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Differential contribution of sensory transient receptor potential channels in response to the bioactive lipid sphingosine-1-phosphate

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Abstract

Somatosensation encompasses a wide range of sensations including pain, itch, touch, and temperature and is essential for the detection of environmental stimuli, ultimately allowing an organism to escape, communicate, and adapt to its environment. Such sensations are detected by primary sensory neurons whose nerve endings are located in the skin. Compared to external stimuli, mechanisms underlying endogenous stimulation of primary sensory neurons, such as by lipids, are still largely unknown. Here, we focus on one of the endogenous bioactive lipids, sphingosine-1-phosphate (S1P), to investigate the physiological roles of S1P in pain and itch. We showed that S1P-induced calcium responses in sensory neurons through S1P receptors. Transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1) are nonselective calcium-permeable ion channels that are known to be involved in pain and itch. Neurons that respond to S1P show reduced responsiveness when treated with antagonists that block either TRPA1 or TRPV1 alone or in combination. In addition, using single and double knockout mice (TRPA1; TRPV1; TRPA1/TRPV1) with loss of function of these channels, we demonstrated that both TRP channels are involved in S1P-induced neuronal responses in vitro. Next, we examined the effects of S1P on pain and itch responsiveness in freely behaving mice post-S1P injection into the cheek, neck, and hind paw. Our findings reveal that S1P induces both pain and itch in vivo and that these responses are partially dependent upon the TRPV1, but not TRPA1 channels.

Keywords

Itch, pain, sphingosine 1-phosphate, TRPA1, TRPV1

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Introduction

Pain and itch are unpleasant sensations that are processed by a complex system of sensory neurons whose afferents innervate the skin.[1] While pain sensation initiates withdrawal responses to protect the body from potentially damaging thermal, mechanical, and chemical stimuli, itch evokes a desire to incite damage through scratching the skin.[2,3] The signals encoding both pain and itch are initiated at primary sensory neurons by endogenous and exogenous mediators or stimuli.[4] Endogenous mediators of pain include bioactive molecules produced by certain cancers (bone cancer), prostaglandin (PGE_2) produced during inflammation and H^+ ions released from tissue injury,[1,4–10] whereas exogenous mediators of pain originate from certain chemicals of plant origin, and several toxins.[11,12] Similarly, itch associated with skin diseases like atopic dermatitis and psoriasis is triggered by certain

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endogenous cytokines and biogenic amines,[13–18] while exogenous mediators of itch may include insect bites or side effects of drugs like chloroquine that is used to treat malaria.[2,19]

It has been challenging to uncover the mechanisms that govern how lipids control these behaviors due to the wide variety of molecular species and their complex metabolic profiles. However, some bioactive lipids derived from the plasma membrane through arachidonate metabolism cascades have been well characterized, and there are drugs targeting these metabolic pathways currently being used in the clinical setting to treat pain.[20,21] However, the physiological roles and mechanisms of action on sensory neurons of the by-products of the first step of this metabolic cascade, lysophospholipids (LPLs), remain mostly unknown. The physiological role of one of the two major LPLs,[22] lysophosphatidic acid (LPA), was recently reported for pain and itch.[23,24] The other major LPL, sphingosine-1-phosphate (S1P), is produced and released from red blood cells, platelets,[25,26] and endothelial cells[25,27] and plays a vital role in various physiological events and diseases.[25] Recently, it is shown that S1P induced pain behavior in wild-type (WT) mice, which is substantially reduced in $S1P_3$ receptor subtype knockout (KO) mice.[28] In the same study, authors have shown that S1P evoked a small excitatory current resulted in nociceptor depolarization and action potential firing. Lastly, no alterations in both in vitro and in vivo responses to S1P have shown using conditional $S1P_1$ floxed mice suggesting acute activation of nociceptive nerve terminals by S1P receptor subtype $S1P_3$. In a separate research lately, other researchers have shown the role of S1P in both itch and pain in a concentration-dependent manner with low doses triggering itch and high doses triggering both itch and pain.[29] Based on calcium imaging, electrophysiology, and behavior data, researchers concluded that S1P signals via different cellular and molecular mechanisms to trigger itch and pain.[29] In another study, Hill et al. reveal the role of $S1P_3$ signaling that regulates mechanonociceptor excitability via modulation of KCNQ2/3 channels.[30]

Transient receptor potential (TRP) channels are a family of cation channels, some of which play critical roles in sensing exogenous and endogenous physical, chemical, and biological stimuli in the somatosensory system.[31] Transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1) are calcium-permeable nonselective cation channels that are expressed in dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons.[32,33] These ion channels are involved in both pain and itch.[19] The TRPA1 channel senses various pungent chemicals (cinnamaldehyde and mustard oil) causing pain or irritation[34] and also functions as an itch inducer downstream of receptors for chloroquine[35] and serotonin.[36] The TRPV1 channel is a receptor for capsaicin, acidic pH, and heat[33] ($\geq$43°C) that mediates pain[37] and also works as an effector for histamine-induced itch following histamine receptor activation.[38]

The detailed physiological roles and the mechanisms of S1P action in the somatosensory system, including whether S1P signaling is regulated through TRPA1 and TRPV1 activation, are only beginning to be understood. Here, we determine the physiological roles of S1P in vitro and in vivo by applying a range of technical approaches including calcium imaging, pharmacology, the use of genetic KO mice, and behavior assays in freely moving animals. Taken together, this work supports the role of TRP channels functioning downstream of S1P receptors in mediating pain and itch sensations, highlighting distinctions of TRP channel utilization in vivo.

Materials and methods

Animals

Male and female adult WT C57BL/6J mice, TRPA1 (Jax ID #006401), and TRPV1 (Jax ID # 003770) KO mice were used in this research and were obtained from The Jackson Laboratory (ME, USA). TRPA1/TRPV1 double knockout mice (DKO) were bred from crossing TRPA1 KO and TRPV1 KO mice. All procedures were performed in accordance with North Carolina State University laboratory animal care protocols approved by an Institutional Animal Care and Use Committee (IACUC).

Chemicals

S1P (d18:1), HC-030031, AMG 9810, SEW 2871, CYM 5520, CYM 5541, JTE 013, and TY 52156 were purchased from Cayman Chemical (MI, USA). Ex 26, CYM 50260, and A 971432 were purchased from Tocris Bioscience (MN, USA). Allyl isothiocyanate (AITC) and capsaicin were purchased from MilliporeSigma (MA, USA). S1P stock (10 mM, in DMSO) was used for in vitro experiments and 10 mM S1P stock in H_2O filtered by 20 µm nylon filter (VWR International, LCC., PA, USA) was used for in vivo experiments. HC-030031 and AMG 9810 were stocked in DMSO (27.6 mM and 29.0 mM, respectively). Stock solutions were prepared as described. SEW2871 was dissolved in DMSO (50 mM). CYM 5520 and CYM 50260 were dissolved in 10 mM in DMSO. CYM5541, JTE 013, TY 52156, Ex 26, and A 971432 were prepared in 100 mM in DMSO. AITC and capsaicin were dissolved in ethanol (100 mM and 1 mM, respectively). All stocks were stored at −20°C.

Primary culture of DRG and TG neurons

Primary culture of DRG and TG neurons was performed as described before.[24] Briefly, DRGs and TGs were isolated from mice, digested by 2.5 mg/ml collagenase (C7657; MilliporeSigma, MA, USA), dispersed by fire-polished Pasteur pipette and the neurons were cultured on glass coverslips (VWR) coated with 20 µl/coverslip of 0.4 µg/ml laminin (MilliporeSigma) and 0.01% poly-L-lysine (MilliporeSigma). DRG and TG neurons were cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc., VA, USA) containing 10% fetal bovine serum (VWR), 100 units/ml penicillin and 100 µg/ml streptomycin (VWR) under a condition at 37°C in 5% CO_2. Cultured DRG and TG neurons were used for calcium imaging experiments 24–48 h after dissection.

Calcium imaging

Calcium imaging using the DRG and TG neurons was performed at 37°C as described before.[24] Briefly, DRG and TG neurons were incubated for longer than 30 min at 37°C with 5% CO_2 atmosphere and protected from light in DMEM containing 1 µM of a fluorescent indicator Fura-2 AM (Enzo Life Sciences, Inc., NY, USA). The neurons were perfused in a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM D-glucose at pH 7.4 adjusted with NaOH. A calcium-free bath solution was prepared by omitting 2 mM $CaCl_2$ from the standard bath solution instead of adding 5 mM EGTA. Fura-2 fluorescence was excited at 340 and 380 nm and emission was monitored at 510 nm and collected every 5s with a digital CCD camera (Andor Clara DR-4152, Andor Technology Ltd, UK) attached to a Lambda LS lamp and a Lambda optical filter changer. Data were obtained using imaging software (NIS-Elements AR 4.13.04, Nikon Corporation, Japan) and analyzed by Microsoft Excel (Microsoft, WA, USA). Values of calcium responses were normalized by dividing measured values (F) with average values of initial five frames (F_0) of each cell and described as F/F_0. In this study, changes in each response to an application with $F/F_0 > 0.1$ were regarded as a positive response. Cells not responding to 100 mM KCl, which was applied at the end of each experiment, were regarded as non-neuronal cells and excluded from the analysis.

Behavioral assays

Before injections, the mice were acclimated in an acrylic chamber prior to video recording for at least 30 min. Spontaneous behaviors following injection into the nape of the neck (0.1, 1, and 10 nmol S1P/10 µl saline) or left hind paw (1 and 10 nmol S1P/10 µl saline) were recorded by a video camera (EX-ZR1700, CASIO, Japan) for 30 min and 10 min, respectively, and evaluated for scratching or nocifensive behaviors. The number of scratching bouts to the nape of the neck was counted as itch-related behaviors, and licking and flinching of the ipsilateral hind paw were counted as pain-related behaviors. Heat and mechanical hypersensitivities were measured by plantar Hargreaves and von Frey units (catalog # 37370 and 37450, Ugo Basile, Italy). Nocifensive behaviors against noxious cold stimuli were induced by dry ice, and cold hypersensitivity was evaluated by the latency of the behaviors.[39] In a separate experiment, S1P (1 nmol/10 µl and 10 nmol/10 µl) was injected in the cheek and both wiping and scratching bouts were recorded and counted. For all behavioral assays, the investigator was blinded to group allocation and treatments.

Statistics

No power analysis calculation was used to predetermine sample sizes. All data were represented as mean ± standard error of the mean (SEM). The numbers in the parenthesis showed the number of coverslips from multiple preparations except Figure 4(e) or (g), and the number of cells was described in figure legends appropriately. Statistical significance was calculated by Student's t-test, Mann–Whitney U test to compare two groups, Kruskal–Wallis test followed by Steel test, and Bonferroni test for multiple comparison. GraphPad Prism software was used for statical analysis and $p < 0.05$ was considered as significant.

Results

Mouse sensory neurons response to the bioactive lipid S1P

In order to examine whether sensory neurons respond to S1P, we applied 10 µM of S1P to primary cultured mouse DRG neurons and observed responsive cells (Figure 1(a) and (b)), suggesting that S1P plays a functional role in DRG sensory neurons. Approximately 30% of cultured DRG neurons responded to 10 µM of S1P (Figure 1(c)). Cells were exposed to S1P to single concentration instead of multiple to avoid any carryover from S1P in the susequent application. Response to 0 is a negative control. S1P signals was smaller compared to signals obtained with 100 mM KCl.

Next, we investigated the functional involvement of the five S1P receptors, $S1P_{1\text{-}5}$,[40] which are G protein-coupled receptors. The presence of these S1P receptors in rodent DRGs is reported mainly by focusing on mRNA expression.[41–46] $S1P_1$, $S1P_2$, and $S1P_3$ receptors are highly expressed in sensory neurons.[28,41,43,44,46] Similarly, recent transcriptomic profiling of single-cell mouse DRG neurons confirmed the expression of S1P

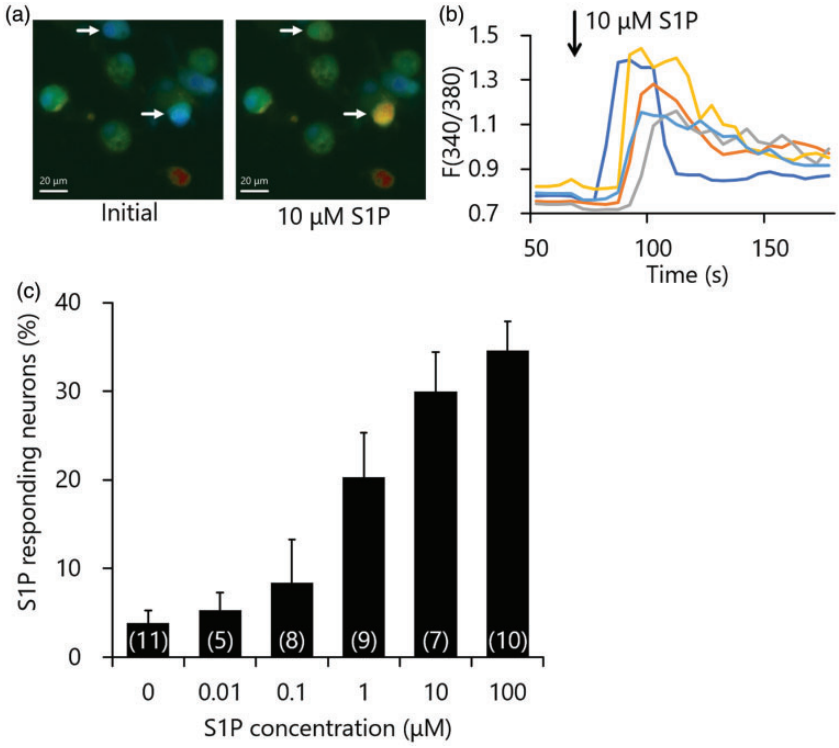

Figure 1. DRG neurons directly respond to S1P. (a) Representative images showing changes in intracellular calcium concentration of primary cultured mouse DRG neurons. Before (left) and after 10 μM S1P application (right). Arrows indicate neurons responding 10 μM S1P, scale bar = 20 μM. (b) Representative traces of DRG neurons responding to 10 μM S1P (n = 5 cells). (c) DRG neurons responding to increasing concentration of S1P (453 cells, 146 cells, 344 cells, 393 cells, 509 cells, and 561 cells, respectively). The numbers in parenthesis indicate the replication of the experiments from independent preparation, N ≥3 mice. Data are represented as mean ± SEM.
S1P: sphingosine-1-phosphate.

receptors.[47] Because we found calcium influx in DRG neurons in response to S1P by calcium imaging, we pharmacologically screened the functional expression of these S1P receptors by their selective agonists.[48–52] We have applied a 100-μM dose of each receptor-specific agonist to compare the receptor-specific activation (Figure 2) in sensory neurons. $S1P_1$ and $S1P_2$ agonists (100 μM each) significantly induced calcium responses in DRG (Figure 2(a), (b), and (f)), but responses to other three agonists specific for each receptor $S1P_3$, $S1P_4$, and $S1P_5$ (100 μM each) were not significant (Figure 2(c) to (f)). As for $S1P_3$, several reports showed the involvement in pain and itch in detail.[28–30] Based on our results and the fact that the potency of $S1P_1$, $S1P_2$, and $S1P_3$ receptor agonists are 10–100 times weaker than the natural ligand S1P, we then used selective antagonists for $S1P_1$ (10 μM), $S1P_2$ (100 μM), and $S1P_3$ (10 μM)[53–55] against the S1P-induced calcium influx (Figure 2(g) to (j)). $S1P_2$ and $S1P_3$ antagonists significantly inhibited the S1P-induced response, but $S1P_1$ antagonist did not show any effect. Together with agonist and antagonist profiling, $S1P_1$, $S1P_2$, and $S1P_3$ were suggested to be involved in S1P-induced calcium responses in DRG neurons.

To investigate S1P-induced calcium response in further detail and determine whether an increase of cytosolic calcium concentration is caused by the influx of extracellular calcium or by cytosolic calcium release from organelles such as the endoplasmic reticulum or mitochondria,[56] S1P (10 μM) was applied to DRG neurons in a calcium-free condition. DRG neurons did not respond to S1P using calcium-free solution (Figure 3(a) and (b)), suggesting that S1P induces calcium influx through the plasma membrane of DRG neurons. Because TRPA1 and TRPV1 are calcium-permeable nonselective cation channels expressed in DRG neurons and are two well-investigated proteins involved in pain and itch signaling,[24,31] we examined whether S1P-responding neurons are included in subpopulations of TRPA1- and/or TRPV1-positive sensory neurons. Following S1P application, selective agonists of TRPA1 (AITC, 100 μM) and TRPV1 (capsaicin, 1 μM) were applied to DRG neurons (Figure 3(c)). Several S1P-responding neurons also responded to AITC and/or capsaicin in a complex manner, and all responses were characterized in the Venn diagram (Figure 3(d)). The majority of the S1P-responding DRG neurons were

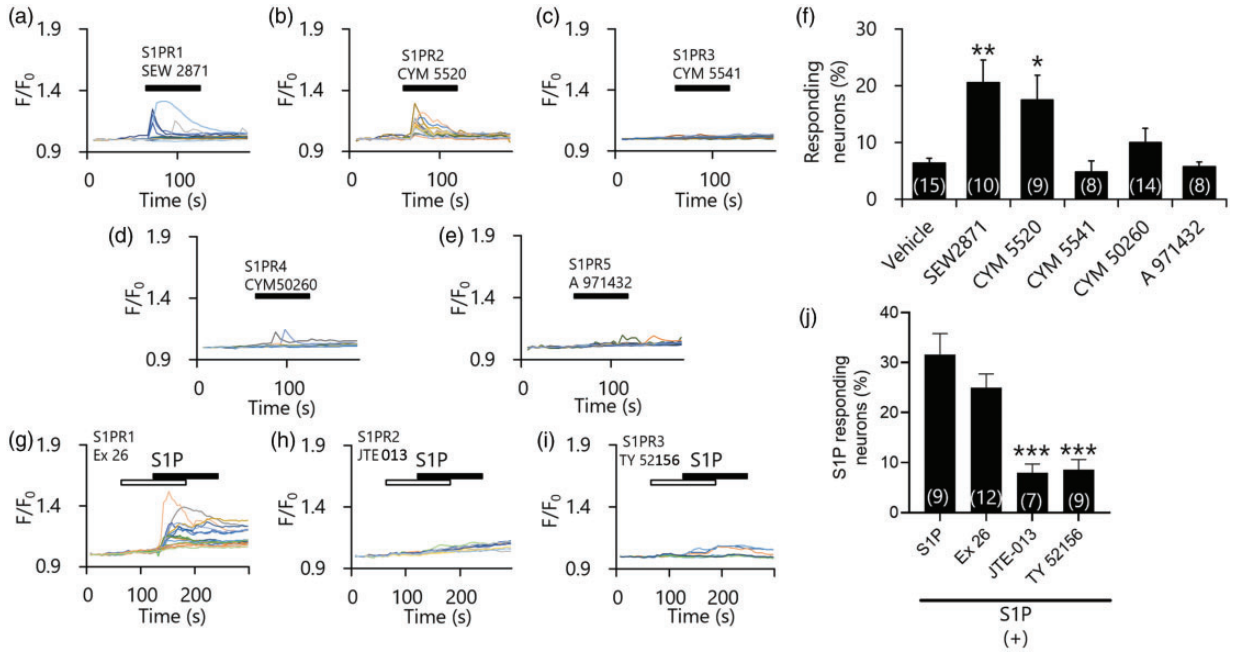

Figure 2. Functional screening of S1P receptors on DRG neurons using agonists and antagonists. (a)–(e) Representative traces of $S1P_{1\text{-}5}$ receptor agonist (31 cells, 19 cells, 9 cells, 15 cells, and 28 cells). (f) Quantification of S1P receptors agonist SEW2871 ($S1P_1$ agonist, 100 μM), CYM 5520 ($S1P_2$ agonist, 100 μM), CYM 5541 ($S1P_3$ agonist, 100 μM), CYM 50260 (S1P4 agonist, 100 μM), and A 971432 ($S1P_5$ agonist, 100 μM), 1272 cells, 596 cells, 562 cells, 334 cells, 953 cells, and 530 cells, respectively. Number of mice used >2. One-way ANOVA multiple comparison Bonferroni test was performed to determine significance, $^{*}p < 0.05$ and $^{**}p < 0.01$. (g)–(i) Representative trace of S1P-induced response in cells treated with $S1P_{1\text{-}3}$ antagonist (19 cells, 12 cells, and 7 cells). (j) Quantification of S1P-induced response with Ex26 ($S1P_1$ antagonist, 10 μM), JTE 013 ($S1P_2$ antagonist, 100 μM), and TY52156 ($S1P_3$ antagonist, 10 μM), 489 cells, 783 cells, 393 cells, and 334 cells, respectively. Data are represented as mean ± SEM. Number of mice used >2. One-way ANOVA multiple comparison Bonferroni test was performed to determine significance, $^{***}p < 0.001$. S1P: sphingosine-1-phosphate.

also responsive to AITC and/or capsaicin, suggesting that S1P-responding sensory neurons functionally express TRPA1 and/or TRPV1 channels. According to several reports that found potential overlap between pruritogens[15,29,36] in sensory neurons, we characterized DRG neurons responding to various pruritogens by calcium imaging. We investigated the relationship between S1P, histamine, and chloroquine (Figure 3(e) to (h)). We found that S1P responders are not completely independent or included in histamine (100 μM)-responding neurons ($S1P^+$/histamine$^+$=12.6%). Similarly, S1P responsive neurons are not excluded or included from chloroquine (1 mM)-responding neurons ($S1P^+$/chloroquine$^+$ = 4.8%, Figure 3(f) and (h)). From our results, we speculate that the mechanism of these pruritogens might be shared in some itch-related subpopulations, and it indicates that this type of analysis for these molecules in our case might not simplify the G-protein-coupled receptor signaling segregation in DRG neurons, as is the case with separation of histaminergic versus nonhistaminergic itch.

Pain and itch have been evaluated by intraplantar and nape of the neck injections, respectively, where stimuli are detected by DRG neurons. In rodents, both pain and itch can be evaluated at the same injection site by cheek injection,[57,58] where stimuli are detected by TG neurons. Following S1P application, selective agonists of TRPA1 (AITC, 100 μM) and TRPV1 (capsaicin, 1 μM) were applied to TG neurons. The majority of the S1P-responding TG neurons were also responsive to AITC and/or capsaicin, suggesting that S1P responding to TG sensory neurons functionally express TRPA1 and/or TRPV1 channels (Figure 3(i) and (j)). In brief, we found that S1P-induced neuronal activation shares similar functional profiles in both DRG and TG ganglia.

Pharmacological and genetic basis of S1P-induced responses are dependent on TRPA1 and/or TRPV1 in mice

We hypothesized that increases in intracellular calcium influx induced by S1P in DRG neurons are mediated by ion channel activity of TRPA1 and/or TRPV1. First, we assessed the efficacy of antagonists to inhibit AITC and capsaicin responses by using selective antagonists of TRPA1 (HC-030031, 100 μM) and TRPV1 (AMG 9810, 10 μM). We observed a significant reduction (91%) in AITC responses to HC-030031 treated compared to

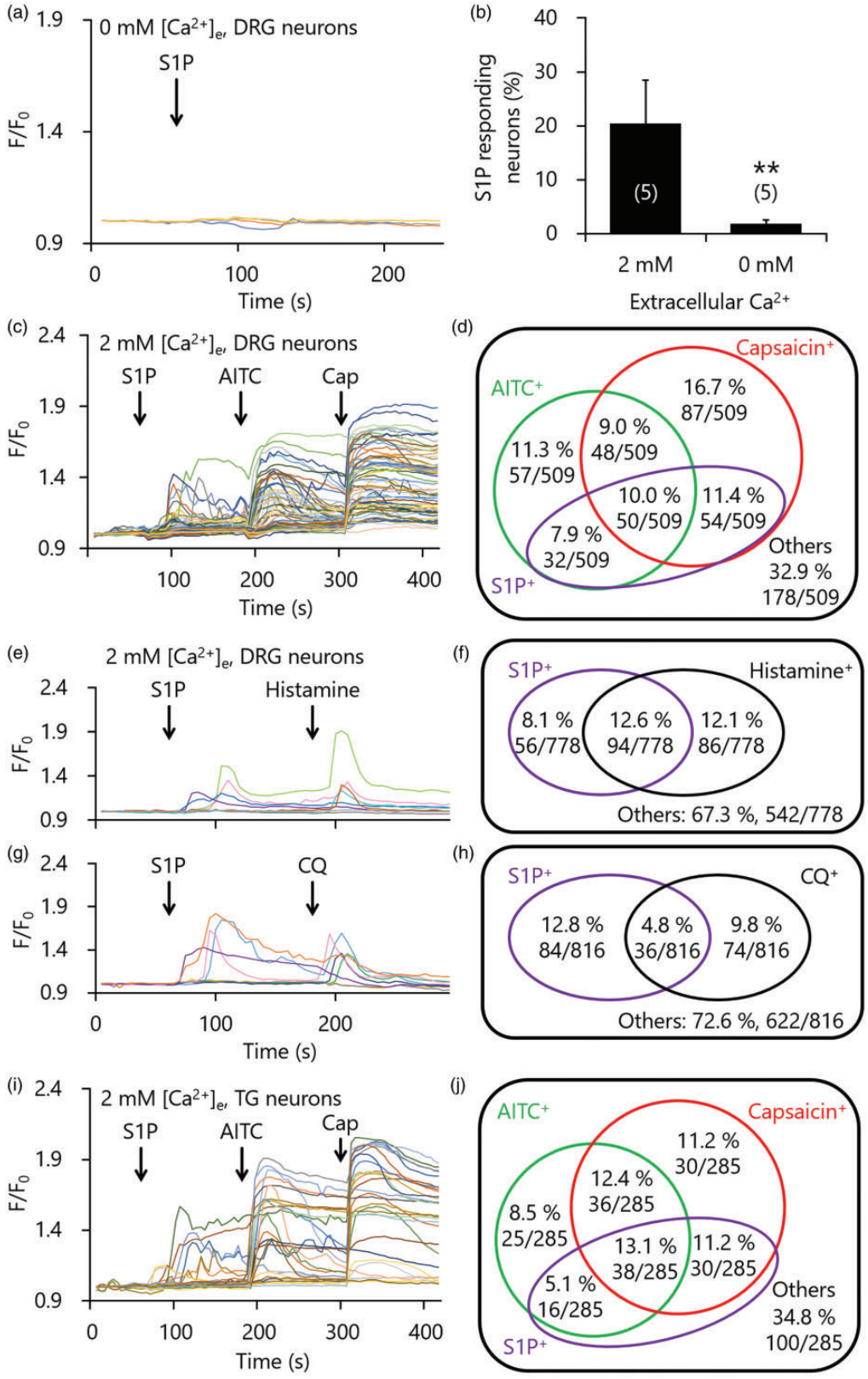

Figure 3. S1P-induced calcium influx in DRG and TG neurons. (a) Representative traces of changes in intracellular calcium concentration of DRG neurons upon 10 μM S1P application in a calcium-free condition (four cells). (b) S1P (10 μM)-responding neurons in 2 mM and 0 mM extracellular calcium conditions, 238 cells, and 286 cells. $^{**}p < 0.01$ by Mann–Whitney U test. Data were represented as mean ± SEM, n ≥ 3. (c) Representative traces of calcium responses in DRG neurons responding to 10 μM S1P, 100 μM allyl isothiocyanate

(continued)

control without antagonist HC-030031. Similarly, we found that TRPV1 antagonist AMG 9810 significantly inhibits (95%) capsaicin response compared to without AMG 9810. Next, we examined the responses induced by the S1P application. These antagonists reduced the S1P-induced increases of intracellular calcium influx as compared with the S1P response without antagonists (Figure 4(a) to (e)). Here, we showed that both population and amplitude of S1P-responding DRG neurons were significantly reduced (50%) in the condition with HC-030031, AMG 9810, and combined HC-030031 plus AMG 9810 (Figure 4(d) and (e)), which suggests that calcium influx caused by S1P is mediated by TRPA1 and TRPV1 channels. Also, we observed that TRPA1 and TRPV1 antagonists washed out during perfusion, which may account for the lack of effect on AITC and capsaicin responses (Figure 4(a) to (c)).

S1P-induced calcium influx was inhibited by the applications of HC-030031, AMG 9810, and HC-030031 in combination with AMG 9810 in WT DRG neurons (Figure 4(a) to (d)). Blocking the response by each of these antagonists was similar to the population obtained by inhibition of both TRPV1 and TRPA1 ion channels (Figure 4(d)). The residual S1P sensitive population (approximately 65%, Figure 4(d)) might consist of neurons expressing TRPs with unknown S1P-sensitive calcium signaling or even non-TRP expressing neurons other than TRPA1 and TRPV1 channels. To further confirm our pharmacological results, we examined S1P-induced responses in single and double KO mice with loss of function of TRPA1 and TRPV1 channels. Our calcium imaging data demonstrated that AITC responses in A1KO and A1/V1 DKO mice neurons (Figure 5(a), (c), and (d)) and capsaicin responses in V1KO and A1/V1 DKO were negligible (Figure 5(b), (c), and (e)). S1P-induced intracellular calcium influx was decreased (approximately 50%) in A1KO (Figure 5(a), (c), and (f)), V1KO (Figure 5(b), (c), and (f)), and A1/V1 DKO mice (Figure 5(c) and (f)) compared to WT DRG neurons. We further analyzed and quantitated the amplitude of in vitro calcium responses induced by S1P in WT and KO mice. We found that S1P-induced calcium response amplitude/intensity was not impaired in A1KO DRG neurons; however, those of V1KO and A1/V1 DKO DRG neurons were significantly reduced by approximately 25% (Figure 5(g)), but still a large part of the amplitude/intensity remains unchanged. Similar to the pharmacological approach, we found a remaining S1P-responding population (around 50%, Figure 5(f)) in single and double KO mice, demonstrating congruency between our pharmacological and genetic approaches. Our data further support the idea that TRPA1 and TRPV1 channels may be required for sensory neuron responses caused by S1P in an in vitro system, but we cannot exclude the possible involvement of other ion channels.

S1P induces pain and itch behavior in mice

We next examined some physiological responses of S1P and the contributions of TRPA1 and TRPV1 to the physiology of S1P in vivo. We first injected S1P (1 and 10 nmol/10 µl saline) into the cheek of mice to demonstrate if S1P-induced pain and itch behaviors happen simultaneously.[57] Here, we found that no responses to S1P-induced wiping, indicating pain, were not observed at several doses of S1P in the cheek assay (Figure 6(a)). Next, we injected S1P (10 nmol/10 µl) in the cheek of A1 and V1KO mice to find if they have any observable effect on wiping behavior, and not to our surprise we found no response (Figure 6(a)). In contrast, injection of S1P directly into the cheek showed a significant increase in scratching bouts at both 1 and 10 nmol compared to the vehicle (Figure 6(b)). However, when we injected S1P into the cheek of A1 and V1 KO mice, we found no significant decrease in itch behavior in A1 and V1KO mice (Figure 6(b)). Since the response of pain and itch behaviors in the cheek assays were too low in our hands, we next moved to dorsal nape of the neck for itch and hind paw for pain assay. S1P (10 nmol/10 µl saline) injected directly into the nape of the neck of WT mice significantly induced scratching behaviors compared to the saline-treated WT mice (Figure 6(c) and (d)). However, lower doses of S1P (0.1 and 1 nmol) had no significant effect on itch response (Figure 6(d)). Because the in vitro data indicated the involvement of TRPA1 and TRPV1 in sensory neurons activation induced by S1P, we explored whether S1P-induced itch is decreased

Figure 3. Continued.
(AITC), and 1 µM capsaicin (Cap) in 2 mM extracellular calcium condition (63 cells). (d) A Venn diagram showing subpopulation overlapping among S1P-, AITC-, and capsaicin-responding DRG neurons (509 cells; n = 7 mice). Numbers of small subpopulations are not described. (e) Representative traces of calcium responses in DRG neurons responding to 10 µM S1P and 100 µM histamine in 2 mM extracellular calcium condition. (f) A Venn diagram showing subpopulation overlapping among S1P and Histamine 10 µM S1P (788 cells for histamine). (g) Representative traces of 10 µM S1P and 1mM chloroquine in 2 mM extracellular calcium condition. (h) A Venn diagram showing subpopulations overlapping among S1P and chloroquine-responding DRG neurons (816 cells for chloroquine). Numbers of small subpopulations are not described. (i) Representative traces of calcium responses in TG neurons responding to 10 µM S1P, 100 µM AITC, and 1 µM Cap in 2 mM extracellular calcium condition (28 cells). (j) A Venn diagram showing subpopulations overlapping among S1P-, AITC-, and capsaicin-responding TG neurons (285 cells; n=5 mice). Numbers of small subpopulations are not described. S1P: sphingosine-1-phosphate; AITC: allyl isothiocyanate; DRG: dorsal root ganglion; TG: trigeminal ganglion.

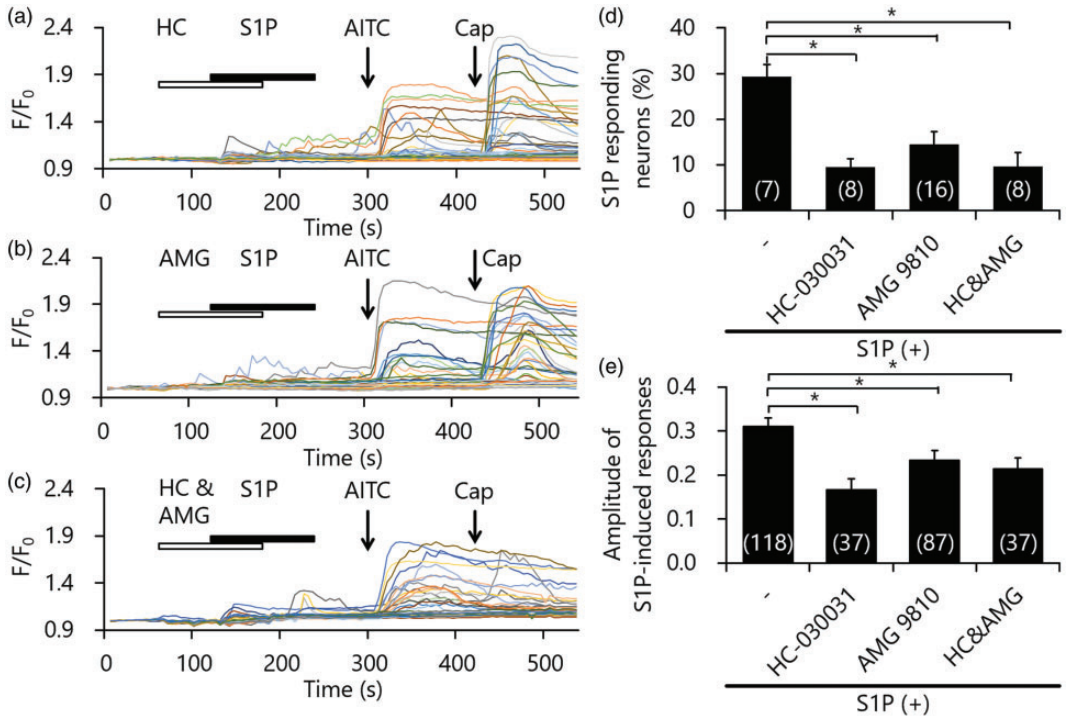

Figure 4. TRPA1and TRPV1 are involved in S1P-induced calcium influx is illustrated using receptor antagonists. (a) Representative traces of calcium responses in WT mouse DRG neurons induced by 10 μM S1P with 100 μM HC-030031 (HC), an antagonist for transient receptor potential ankyrin 1 (TRPA1) (38cells). (b) Representative traces of calcium responses in WT mouse DRG neurons induced by 10 μM S1P with 10 μM AMG 9810 (AMG), an antagonist for transient receptor potential vanilloid 1 (TRPV1) (39 cells). (c) Representative traces of calcium responses in WT mouse DRG neurons induced by 10 μM S1P with combined 100 μM HC-030031 (HC) and 10 μM AMG 9810 (AMG), an antagonist TRPA1 and TRPV1 receptors (31 cells). (d) S1P-responding DRG neurons without TRP antagonists (S1P) and with HC-030031 or AMG 9810 and combined HC + AMG (393, 413, 854, and 232 cells, respectively). Data are represented as mean ± SEM, N ≥ 3 mice *$p < 0.05$ versus S1P group by Steel multiple comparison test. (e) Normalized amplitude from S1P-responding DRG neurons without TRP antagonists (S1P) and with HC-030031 or AMG 9810 and combined HC-030031 or AMG (393, 413, 854, and 232 cells, respectively). Data are represented as mean ± SEM, N ≥ 3 mice. *$p < 0.05$ versus S1P group by Steel multiple comparison test. AITC: allyl isothiocyanate; S1P: sphingosine-1-phosphate; Cap: capsaicin.

in A1KO and V1KO mice. Interestingly, S1P-induced scratching behaviors decreased in V1KO mice (Figure 6(d)), but not in A1KO mice, indicating that TRPV1 is involved in the S1P-induced itch response.

To assess pain, S1P (10 nmol/10 μl saline) was injected into the left hind paw of WT mice to observe whether S1P induces pain-related behavior. Over a 10 min period, S1P induced approximately threefold more licking and flinching behavior in S1P-injected WT mice as compared to saline-injected WT mice (Figure 6(e) and (f)). S1P-induced pain-related behaviors were decreased in V1KO mice, but not in A1KO mice (Figure 6(f)), indicating that TRPV1 is involved in S1P-induced acute pain response. Furthermore, we assessed the hot, cold, and mechanical hypersensitivity by direct injection of S1P (10 nmol/10 μl saline) into the hind paw post 3-h for hot and cold and post 1-h post for mechanical allodynia (Figure 7(a) to (c)). Our pilot data (not shown) suggest that mice were hypersensitive to heat and cold maximally at 3-h, and mechanical sensation at 1-h; therefore, the rest of the experiments were performed at only one time-point at 3- and 1-h to minimize the stress to the mice. We found S1P-induced pain hypersensitivity for heat, cold, and mechanical responses in WT mice. Interestingly, S1P-induced heat hypersensitivity is reversed in V1KO mice, but it did not change in A1KO mice (Figure 7(a)), indicating that V1 is involved in heat-induced pain. S1P-induced mechanical and cold responses remained unchanged in both A1KO or V1KO mice (Figure 7(b) and (c)) when compared with control, suggesting the involvement of other channels for mechanical allodynia and cold hypersensitivity in mice.

Discussion

S1P is released from endothelial cells,[27] erythrocytes,[26] and platelets[25] and plays a role in regulating lymphocyte trafficking and inflammation.[59] Although S1P is known to be involved in allergic responses during anaphylaxis,[59] acute pain mediated by the $S1P_3$ receptor,[28] and

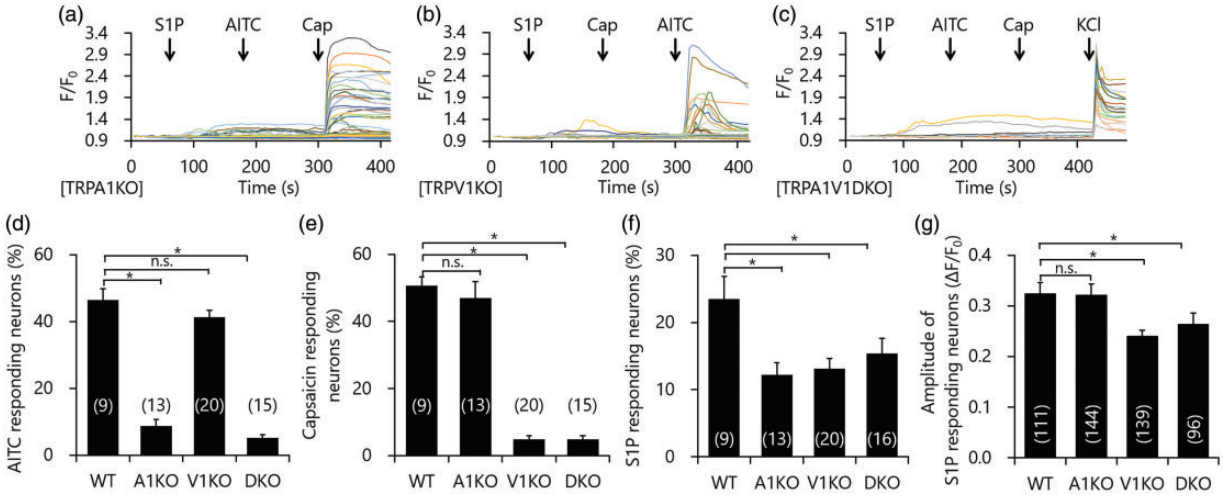

Figure 5. TRPA1and TRPV1 are involved in S1P-induced DRG neurons response as illustrated using knockout mice. (a) Representative traces of calcium responses in TRPA1, (b) TRPV1 KO, (c) TRPA1/TRPV1 double KO mice neurons upon application of 10 μM S1P, 100 μM AITC and 1 μM Cap (39 cells, 45 cells, and 22 cells, respectively). (d) Quantification of AITC response in WT and TRPA1 KO (A1KO), TRPV1 KO (V1KO), and double KO mice. Data are represented as mean ± SEM, N ≥ 3 mice. (e) Quantification of capsaicin response in WT and TRPA1 KO (A1KO), TRPV1 KO (V1KO), and double KO mice (n = 431, 1126, 645, and 705 cells, respectively). Data are represented as mean ± SEM, N ≥ 3 mice. *$p < 0.05$ versus WT group by Steel multiple comparison test. (f) S1P-responding DRG neurons in WT, TRPA1 KO (A1KO), TRPV1 KO (V1KO) mice, and double KO (DKO) (n = 488, 1126, 1172, and 705 cells, respectively). Data are represented as mean ± SEM, N≥3 mice *$p < 0.05$ versus WT group by Steel multiple comparison test. (g) Normalized amplitude response to S1P in WT, TRPA1 KO (A1KO), TRPV1 KO (V1KO), and double KO mice (n = 488, 1126, 1172, and 705 cells, respectively). Data are represented as mean ± SEM, N ≥ 3 mice. *$p < 0.05$ versus WT group by Steel multiple comparison test. AITC: allyl isothiocyanate; Cap: capsaicin; S1P: sphingosine-1-phosphate.

mechanical nociceptor excitability via modulation of KCNQ2/3 channels,[30] other physiological roles of S1P and downstream signaling mechanisms are not fully understood. We found that S1P is a molecule that activates primary sensory neurons. Almost all of the S1P-responding neuronal subpopulations of sensory neurons are included in AITC and/or capsaicin-responding subpopulations in vitro, suggesting that S1P induces pain and/or itch through TRPA1 and/or TRPV1 in vivo. Moreover, the characterization of S1P-induced calcium responses in DRG and TG neurons are almost equivalent, consistent with the fact that pain and itch can be similarly evaluated by the injection into the dorsal nape of the neck[60] and the hind paw,[61] where DRGs relay somatic sensations, and into the cheek,[57,58] where TGs transmit somatic sensations. Our data illustrate low levels of pain through DRG neurons (Figure 6(f)), but we found no pain response to S1P in the cheek assay (Figure 6(a)). This suggests that TG neurons relay pain differently than DRG neurons due to the physiologically heterogeneous rather than highly parallel lines of sensory input.[62] Interestingly, scratching response to S1P injections (Figure 6(d)) in the dorsal nape of the neck and licking and flinching pain behavior directed at the paw (Figure 6(f)) were observed in mice through DRG neurons. Our behavioral data are consistent with other behavioral findings.[28,29] It is also consistent with our own in vitro studies where we found that S1P is much less effective in triggering calcium influx in DRG neurons (Figure 3) than canonical agonists of TRPA1 and TRPV1.

TRPA1 and TRPV1 in DRG neurons are activated by both pain and itch mediators.[24,31] In our data, S1P-induced responses in DRG neurons are partially inhibited by TRPA1 and TRPV1 antagonists and in A1KO and V1KO DRG neurons, indicating that TRPA1 and TRPV1 activity could be induced either directly/indirectly by S1P. Regarding the molecular target of S1P in sensory neurons, some possibilities can be considered. First, S1P could activate one or some of the S1P receptors, $S1P_1$-$S1P_5$.[63] Our pharmacological screening demonstrates that $S1P_1$, $S1P_2$, and $S1P_3$ receptors are expressed in the DRG and are functional. We did not find $S1P_4$ and $S1P_5$ functional activation by specific agonists for these receptors in the DRG, which is largely consistent with a previous report that found lower levels of $S1P_4$ and $S1P_5$ in mouse DRG explant and absent in isolated neurons.[43] Using antagonists specific for $S1P_1$, $S1P_2$, and $S1P_3$, we found no inhibition of $S1P_1$ in the DRG in response to S1P; however, $S1P_2$ and $S1P_3$ responses were significantly inhibited (Figure 2(j)).

Second, S1P could directly activate TRPA1 and TRPV1 channels. Lipids are known to directly activate or modulate ion channels[64] including TRP channels.[65]

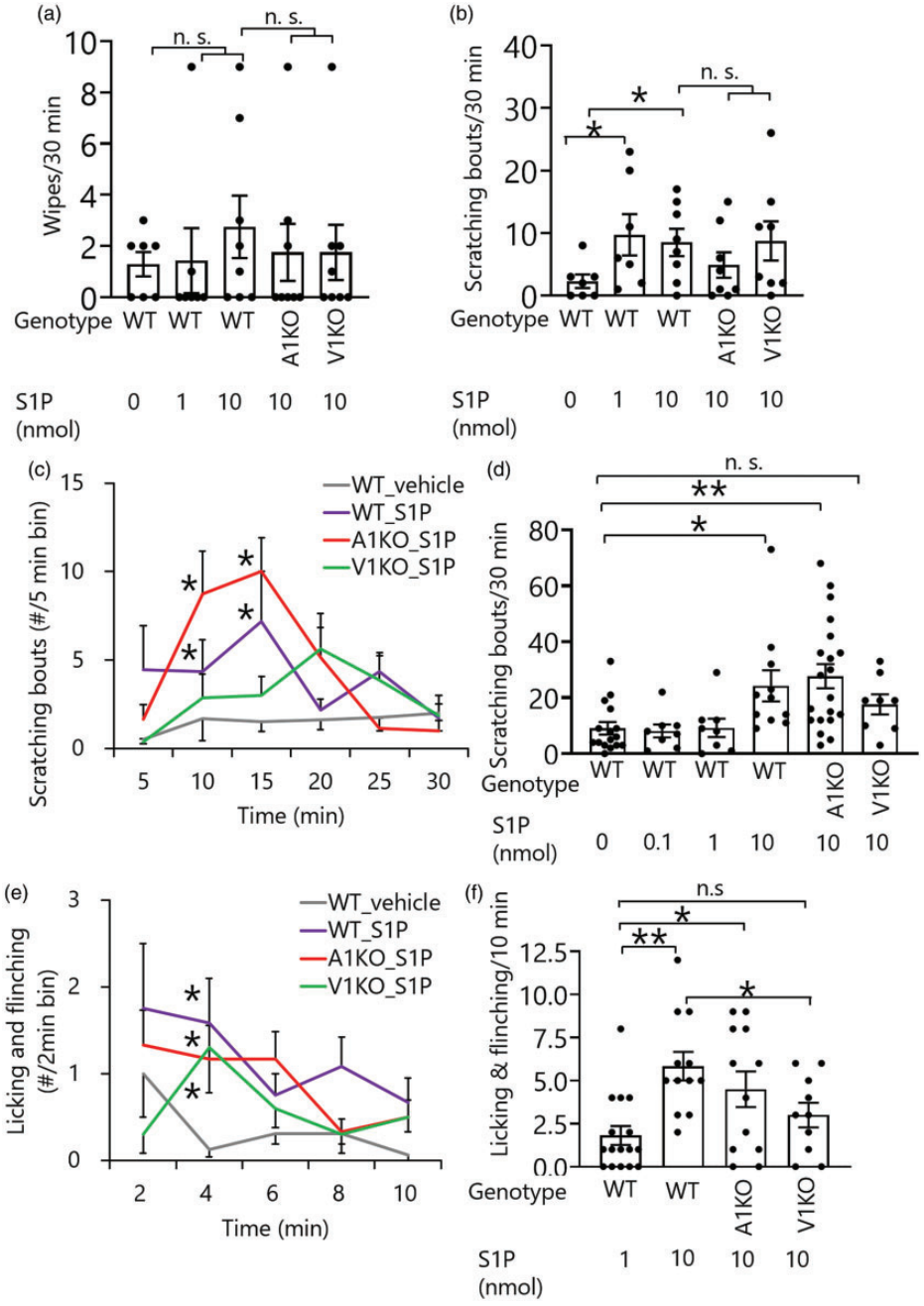

Figure 6. S1Pinduces acute nocifensive and itch-related behaviors in mice. (a) and (b) Pain- and itch-related behaviors were measured by counting wiping and scratching bouts. Mice were injected subcutaneously with varying concentrations of S1P, n ≥ 7–8. (c) Time-dependent changes of scratching behaviors of mice recorded in 5-min intervals. Saline (10 μl) was injected into the nape of the neck of WT mice (WT, saline) and S1P (10 nmol/10 μl saline) was injected to WT mice (WT, S1P), TRPA1 KO mice (A1KO, 10 nmol S1P), and TRPV1 KO mice (V1KO, 10 nmol S1P). At 10 min, $p = 0.044$ by ANOVA and $p < 0.05$ between vehicle-treated WT and S1P-treated TRPA1 KO. At 15 min, $p = 0.03$ by Kruskal–Walls test and $p < 0.05$ between vehicle-treated WT and S1P-treated WT and vehicle-treated WT between S1P-treated TRPA1 KO. No significant differences were observed among the groups by multiple comparisons. (d) Scratching behaviors of each group were recorded for 30 min following injections. Saline (10 μl) was injected into the nape of the neck of WT mice (WT, saline) and S1P (0.1, 1, and 10 nmol/10 μl saline) was injected to WT mice (WT, S1P), TRPA1 KO mice (A1KO, 10 nmol S1P) and TRPV1 KO mice (V1KO, 10 nmol S1P), *$p < 0.05$ versus saline-injected WT group by Steel multiple comparison test. (e) Time-dependent changes of licking and flinching behaviors of mice recorded in 2-min intervals. Saline (10 μl) and S1P (10 nmol/10 μl saline) were injected into the left hind paws of WT mice, WT (saline), (WT, S1P), TRPA1 KO mice (A1KO, S1P), and TRPV1 KO mice (V1KO, S1P). No significant differences were observed among the groups by multiple comparisons. (f) Licking and flinching behaviors of each group were recorded for 10 min post S1P injections. Saline as a negative control (10 μl) and S1P (10 nmol/10 μl saline) were injected into the left hind paws of WT mice (WT, S1P), TRPA1 KO mice (A1KO, S1P), and TRPV1 KO mice (V1KO, S1P) S1P (n = 8 mice). All data are represented as mean ± SEM. *$p < 0.05$ versus S1P-injected WT group by one-tailed Steel multiple comparison test. AITC: allyl isothiocyanate; Cap: capsaicin; S1P: sphingosine-1-phosphate; DKO: double knockout mice.

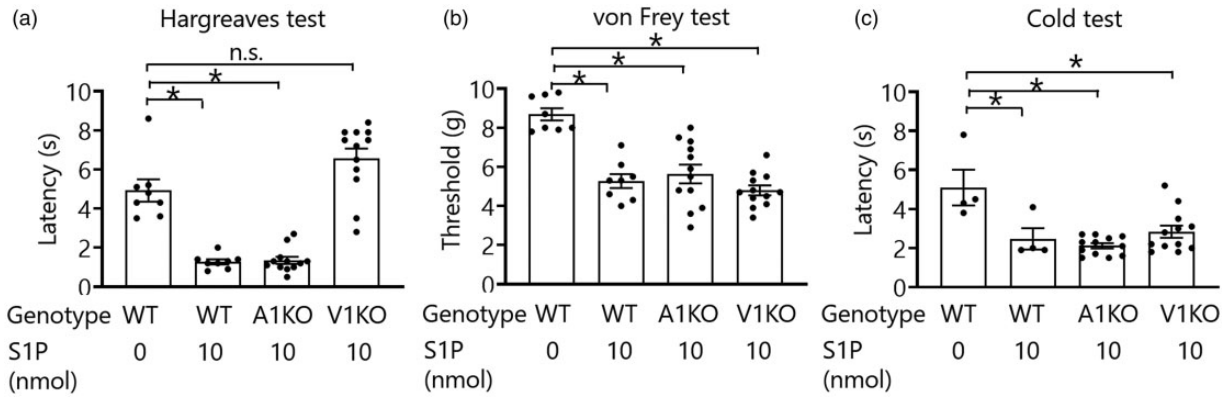

Figure 7. S1Pinduces hypersensitivity to heat, pressure, and cold. (a) Latencies until mice show nocifensive behaviors induced by noxious heat stimulation 3-h after injection of saline (10 μl) in WT mice (Veh) and S1P (10 nmol/10 μl saline) in WT (S1P), TRPA1 KO (A1KO), and TRPV1 KO (V1KO) mice. *$p < 0.05$ versus Vehicle group by Steel multiple comparison test. The baseline value of TRPV1 KO mouse was observed at 6.6 ± 0.9. (b) Mechanical thresholds for avoidance behaviors to mechanical stimulation of mice 1-h after injection of saline and S1P as in Figure 6(a). *$p < 0.05$ versus Veh group by Steel multiple comparison test. Latencies until mice show nocifensive behaviors induced by noxious cold stimulation 3-h after injection of saline and S1P as in Figure 6(a). *$p < 0.05$ versus Veh group by one-tailed Steel multiple comparison test. All data are represented as mean ± SEM. Hargreaves assay, n = 8–12; von Frey, n = 8–12; cold test, n = 4–12. WT: wild type.

For example, some prostaglandins directly activate the TRPA1 channel[66] and some lipoxygenase products from arachidonate activate the TRPV1 channel.[67] Regarding S1P, it has been suggested that S1P directly activates TRPC1 and TRPC5 channels.[68,69] Similar to S1P, another LPL, LPA, was also recently reported to directly activate both TRPA1 and TRPV1 channels.[24] Most cellular heterologous expression systems endogenously express S1P receptors. Therefore, it is difficult to evaluate using heterologous expression system whether S1P activates TRPV1 and TRPA1 channels.

Third, S1P could act on sensory neurons and cause calcium influx partially through TRPA1 and TRPV1 channels by unknown mechanisms or signaling. It might be nonspecific effects on the plasma membrane that change membrane lipid composition[70] or some cytosolic effects through the plasma membrane permeation. If S1P directly activates TRPA1 or TRPV1 and the responses can be regarded close to saturation, the S1P positive population should be as large as AITC or TRPV1 positive populations, respectively. Therefore, TRPA1 and TRPV1 are likely activated by S1P indirectly downstream of one or multiple S1P receptors mentioned above,[22,63] which might be expressed in a subset of the AITC- or capsaicin-responding subpopulation.

In our behavior tests, S1P induces both pain and itch. TRPV1 is involved in pain and itch via multiple pathways having been elucidated in vitro and in vivo.[24] Considering our in vitro results, S1P-induced itch appears to be mediated by a S1P/TRPV1 axis similar to the H1/TRPV1 axis in histamine-induced itch.[71] The involvement of S1P receptors and their mode of signaling will need to be clarified. Both TRPA1 and TRPV1 are involved in acute nocifensive behaviors caused by their chemical ligands and physical stimuli.[31] Although our in vitro data imply that TRPA1 and TRPV1 are required for S1P-induced neuronal responses, our acute nocifensive behavior tests indicate that S1P-induced acute pain and itch are partially dependent on TRPV1, but not on TRPA1.

An important question raised by our result is: why is TRPV1 and not TRPA1 required for pain and itch in freely behaving mice? Our in vitro data from DRG neurons revealed that both TRPV1 and TRPA1 are required for S1P-induced calcium response in vitro (Figures 4 and 5). Nonetheless, we found that S1P-induced pain and itch behavior in vivo is mainly due to TRPV1 but remains unaffected by TRPA1. The potential answer for this difference is not trivial, and we must consider the ways sensory stimuli are processed in vivo and the differences between unique subsets of neurons in vitro. For instance, S1P can activate multiple receptors in different classes of neurons, and there are multiple receptors in different cell types with nonoverlapping downstream signaling pathways including phospholipase C signaling partner G_q[29,72] and $G_{\beta\gamma}$.[29,35] In addition, although culturing sensory neurons is a powerful tool in neuroscience, in vitro responses can differ slightly from in vivo responses because of culture-induced changes in gene expression, differences in the way neurons are activated (response to soma which is not the nerve terminals), differences in calcium response versus action potential firing, and the potential for processing of sensory signals in the spinal cord. Our data are largely consistent with

the reports that have recently shown the induction of pain and itch behavior occurring in a dose-dependent manner.[29,73] One study is based on the chemical-induced activation of pruriceptors and nociceptors in zebrafish and mice.[73] The authors used imiquimod, a weak activator of TRPA1 channel, followed by AITC, a strong activator, and observed this phenomenon of affinity driven activation of the TRPA1 channel. Another study used S1P to show that low doses of S1P induce itch alone and high concentrations of S1P induce both pain and itch.[29] Despite the fact that high doses of S1P have effects on both pain and itch, our results are very similar to those reported by Hill et al.[29] In general, our study provides a mechanism that is not completely based on S1P/TRPV1 signal transduction. Therefore, examining the interaction between S1P signaling and TRPV1 activation is important for further understanding of physiological roles of S1P in vivo and perhaps including other TRP channels may also provide more mechanistic information downstream of S1P receptors.[74]

S1P also induces hypersensitivity to heat, mechanical stimuli, and cold. TRPV1 mediates heat hypersensitivity, but not mechanical allodynia induced by pungent chemicals or inflammatory conditions.[37] TRPV1 is not activated by cold stimulation. This is all consistent with our results. If S1P receptors are involved in TRPV1-dependent heat hypersensitivity, it is possible that TRPV1 channels are sensitized with phosphorylation by protein kinase A, cAMP-dependent kinase, or by protein kinase C, which is activated by DAG.[75] Contrary to TRPV1, TRPA1 is involved in mechanical allodynia but not thermal allodynia in an inflammatory condition,[76] and its role in mammalian cold nociception is controversial. Unexpectedly, our data revealed that TRPA1 was not involved in any nociception initiated by S1P observed in WT mice (Figures 6 and 7). Other receptors or ion channels for noxious, mechano- or cold stimuli could be involved in the nociception.[77,78]

In this study, we showed the physiological roles and the cellular mechanisms governing S1P's role in pain and itch transmission. However, there are several points to be further examined including (1) whether S1P signaling through TRPA1 and TRPV1 still requires S1P receptors, (2) the physiological source of S1P and its metabolism, (3) S1P signaling in chronic pain models induced by neuronal damage or diabetic neuropathy, and, finally, (4) the consequences of S1P signaling in chronic itch models such as atopic dermatitis, psoriasis, and cholestasis.

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Declaration of Conflicting Interests

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