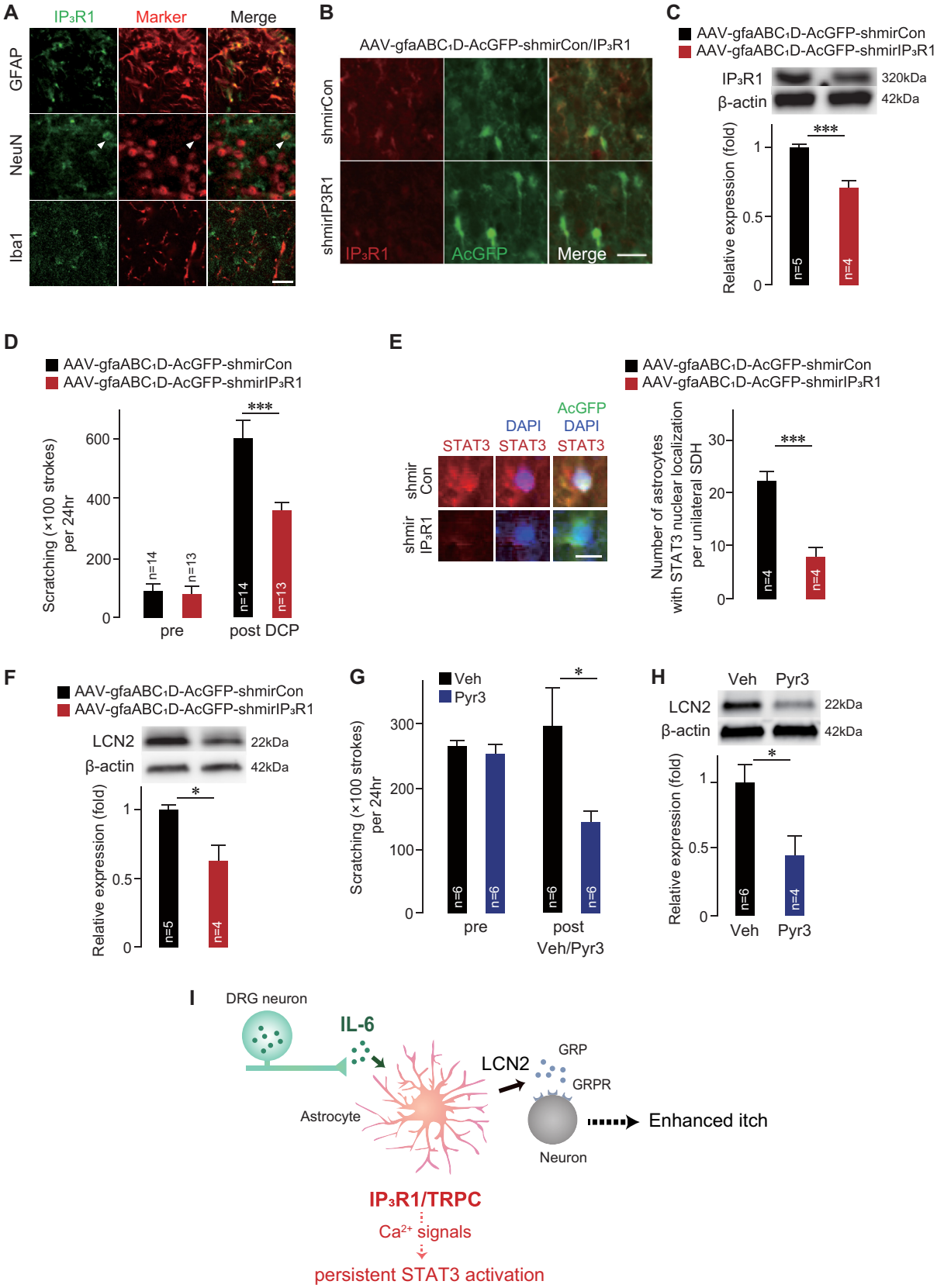

FIG 6. IP_3R1 and TRPC channels are required for LCN2 upregulation and chronic itch. **A,** Immunofluorescence of IP_3R1, glial fibrillary acidic protein (GFAP) (an astrocytic marker), neuronal nuclei (NeuN) (a neuronal marker), and Iba1 (a microglial marker) in the SDH. An arrowhead indicates IP_3R1 signals

STAT3 is generally known to be transiently activated via direct phosphorylation by JAK following activation of IL-6 family cytokine receptors.[13] However, this study revealed a novel mechanism for STAT3 activation involving IP_3R1-dependent Ca^{2+} signals. In general, IP_3Rs are activated by IP_3, which is increased with activation of G_q-coupled receptors. However, IL-6 receptors are not coupled to G_q proteins. Whether IL-6–stimulated astrocytes increase the level of IP_3 remains unclear, but considering previous findings indicating that IP_3R1 has several phosphorylation sites, some of which enhance the activity of IP_3R1 by phosphorylation,[54] it is conceivable that IL-6 may induce phosphorylation of IP_3R1 via intracellular protein kinases downstream of IL-6 receptors such as JAK, mitogen-activated protein kinases, and phosphatidylinositol-3-kinase/Akt and may enhance activity of IP_3R1.[55] The mechanism underlying how Ca^{2+} signals activate STAT3 remains to be determined, but proline-rich tyrosine kinase 2 (PYK2), a nonreceptor tyrosine kinase that is activated in a Ca^{2+}-dependent manner, has been shown to mediate STAT3 activation in multiple tumor cell types,[56-58] suggesting possible involvement in IP_3R1-dependent STAT3 activation in astrocytes.

Our study identified IL-6 in DRG neurons as an activator of SDH astrocytes under chronic itch conditions. The selective increase in IL-6 expression in the DRG but not in the SDH of DCP-treated mice and the suppression of astrocytic LCN2 upregulation and chronic itch by DRG-specific knockdown of IL-6 raise the possibility that the increased IL-6 protein in the cell bodies of DRG neurons is axonally transported to their terminals in the SDH, which in turn activates the IP3R1/TRPC-Ca^{2+}-STAT3 signaling pathway to upregulate LCN2 in SDH astrocytes and contributes to chronic itch (Fig 6, *I*). Although we could not detect IL-6 protein in the SDH by ELISA-based assay (data not shown) (which might be due to the difficulty in detecting presynaptic IL-6), intrathecal injection of IL-6 in naive mice induced astrocytic STAT3 activation and LCN2 upregulation (see Fig E9, *A* and *B* in this article's Online Repository at www.jacionline.org), indicating that spinal IL-6 is sufficient to induce these 2 astrocytic responses *in vivo*. In contrast, intrathecal IL-6 failed to produce scratching behavior (see Fig E9, *C*). However, this is not surprising because our previous studies have shown that mice expressing a constitutive active form of STAT3 in spinal astrocytes do not exhibit spontaneous itch-like behaviors (biting and scratching)[16] and that LCN2 alone does not induce scratching (when given intrathecally in naive mice)[11] and has no effect on basal excitability of itch-transmission SDH neurons.[12] Given that LCN2 is capable of enhancing both neuronal excitation and scratching behavior evoked by gastrin-releasing peptide[11,12] (GRP), which is an itch inducer in the SDH,[2,3] it seems likely that spinal IL-6 or astrocytic STAT3 activation might produce scratching behavior in a condition wherein spinal GRP-GRP receptor signaling is active.[11] In addition, a recent study reported that spinal IL-33 is involved in activation of STAT3 in SDH astrocytes in a model of chronic itch associated with allergic contact dermatitis.[59] Because IL-33 has been reported to induce production of IL-6,[60,61] IL-33 may also play a role in IP_3R1-dependent astrocytic STAT3 activation via IL-6 production under chronic itch conditions.

In the present study, by using a mouse model of dermatitis, we demonstrated a new mechanism whereby astrocytic STAT3 activation and chronic itch require IP_3R1/TRPC-dependent Ca^{2+} signals. The relevance of the mouse model for chronic itch in human atopic dermatitis remains unclear, but as an itch-scratch vicious cycle that is a critical component of chronic itch seems to be a commonly observed phenomenon in mouse models and in patients with inflammatory skin disease, including those with atopic dermatitis,[11,62] the implications of our findings would provide valuable information regarding the mechanism underlying chronic itch associated with atopic dermatitis. Furthermore, STAT3 activation in astrocytes is also observed in many CNS conditions, such as trauma, multiple sclerosis, Alzheimer disease, and Parkinson disease. In many cases, the activation appears to be long-lasting.[9] Moreover, in addition to the chronic itch model used here, IL-6 has been shown to be upregulated in various brain regions under neuropathologic conditions.[63,64] Furthermore, mutant huntingtin, which induces astrocytic STAT3 activation, causes abnormal activity of IP_3R1.[65,66] According to these findings, it is possible that IP_3R1/TRPC-dependent persistent STAT3 activation in astrocytes occurs not only in chronic itch but also in many CNS diseases, which may shed new light on our understanding of mechanisms underlying the reactive astrocyte-dependent neuroinflammation that plays a critical role in CNS pathologic conditions, including chronic itch, and may provide a potential therapeutic target.

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colocalized with NeuN signals. Scale bar = 10 μm. **B,** Immunofluorescence of IP_3R1 (*red*) and fluorescence of green fluorescent protein (GFP) in the SDH of DCP-treated mice injected with AAV-gfaABC$_1$D-AcGFP-shmirCon or IP_3R1. Scale bar = 20 μm. **C**, IP_3R1 protein in the SDH of DCP-treated mice injected with AAV-gfaABC$_1$D-AcGFP-shmirCon or IP_3R1 (n = 4 and 5; 2-tailed unpaired *t* test; $P = .0001$). **D,** Scratching in DCP-treated mice with SDH astrocyte–specific knockdown of IP_3R1 (n = 13 and 14; 2-way repeated measures ANOVA). **E**, Immunofluorescence of STAT3, DAPI, and GFP fluorescence (*left*) and the number of astrocytes with nuclear localized STAT3 (*right*) (n = 4; 2-tailed unpaired *t* test; $P = .0005$) in the SDH of DCP-treated mice injected with AAV-gfaABC$_1$D-AcGFP-shmirCon or IP_3R1. Scale bar = 10 μm. **F**, LCN2 protein in the cervical SDH of DCP-treated mice with SDH astrocyte–specific knockdown of IP_3R1 (n = 4 and 5; 2-tailed unpaired *t* test). **G** and **H,** Effect of intrathecal treatment with Pyr3 on scratching in DCP-treated mice (**G**) (n = 6; 2-way repeated measures ANOVA) and the expression level of LCN2 protein in the SDH of DCP-treated mice (**H**) (n = 4 and 6; 2-tailed unpaired *t* test). **I**, Schematic illustration of the mechanisms of STAT3 activation in the SDH astrocytes under chronic itch conditions. $*P < .05$; $**P < 01$; $***P < 001$. Data are means ± SEMs. *hr*, Hour; *Veh*, vehicle.

Key messages

- **IL-6 induces persistent activation of STAT3 and upregulation of LCN2, an astrocytic STAT3-dependent factor that is necessary for chronic itch, via IP_3R1/TRPC-dependent Ca^{2+} signals in astrocytes.**
- **Primary afferent sensory neuron–derived IL-6 and IP_3R1/TRPC-dependent Ca^{2+} signals in astrocytes play a pivotal role in chronic itch via upregulation of LCN2 in the SDH.**

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