Hydrogen sulfide-induced itch requires activation of Ca\textsubscript{v}3.2 T-type calcium channel in mice

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The contributions of gasotransmitters to itch sensation are largely unknown. In this study, we aimed to investigate the roles of hydrogen sulfide (H\textsubscript{2}S), a ubiquitous gasotransmitter, in itch signaling. We found that intradermal injection of H\textsubscript{2}S donors NaHS or Na\textsubscript{2}S, but not GYY4137 (a slow-releasing H\textsubscript{2}S donor), dose-dependently induced scratching behavior in a \mu-opioid receptor-dependent and histamine-independent manner in mice. Interestingly, NaHS induced itch via unique mechanisms that involved capsaicin-insensitive A-fibers, but not TRPV1-expressing C-fibers that are traditionally considered for mediating itch, revealed by depletion of TRPV1-expressing C-fibers by systemic resiniferatoxin treatment. Moreover, local application of capsaizapine (TRPV1 blocker) or HC-030031 (TRPA1 blocker) had no effects on NaHS-evoked scratching. Strikingly, pharmacological blockade and silencing of Ca\textsubscript{v}3.2 T-type calcium channel by mibefradil, ascorbic acid, zinc chloride or Ca\textsubscript{v}3.2 siRNA dramatically decreased NaHS-evoked scratching. NaHS induced robust alloknesis (touch-evoked itch), which was inhibited by T-type calcium channels blocker mibefradil. Compound 48/80-induced itch was enhanced by an endogenous precursor of H\textsubscript{2}S (L-cysteine) but attenuated by inhibitors of H\textsubscript{2}S-producing enzymes cystathionine \gamma-lyase and cystathionine \beta-synthase. These results indicated that H\textsubscript{2}S, as a novel nonhistaminergic itch mediator, may activates Ca\textsubscript{v}3.2 T-type calcium channel, probably located at A-fibers, to induce scratching and alloknesis in mice.

Itch (pruritus) is an unpleasant cutaneous sensation that elicits scratch reflex\textsuperscript{1}. Although itch and pain share many similarities, recent studies revealed that itch has its own unique molecular, cellular and circuitry mechanisms\textsuperscript{2}. Acute itch serves as a self-protective mechanism to prevent our bodies from harmful external irritants\textsuperscript{1}. However, chronic itch is a debilitating symptom that accompanies numerous skin and systemic diseases, including atopic dermatitis and psoriasis, chronic kidney failure and cholestasis, diabetes and some cancer\textsuperscript{3}. Antihistamines are the first choice for treating allergic itch. However, they are inefficient for many other chronic itch conditions\textsuperscript{4}, suggesting that histamine-independent mechanisms are involved in\textsuperscript{2}. Although itch sensation can be transiently relieved by scratching\textsuperscript{2}, itch-scratch-itch cycles often exacerbate skin problems\textsuperscript{4}. Chronic itch disrupts sleep and substantially reduces the quality of life of patients. Thus, there is an urgency to identify novel non-histaminergic itch mediators, which may be involved in the pathogenesis of chronic itch.

Hydrogen sulfide (H\textsubscript{2}S) is now considered to be the third gasotransmitter in addition to nitric oxide (NO) and carbon monoxide (CO)\textsuperscript{7}. H\textsubscript{2}S is endogenous produced enzymatically mainly by cystathionine \gamma-lyase (CSE) and cystathionine \beta-synthase (CBS) from L-cysteine or by 3-mercaptopyruvate sulfurt-ransferase (MPST) with cysteine aminotransferase (CAT) from 3-mercaptopyruvate\textsuperscript{8}. In recent years,
H₂S is becoming a molecule of high interest and getting more attention to its physiological and pathological functions involved in the regulation of cardiovascular system, nervous system, gastrointestinal system, renal function and inflammatory responses⁸⁻¹³. Increasing evidence supports that H₂S is involved in modulation of pain processing¹⁴,¹⁵. Although itch and pain are distinct sensations and have separate neural pathways⁷, they might share similar mediators and receptors¹⁶. Interestingly, recent studies emphasized the crucial contribution of NO in itch signaling elicited by chloroquine, serotonin and substance P¹⁷⁻¹⁹, suggesting an important role of gasotransmitter in itch signaling. In sharp contrast, the roles of H₂S in itch regulation remain elusive.

The aim of the present study is to test whether H₂S can induce itch behaviors in mice and further elucidate the underlying molecular mechanisms. Itch behavior can be quantitatively evaluated by measuring the scratching behavior elicited by pruritogens and can be differentiated from pain by using cheek model in rodents²⁰,²¹. In this study, we investigated the behavioral responses in mice induced by intradermal (i.d.) injection of NaHS or Na₂S, two commonly used H₂S donors. We firstly found that H₂S could elicit robust scratching behavior, which required activation of Caᵥ₃.₂ T-type calcium channel, but not TRPV1 and TRPA1. In contrast, H₂S-induced pain required activation of both T-type calcium channel and TRPV1. We next revealed that endogenous production of H₂S contributes to compound 48/80-induced itch sensation by using CBS inhibitor aminooxyacetic acid (AOAA) and CSE inhibitor dl-Propargylglycine (PAG). Thus, our results identified H₂S as a novel itch mediator and indicated Caᵥ₃.₂ T-type calcium channel inhibitors or H₂S synthesis inhibitors may be novel promising strategies for management of itch, although the precise roles of H₂S in chronic itch need further investigation.

Materials and Methods

Animals. Adult male CD1 mice (8–10 weeks old upon arrival) used in this study were obtained from Laboratory Animal Center of Chinese Academy of Sciences. Animals were housed with food and water available ad libitum and kept in controlled room temperature (22 ± 2 °C) and humidity (60–80%) under a 12 h/12 h light/dark cycle. All the behavioral tests were done in blind respect to the drug treatment. All experimental procedures and animal handling were performed in accordance with the guidelines of the International Association for the Study of Pain and the animal protocols were approved by Soochow University Animal Committee. The authors tried all efforts to minimize the number of animals used.

Drugs and administration. We purchased NaHS, Na₂S, GYY4137, compound 48/80, chloroquine, resiniferatoxin (RTX), chlorpheniramine maleate, and capsazepin from Sigma-Aldrich (St. Louis, MO, USA). Zinc chloride was obtained from China Sinopharm Chemical Reagent Co., Ltd (Shanghai, China), and ascorbic acid was obtained from China Shanghai Xiandai Hasen (Shanggu) Pharmaceutical Co., Ltd (Shanghai, China). Naloxone hydrochloride was obtained from China National Medicines Guorui Pharmaceutical Co., Ltd (Huainan City, Anhui Province, China). Mibebradil dihydrochloride, capsazepine and HC-030031 was obtained from Tocris (Bristol, UK). Morphine hydrochloride was obtained from China Northeast Pharmaceutical Group Shenyang No.1 Pharmaceutical Co., Ltd (Shenyang City, Liaoning Province, China). Lidocaine(2%) was obtained from China Otsuka Pharmaceutical CO., Ltd (Huainan City, Anhui Province, China). Capsaicin, Resiniferatoxin (RTX), capsazepine, mibebradil and HC-030031 was dissolved in 10% DMSO. Other reagents were dissolved in sterile saline if not specified.

Selective Caᵥ₃.₂ siRNA (sc-42707) and scrambled Caᵥ₃.₂ control siRNA (sc-37007) were synthesized by Santa Cruz (Shanghai, China). siRNA was dissolved in RNase-free water at the concentration of 1 μg/μl as stock solution, and mixed with polyethyleneimine (PEI, Fermentas) (Shanghai, China), 10 min before injection, to increase cell membrane penetration and reduce the degradation. PEI was dissolved in 5% glucose, and 1 μg of siRNA was mixed with 0.18 μl of PEI. We intrathecally injected 10μl of siRNA (2μg) once a day for 2 days to knockdown Caᵥ₃.₂ expression. Intrathecal injection was performed by a lumbar puncture to deliver reagent into cerebral spinal fluid. A successful spinal puncture was evidenced by a brisk tail-flick after the needle entry into subarachnoid space²². Capsaicin (10μg in 20μl 2.5% DMSO) or NaHS (200μg in 20μl saline) was intraplantarly injected into one hindpaw, and the number of flinches was counted for the first 10 min.

Neck model of itch. As described previously²³, mice were shaved at the nape of the neck at least 2 day before experiments. On the day of behavioral testing, mice were individually placed in small plastic chambers (10 × 10 × 12.5 cm) on an elevated metal mesh floor and allowed at least 30 min for habituation. Under brief anesthesia of isoflurane, mice were given an intradermal injection of 50 μl of agents via a 26G needle into the nape of the neck. Immediately after the injection, mice were returned to their chambers and were video recorded for 30 min. The video was subsequently played back offline and itch behavior was quantified by counting the number of scratches in a blinded manner. A scratch was counted when a mouse lifted its hindpaw to scratch the shaved region and returned the paw to the floor or to the mouth for licking.

Cheek model of itch. To distinguish itch and pain behavior, we used the cheek model by injection of chemicals into the cheek of mice. Mice were shaved on cheeks (approx. 5 × 8 mm area) at least 2 day before the experiment. On the day of experiment, mice were intradermally injected of 20 μl of reagent (NaHS or Na₂S) via a 26G needle into the cheek under brief anesthesia with isoflurane.
after the injection, mice were returned to their chambers and were video recorded for 30 min. The video was played back and the number of wipes and scratches were quantified by counting their number. One wipe was defined when mouse unilaterally wipes the injected site with the forelimb, which was not part of grooming behavior. One scratch was defined as a lifting of the hind paw toward the injection site on the cheek and then returning the paw to the floor or to the mouth.

**Alloknesis assay.** According to a previous report, alloknesis after intradermal injection of pruritogens was evaluated. Briefly, 30 min after the injection of NaHS or Na$_2$S, a von Frey filament (0.7 mN) was applied to the affected skin site. A scratch bout directed to the site of mechanical stimulation was considered as a positive response. The alloknesis score was determined by calculating the total number of scratches elicited by three mechanical stimuli and was evaluated at 5-min intervals.

**Tail immersion test.** As previously described, tail immersion test was employed to assess heat pain sensitivity in mice. Briefly, the terminal 3 cm of a mouse's tail was immersed in hot water bath at 52°C and the latency of tail flick was recorded with a cutoff time of 10 seconds to avoid potential tissue injury.

**Rotarod test.** The mouse motor function was tested using DXP-2 Rota-Rod equipment (Institute of Materia Medica, Chinese Academy of Medical Sciences). Each mouse was trained for two consecutive days (6 trails per day) where the speed of the rotor was accelerated from 4 to 25 rpm with an acceleration of 0.2 rpm/sec. One day after the last training session, the mouse was tested at the speed of the rotor (25 rpm) for three times and the longest duration of running time was recorded.

**Pharmacological treatments.** To test the possible effects of $\mu$-opioid receptor agonist or antagonist on H$_2$S donors-induced scratching, $\mu$-opioid receptor agonist morphine (1 mg/kg) or antagonist naloxone (1 mg/kg) was i.p. injected into mice 20 min before i.d. injection of 200 $\mu$g NaHS or Na$_2$S in mice. To assess the possible effects of antihistamines on H$_2$S donors-induced scratching behavior, chlorpheniramine (10–50 $\mu$g/kg), the selective histamine H1 receptor antagonist, was i.p. injected 20 min before i.d. injection of NaHS or Na$_2$S in mice. To examine the role of mast cells in the H$_2$S donors-induced itch, we deplete mast cells by daily treatment with compound 48/80 (1, 3, 10 and 10 $\mu$g per site on the 1st, 2nd, 3rd and 4th days, respectively) before injection of NaHS. As we described previously, to examine the role of TRPV1-expressing C-fibers in the H$_2$S donors-induced itch, we destroyed these C-fibers by daily treatment with the ultra-potent TRPV1 receptor agonist resiniferatoxin (RTX, 30, 70 and 100 $\mu$g/kg, subcutaneously for 3 consecutive days), one week before injection of H$_2$S donors. Intradermal injection of lidocaine (2%) was also used for testing the possible role of A-fibers in H$_2$S donors-induced scratching in both vehicle and RTX-treated mice. To assess the involvement of TRPV1 or TRPA1 in H$_2$S donors-induced scratching behavior, capsazepine (10–50 $\mu$g) or the selective TRPV1 antagonist HC-030031 (10–50 $\mu$g) and the selective TRPA1 agonist HC-030031 (10–50 $\mu$g) were administrated. To assess the involvement of Ca$_v$3.2 T-type calcium channel in NaHS-induced scratching behavior, mibebradil (i.d. 5–25 nmol; T-type calcium channel blocker), ascorbic acid (i.d. 1 nmol; i.p. 1 mg/kg; selective Ca$_v$3.2 blocker) or zinc chloride (i.d. 5 nmol; i.p. 1 mg/kg; selective Ca$_v$3.2 blocker) were administrated.

**Real-time quantitative RT-PCR.** We collected cervical DRGs and spinal cord, isolated total RNAs using RNeasy Plus Mini kit (Qiagen, Valencia, CA). One microgram of RNA was reverse transcribed for each sample using Omniscript reverse transcriptase according to the protocol of the manufacturer (Qiagen). Q-RT-PCR sequences for Ca$_v$3.2: forward: TCTCGCTACCCAATGACAGC; reverse: CTCGCCGTAGTCTGGGATGC; Ca$_v$3.1: forward: ACATTGGAGCAGCCTCTTCAG; reverse: CTGCCGTGTTGGGAGTGACA. Triplicate qPCR analyses were performed using the SYBR Green master mix (KAPA) and Opticon real-time PCR Detection System (ABI Life7500) as described previously.

**Immunohistochemistry.** Mice were terminally anesthetized with isoflurane and perfused through the ascending aorta with saline followed by 4% paraformaldehyde. The L4-6 lumbar spinal cords were collected and postfixed in the same fixative overnight. The spinal cord sections were cut at the thickness of 14-μm in a cryostat. The tissue sections were blocked with 10% goat serum, and incubated over night at 4°C with the primary antibodies guinea pig anti-TRPV1 antibody (1:1000, Neuroemics) (Edina, MN, USA). The sections were then incubated for 1 h at room temperature with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Immunostained tissue sections were examined under a Zeiss fluorescence microscope AXIO SCOPE A1 (Oberkochen, Germany), and images were analyzed with NIH Image software or Adobe PhotoShop.

**Western blotting.** Mice were terminally anesthetized with isoflurane and transcardially perfused with PBS, and the DRGs were rapidly removed and homogenized in a lysis buffer containing a cocktail of protease inhibitors and phosphatase inhibitors for total protein extraction and assay according to our previous report. The protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL, USA), and 25 μg of proteins were loaded for each lane and subjected to SDS-PAGE. After the transfer, the blots were blocked with 5% nonfat milk in TBST and PVDF membranes were incubated overnight at
4°C with primary polyclonal antibody against Ca v3.2 (goat, 1:500; Santa Cruz Biotechnology). For loading control, the blots were probed with Tubulin antibody (mouse, 1:1000, Vazyme, Suzhou City Jiangsu Province, China). The blots were washed and incubated in horseradish peroxidase–conjugated donkey anti-goat or goat anti-mouse IgG secondary antibody (1:2000, Santa Cruz Biotechnology). Protein bands were visualized using an enhanced chemiluminescence detection kit (Pierce) and the band densities were detected and analyzed using Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Shanghai, China). Data from five mice were used for statistical analysis.

Statistical analysis. All values were presented as the mean ± S.E.M. Student's t test was used for two group comparison. One-way ANOVA followed by post-hoc Bonferroni test was used for multiple comparisons. Two-way repeated-measured ANOVA was also used to analyze the data with multiple time points. Differences with $p<0.05$ were considered as statistical significance.

Results

Intradermal injections of H$_2$S donors induce scratching behavior in mice. We firstly investigated whether intradermal (i.d.) injection of NaHS and Na$_2$S, two commonly used H$_2$S donors, are able to induce scratching behavior in CD1 mice. Injection of NaHS (i.d., 20–800 μg; Fig. 1A) or Na$_2$S (i.d., 20–800 μg; Fig. 1B) into the nape of neck produced robust scratching behavior in a dose-dependent manner in mice. NaHS or Na$_2$S began to elicit scratching at the dose of 20 μg and reached a platform at the doses of 200–800 μg. Thus, the dose of 200 μg NaHS or Na$_2$S was chosen for the following experiments.

We did not observe any abnormal behaviors of mice following i.d. application of H$_2$S donors in these experiments. In contrast, injection of GYY4137 (i.d., 20–200 μg), a slow-releasing H$_2$S donor, into the nape of neck did not produce obvious scratching in mice (Fig. 1C). We also found that intrathecal (i.t.) injection of NaHS (20–100 μg) did not elicit obvious scratching (Fig. 1D), indicating peripheral, but not central, application of H$_2$S donors elicited itch in mice.

In order to distinguish itch and pain behaviors in rodents, we used the cheek model by i.d. injection of chemicals into cheek of mice. In cheek model, painful agents elicit forelimb wiping behavior, while itchy agents elicit hindlimb scratching behavior. I.d. injection of NaHS (Fig. 2A) or Na$_2$S (Fig. 2B) into the cheek of mice dose-dependently elicited both wiping and scratching behavior, indicating these H$_2$S donors can induce mixed itch and pain sensations. Notably, both NaHS and Na$_2$S induced relative
more scratching than wiping behavior, suggesting that itch may be one of the major sensory modalities induced by these H₂S donors.

Intradermal injections of H₂S donors induce scratching in an opioid receptor-dependent and histamine-independent manner in mice. We next tested whether H₂S donors-induced scratching could be modulated by μ-opioid receptor, which has been implicated in itch for rodents and humans. Morphine, a μ-opioid receptor agonist, is clinical used as analgesic. Systemic morphine (1 mg/kg, i.p.) did not reduce NaHS or Na₂S-induced scratching behavior (Fig. 2C), consistent with clinical observations that morphine did not reduce even exacerbate itch. However, naloxone (1 mg/kg, i.p.), an opioid receptor antagonist, significantly reduced NaHS or Na₂S-induced scratching behavior. The result indicated that NaHS or Na₂S-induced scratching was itch-related behavior in mice and also suggested that endogenous opioids may be involved in H₂S donors-induced itch in mice.

One of the best-known itch mediators is histamine, which is stocked and released from skin cells, such as mast cells and keratinocytes. We subsequently asked whether mast cells and histamine were involved in H₂S donors-induced scratching in mice. Mice were pretreated with compound 48/80 to cause skin mast cells degranulation and reduce the number of mast cells. It was found that NaHS induced comparable scratching in vehicle and compound 48/80-pretreatment mice (Fig. 2E), suggesting mast cells have little effects on NaHS-induced itch. Systemic injection of histamine H₁ receptor antagonist chlorpheniramine (10 mg/kg, i.p.) did not affect NaHS-induced scratching in mice (Fig. 2F), but significantly decreased compound 48/80-induced scratching in mice (200.8 ± 13.4 vs. 105.3 ± 7.0; P < 0.001; Fig. 2F). Thus, H₂S donors-elicited scratching behavior is largely independent of mast cells and histamine.

Figure 2. Pharmacological interventions of H₂S donors-induced scratching behavior in mice. (A,B) Both forelimb wiping and hindpaw scratching could be induced by i.d. 20 μl NaHS (20–200 μg; A) or Na₂S (20–200 μg; B) into cheek, indicating H₂S donors-induced mixed pain and itch sensation. i.d injection of 20 μl saline served as a control. *P < 0.05, **P < 0.01; ***P < 0.001 vs. control, one-way ANOVA following Bonferroni post hoc test. (C,D) Naloxone (i.p. 1 mg/kg; D) but not morphine (i.p. 1 mg/kg; C) significantly reduced NaHS or Na₂S-induced scratching behavior. **P < 0.01; ***P < 0.001 vs. control, Student's t test. (E) In compound 48/80-pretreated mice, NaHS (200 μg) could induce comparable scratching behavior, suggesting mast cells were not critically involved in. (F) Systemic injection of chlorpheniramine (a histamine H₁ antagonist; 10 mg/kg, i.p.) suppressed scratching behavior induced by compound 48/80 (100 μg) but not by NaHS (200 μg), suggesting histamine-independent mechanisms were involved in. ***P < 0.001; vs. control, Student's t test. All data are expressed by means ± SEM. n = 5–8 mice per group.
TRPV1-expressing C-fibers are dispensable for H$_2$S donors-induced scratching behavior in mice.

It is well appreciated that TRPV1-expressing C-fibers mediate itch sensation induced by histaminergic and nonhistaminergic pruritogens$^{34,35}$. To further determine whether TRPV1-expressing C-fibers mediate H$_2$S donors-induced itch, we employed systemic pretreatment of resiniferatoxin (RTX), an ultrapotent TRPV1 agonist, to destroy TRPV1-expressing C-fibers in mice. Consistent with our previous work, immunostaining showed that pretreatment with RTX resulted in lack of TRPV1-positive signals in spinal cord dorsal horn of mice (Fig. 3A). The tail-flick latency of 52°C noxious heat reached cutoff time (10 s) in RTX-treated mice (Fig. 3B). Intraplantar capsaicin-induced flinching was abolished in RTX-treated mice. (D) Compound 48/80 or chloroquine-induced scratching were significantly inhibited in RTX-treated mice. (E) NaHS- or Na$_2$S-induced scratching behavior was comparable in vehicle- and RTX-treated mice. (F) Local application of lidocaine (2%) completely abolished NaHS-induced scratching in both vehicle- and RTX-treated mice. (G) In cheek model, NaHS-induced forelimb wiping, but not hindpaw scratching, was abolished in RTX-treated mice, suggesting capsaicin-sensitive C-fibers were required for NaHS-induced pain but not itch. All data are expressed by means ± SEM. n = 6–9 mice per group. ***P < 0.05 vs. control, Student’s t test.

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**Figure 3.** TRPV1-expressing C-fibers were not required for H$_2$S donors-induced itch behaviors in mice. (A) Immunostaining showing that TRPV1-expressing C-fibers are dramatically reduced in RTX-treated mice comparing with vehicle-treated mice. (B) Tail-flick latency of RTX-treated mice reached cutoff time (10 s) in 52°C hot water bath. ***P < 0.001 vs. control, Student’s t test. (C) Flinches induced by capsaicin (10 μg) were abolished in RTX-treated mice. (D) Compound 48/80 or chloroquine-induced scratching were significantly inhibited in RTX-treated mice. (E) NaHS- or Na$_2$S-induced scratching behavior was comparable in vehicle- and RTX-treated mice. (F) Local application of lidocaine (2%) completely abolished NaHS-induced scratching in both vehicle- and RTX-treated mice. (G) In cheek model, NaHS-induced forelimb wiping, but not hindpaw scratching, was abolished in RTX-treated mice, suggesting capsaicin-sensitive C-fibers were required for NaHS-induced pain but not itch. All data are expressed by means ± SEM. n = 6–9 mice per group. ***P < 0.05 vs. control, Student’s t test.
Capsaicin-insensitive A-fibers might mediate H2S donors-induced itch, while capsaicin-sensitive C-fibers mediated H2S donors-induced pain in mice.

Neither TRPV1 nor TRPA1 is required for H2S donors-induced itch in mice. TRPV1 and TRPA1 were well-demonstrated to mediate histamine-dependent and -independent itch, respectively, in mice35,36. Recent work showed that excitatory effect of H2S on gastrointestinal motility may be attributed to the activation of TRPV137. H2S-induced mechanical pain is also partially mediated by the activation of TRPA1 in mice38. Thus, it suggested H2S may act on TRPV1 and/or TRPA1 to produce pain in mice. In this study, we subsequently examined the role of TRPV1 and TRPA1 in H2S donors-induced itch using pharmacological methods. Co-administration of TRPV1 selective blocker capsazepine (10–50μg) in either neck or cheek did not affect NaHS-induced scratching in mice (Fig. 4A,B). Capsazepine (50μg) significantly reduced intradermal NaHS-induced forelimb wiping, but not hindpaw scratching. (C) Intraplantar co-administration of capsazepine (50μg) attenuated NaHS-induced flinching. (D) Local administration of TRPA1 antagonist HC-030031 (10–50μg) did not affect NaHS-induced scratching. (E) In cheek model, co-administration of HC-030031 (50μg) did not affect NaHS-induced both forelimb wiping and hindpaw scratching. (F) Intraplantar co-administration of HC-030031 (50μg) had no effects on NaHS-induced flinching. All data are expressed by means ± SEM. n = 6–8 mice per group. *P < 0.05; **P < 0.01, ***P < 0.001 vs. vehicle control, Student’s t test.

Figure 4. TRPV1 and TRPA1 were not required for NaHS-induced itch, while TRPV1 was required for NaHS-induced pain in mice. (A) Local administration of TRPV1 antagonist capsazepine (10–50μg) did not affect NaHS-induced scratching. (B) In cheek model, co-administration of capsazepine (50μg) suppressed NaHS-induced forelimb wiping, but not hindpaw scratching. (C) Intraplantar co-administration of capsazepine (50μg) attenuated NaHS-induced flinching. (D) Local administration of TRPA1 antagonist HC-030031 (10–50μg) did not affect NaHS-induced scratching. (E) In cheek model, co-administration of HC-030031 (50μg) did not affect NaHS-induced both forelimb wiping and hindpaw scratching. (F) Intraplantar co-administration of HC-030031 (50μg) had no effects on NaHS-induced flinching. All data are expressed by means ± SEM. n = 6–8 mice per group. *P < 0.05; **P < 0.01, ***P < 0.001 vs. vehicle control, Student’s t test.
Activation of T-type calcium channels were indispensable H$_2$S donors-induced itch in mice.

Previous reports showed that endogenous and exogenous H$_2$S facilitates T-type calcium channel currents and contributes to pain sensation. We thus investigated whether activation of T-type calcium channels contribute to NaHS-induced itch behaviors in mice. We found that local application of pan-T-type calcium channel blocker mibefradil (Mib) (i.d. 5–25 nmol) dose-dependently inhibited NaHS-induced scratching in naïve mice (Fig. 5A) and RTX-treated mice (184.4 ± 4.8 vs. 96.1 ± 6.7; P < 0.001; Fig. 5B). Mib (i.d. 10 nmol) significantly inhibited NaHS-induced forelimb wipping (24.0 ± 0.7 vs. 8.0 ± 0.9; P < 0.001; Fig. 5C) and hindpaw scratching (64.4 ± 4.3 vs. 30.7 ± 5.6; P < 0.001; Fig. 5C) in cheek model. Mib (i.d. 10 nmol) also significantly inhibited NaHS-induced flinching (41.8 ± 1.0 vs. 18.0 ± 0.9; P < 0.001; Fig. 5D). We further asked whether zinc chloride (ZnCl$_2$ or ascorbic acid (Asc), two selectively inhibitors for Ca$_v$3.2, but not Ca$_v$3.1 or Ca$_v$3.3, isoforms of T-type calcium channels, affects NaHS-induced itch. Systemic (i.p. 1 mg/kg) and local application of ZnCl$_2$ (i.d. 5 nmol) zinc chloride significantly inhibited NaHS-induced scratching in naïve mice (for i.p. injection: 148.8 ± 15.9 vs. 40.0 ± 0.6; P < 0.001; Fig. 5E; for i.d. injection: 162.8 ± 21.4 vs. 50.2 ± 13.5; P < 0.01; Fig. 5F) and RTX-treated mice (i.d. injection: 197.8 ± 33.9 vs. 103.0 ± 13.2; P < 0.05; Fig. 5F). In cheek model, ZnCl$_2$ (i.d. 5 nmol) significantly inhibited NaHS-induced forelimb wipping (22.0 ± 1.7 vs. 8.4 ± 1.5; P < 0.001) and hindpaw scratching (68.2 ± 4.5 vs. 21.2 ± 5.5; P < 0.001; Fig. 5G). ZnCl$_2$ (i.pl. 5 nmol) significantly inhibited NaHS-induced flinching (30.8 ± 2.1 vs. 11.0 ± 1.2; P < 0.001; Fig. 5H). Similarly, systemic (i.p. 1 mg/kg) and local application of ascorbic acid (Asc; i.d. 5 nmol) significantly inhibited NaHS-induced scratching in both naïve mice (for i.p. injection: 148.8 ± 14.2 vs. 55.8 ± 4.4; P < 0.001; Fig. 5I; for i.d. injection: 160.9 ± 21.5 vs. 63.8 ± 8.7; P < 0.001; Fig. 5J) and RTX-treated mice (i.d. injection: 223.2 ± 13.0 vs. 111.2 ± 23.0; P < 0.001; Fig. 5J). In cheek model, Asc (i.d. 1 nmol) significantly inhibited NaHS-induced forelimb wipping (22.5 ± 1.5 vs. 11.0 ± 1.7; P < 0.01) and hindpaw scratching (67.8 ± 3.7 vs. 21.7 ± 5.3; P < 0.001; Fig. 5K). Asc (i.pl. 1 nmol) significantly inhibited NaHS-induced flinching (29.0 ± 2.5 vs. 12.8 ± 1.1; P < 0.001; Fig. 5L). Together, these data indicated Ca$_v$3.2 T-type calcium channels play key roles in H$_2$S donors-induced itch, as well as pain sensation in mice.

Silencing of Ca$_v$3.2 T-type calcium channels in primary sensory neurons abolished NaHS-induced itch in mice.

To avoid the nonspecific effects of pharmacological antagonists of T-type calcium channels, we used intrathecal injection of siRNA specific targeted to Ca$_v$3.2 channel (Ca$_v$3.2-siRNA) to knockdown its expression in primary sensory neurons in dorsal root ganalia (DRG). It was found that repeated intrathecal injection of Ca$_v$3.2-siRNA, but not scrambled control siRNA, selectively knockdown the expression of Ca$_v$3.2, but not Ca$_v$3.1 in DRG (Fig. 6A). Interestingly, the expression of Ca$_v$3.2 in spinal cord was not affected by Ca$_v$3.2-siRNA treatment (Fig. 6A), suggesting DRG is more accessible than spinal cord via intrathecal puncture. Western blotting also confirmed that Ca$_v$3.2 protein expression in DRG was reduced by Ca$_v$3.2-siRNA treatment (Fig. 6B). Behaviorally, NaHS-induced scratching was dramatically decreased in Ca$_v$3.2-siRNA treated mice (106.3 ± 12.4 vs. 26.9 ± 6.9; P < 0.001; Fig. 6C). The motor function of Ca$_v$3.2-siRNA treated mice was not affected (Fig. 6D). Thus, these data further emphasized the crucial roles of Ca$_v$3.2 T-type calcium channels in H$_2$S donors-induced itch in mice.

Activation of T-type calcium channels was required for H$_2$S donors-induced alloknesis (touch-evoked itch) in mice.

Alloknesis is a remarkable feature of chronic itch; however the mechanisms underlying this phenomenon are still unclear. Alloknesis was previously observed following i.d. histamine, 5-HT, protease-activated receptor (PAR)-4 agonist, and MrgprC11 agonist, but not chloroquine or a PAR-2 agonist, suggesting not all pruritogens evoked alloknesis. In this study, we asked whether H$_2$S donors are able to induce alloknesis in mice. The results showed that i.d. NaHS or Na$_2$S induced alloknesis in mice, which lasted for at least 30 min (Fig. 7A). Local co-administration of T-type calcium channels blocker Mib significantly suppressed the development of alloknesis elicited by NaHS (Fig. 7B).

Involvement of endogenous H$_2$S in compound 48/80-induced itch in mice.

We asked whether endogenous H$_2$S is involved in itch responses induced by pruritogens in mice. Compound 48/80, a mast cell degranulator, is known to induce allergic itch via histamine release. Systemic NaHS (10 mg/kg, i.p.) was able to enhanced compound 48/80-induced scratching behavior (Fig. 8A). Systemic administration of l-cysteine (10–30 mg/kg, i.p.), a key precursor for endogenous H$_2$S synthesis, significantly increased compound 48/80-induced scratching behavior in a dose-dependent manner in mice (Fig. 8B). Thus, increased endogenous production of H$_2$S may be able to enhance compound 48/80-induced scratching behavior in mice. We further examined the possible effects of inhibitors of H$_2$S-producing enzymes on itch sensation in mice. Strikingly, local application of the CBS inhibitor AOA or CSE inhibitor PAG dose-dependently reduced compound 48/80-induced scratching behavior in mice (Fig. 8C,D), suggesting the inhibitors of endogenous H$_2$S-producing enzymes are able to relief itch in mice. Together, endogenous H$_2$S may be involved in allergic itch in mice.

Finally, we tested the motor function following systemic administration of drugs used in this study. The results showed that systemic application of naloxone, Asc, ZnCl$_2$, NaHS and L-cysteine did not.
affect the duration of running time using Rotarod test (Fig. 9), suggesting the effects of these drugs on scratching behavior did not attribute to their influence on motor function.

Figure 5. The inhibitory effects of T-type calcium channel blockers on NaHS-induced itch and pain behaviors in mice. (A) Systemic zinc chloride (ZnCl₂; i.p. 1 mg/kg) significantly inhibited NaHS-induced scratching. (B) Local application of ZnCl₂ (i.d. 5 nmol) significantly inhibited NaHS-induced scratching in both RTX- and vehicle-treated mice. (C) ZnCl₂ (i.d. 5 nmol) significantly inhibited NaHS-induced both forelimb wiping and hindpaw scratching in cheek model. (D) ZnCl₂ (i.pl. 5 nmol) significantly inhibited NaHS-induced flinching. (E) Systemic ascorbic acid (Asc; i.p. 1 mg/kg) significantly inhibited NaHS-induced scratching. (F) Asc (i.d. 1 nmol) significantly inhibited NaHS-induced scratching in both RTX- and vehicle-treated mice. (G) Asc (i.d. 1 nmol) significantly inhibited NaHS-induced both forelimb wiping and hindpaw scratching in cheek model. (H) Asc (i.pl. 1 nmol) significantly inhibited NaHS-induced flinching. (I) Local application of mibebradil (Mib) (i.d. 5–25 nmol) dose-dependently inhibited NaHS-induced scratching in mice. (J) Mib (i.d. 10 nmol) significantly inhibited NaHS-induced scratching in RTX-treated mice. (K) Mib (i.d. 10 nmol) significantly inhibited NaHS-induced both forelimb wiping and hindpaw scratching in cheek model. (L) Mib (i.d. 10 nmol) significantly inhibited NaHS-induced flinching. All data are expressed by means ± SEM. n = 6–8 mice per group. *P < 0.05; **P < 0.01, ***P < 0.001 vs. vehicle control, Student’s t test.
Along with pain, touch and thermal sensation, itch is a cutaneous sensation and detected by primary sensory neurons in dorsal root ganglia (DRG) for the body and trigeminal ganglia (TG) for the face. Itch can be acute (e.g., mosquito bite) or chronic (e.g., atopic dermatitis). Acute itch may serve as a warning system, while chronic itch represents a common clinical problem. Antihistamines are first line treatment for allergic itch; however, they are less efficient for many types of chronic itch, suggesting histamine-independent mechanisms are involved. Great progress has been made in recent years to identify series of novel nonhistaminergic itch mediators, such as PAR 2/4 agonists, MrgprA3/C11 agonists and TGR5 (also called Gpbar1) agonists. Interestingly, there were several reports that emphasized the crucial role of nitric oxide (NO) in itch elicited by chloroquine, serotonin and substance P, indicating a key role of gasotransmitters in itch signaling. However, it is largely unknown about the roles of H₂S, the third gasotransmitter, in itch regulation.

**Discussion**

Along with pain, touch and thermal sensation, itch is a cutaneous sensation and detected by primary sensory neurons in dorsal root ganglia (DRG) for the body and trigeminal ganglia (TG) for the face. Itch can be acute (e.g., mosquito bite) or chronic (e.g., atopic dermatitis). Acute itch may serve as a warning system, while chronic itch represents a common clinical problem. Antihistamines are first line treatment for allergic itch; however, they are less efficient for many types of chronic itch (e.g., cholestasis, renal failure and apotic dermatitis), suggesting histamine-independent mechanisms are involved in. Great progress has been made in recent years to identify series of novel nonhistaminergic itch mediators, such as PAR 2/4 agonists, MrgprA3/C11 agonists and TGR5 (also called Gpbar1) agonists. Interestingly, there were several reports that emphasized the crucial role of nitric oxide (NO) in itch elicited by chloroquine, serotonin and substance P, indicating a key role of gasotransmitters in itch signaling. However, it is largely unknown about the roles of H₂S, the third gasotransmitter, in itch regulation.
In the current study, we have identified the critical roles of H₂S (the third gasotransmitter) in itch. We firstly observed that H₂S donors (NaHS and Na₂S) could elicit robust scratching behavior, which is μ-opioid receptor-dependent and histamine-independent in mice. Interestingly, slow-releasing H₂S donor (GYY4137) was not able to elicit scratching in mice. As H₂S donors are potential therapeutic for many diseases, slow-releasing H₂S donors may be a good choice to avoid unwanted side effects, such as itch. We further revealed that activation of Cav3.2 T-type calcium channel possible located in capsaicin-insensitive A-fibers, but not TRPV1 and TRPA1 in capsaicin-sensitive C-fibers, is required for H₂S donors-elicited itch response. In contrast, activation of both Cav3.2 T-type calcium channel and TRPV1 is essential for H₂S-induced pain behavior. We also demonstrated that endogenous H₂S contributes to compound 48/80-induced itch sensation. Thus, our results identified H₂S as a novel non-histaminergic itch mediator and provided several molecular targets for anti-itch treatment, such as T-type calcium channel inhibitors or H₂S synthesis inhibitors.

Primary sensory neurons located in DRGs and TGs are responsible for itch signaling transmission from skin to spinal cord dorsal horn. It is traditionally considered that TRPV1-expressing C-fibers, which include a subset of TRPA1-expressing neurons, are required for both histamine-dependent and independent itch. In the present study, we surprisingly found that ablation of TRPV1-expressing C-fibers, caused by systemic RTX treatment, did not affect H₂S donors-induced itch, but abolished compound 48/80 or chloroquine-induced itch in mice (Fig. 3). Thus, TRPV1-expressing C-fibers are not essential for H₂S donors-induced itch, although they were essential for H₂S donors-induced pain behavior.

Figure 7. H₂S donors induced alloknesis and T-type calcium channel blocker mibefradil suppressed this alloknesis in mice. (A) Time course of alloknesis induced by i.d. injection of H₂S donors NaHS or Na₂S in mice (P < 0.05). (B) Inhibitory effects of local injection of mibefradil on NaHS-induced alloknesis in mice (P < 0.05). All data are expressed by means ± SEM. Two-way repeated-measures ANOVA, n = 7 mice per group.
in mice. Local application of lidocaine (2%) completely abolished NaHS-induced itch in vehicle- and RTX-treated mice (Fig. 3), suggesting capsaicin-insensitive A-fibers may participate in NaHS-induced itch in mice. Previous reports clearly showed that small myelinated A-fibers mediated cowhage-induced histamine-independent itch in human. In this study, we provided strong pharmacological evidence supporting that capsaicin-insensitive A-fibers mediate H₂S donors-induced non-histaminergic itch, while capsaicin-sensitive C-fibers are essential for H₂S donors-induced pain in mice. Consistently, TRPV1

Figure 8. Endogenous H₂S production contributes to compound 48/80-induced itch in mice. (A) Systemic administration of NaHS (1–10 mg/kg; i.p.) increased compound 48/80-induced scratching in mice. (B) Systemic administration of L-cysteine (10–30 mg/kg; i.p.) increased compound 48/80-induced scratching in a dose-dependent manner in mice. (C) Local administration of CBS inhibitor AOAA (10–100 μg; i.d.) dose-dependently attenuated compound 48/80-induced scratching in mice. (D) Local administration of CSE inhibitor PAG (10–100 μg; i.d.) dose-dependently attenuated compound 48/80-induced scratching in mice. All data are expressed by means ± SEM. n = 7–11 mice per group. *P < 0.05, **P < 0.01; ***P < 0.001 vs. control, one-way ANOVA following Bonferroni post hoc test.

Figure 9. Rotarod testing showed the normal motor function following systemic administration of drugs, including naloxone (1 mg/kg), Asc (1 mg/kg), ZnCl₂ (1 mg/kg), NaHS (1 mg/kg) and L-cysteine (10 mg/kg). None of these drugs affected the duration of running time in mice. n = 6 mice per group.
and TRPA1 are dispensable for H$_2$S donors-induced itch, although they might be important for H$_2$S donors-induced pain in mice.

H$_2$S signaling plays different physiological or pathological roles through acting on distinct receptors or channels$^{15}$. We then asked which receptors H$_2$S might act on for mediating itch sensation. Several ion channels or receptors had been identified as possible targets for H$_2$S, such as TRPV1, TRPA1, T-type calcium channels and ATP-sensitive potassium channels etc$^{34,80,51}$. Importantly, a recent study demonstrated that NaHS activated Ca$_{v3.2}$ T-type calcium channels at higher concentration (3–10 mM); however, NaHS selective inhibited Ca$_{v3.2}$ channels at μM concentration$^{35}$. It indicated that the effects of NaHS on Ca$_{v3.2}$ channels are dose-dependent. The doses used in the present study were mM concentration and NaHS at these doses were sufficient to activate Ca$_{v3.2}$ channels. Consistently, inhibition or silencing of Ca$_{v3.2}$ channels abolished NaHS-induced scratching in mice (Figs 5 and 6). To our knowledge, there is no report to directly measure the H$_2$S level in/or around primary sensory neurons. Indeed, there were some reports showed the concentration of H$_2$S in plasma ranged from 20–100 μM$^{35}$. However, the local concentration of H$_2$S may be high enough to activate Ca$_{v3.2}$ channels under pathological condition, such as irritable bowel syndrome$^{29}$ or acute H$_2$S intoxication$^{32}$. Acute exposure H$_2$S also cause eye or skin irritation, including itching$^{52}$. Base on the crucial roles of T-type calcium channels (especially Ca$_{v3.2}$ channel) for itch, the relative lower concentration of H$_2$S may suppress itch via its inhibition of Ca$_{v3.2}$ channel under physiological condition. It needs further investigation on the H$_2$S levels under physiological and pathological conditions and the roles of Ca$_{v3.2}$ channel on chronic itch.

T-type calcium channels, including three difference types (termed Ca$_{v3.1}$, Ca$_{v3.2}$, and Ca$_{v3.3}$), are low voltage activated and play important roles in electrical signaling in nerve, heart and muscle$^{36}$. The isoform Ca$_{v3.2}$ is predominant expressed in primary sensory neurons in DRG, which is activated at low voltage close to the resting membrane potential and controls the bursting firing in sensory neurons and so potently modulates neuronal excitability$^{52}$. Recent studies identified that Ca$_{v3.2}$ is expressed by Aδ-low-threshold mechanoreceptors (LTMRs) and C-LTMRs and is required for light-touch perception and mechanical hypersensitivity (alldynia) under neuropathic pain$^{57}$. Our results not only showed that Ca$_{v3.2}$ mediated H$_2$S donors-induced itch, but also demonstrated that Ca$_{v3.2}$ contributes to NaHS-induced alloknosis (touch-evoked itch). Consistent with the cellular distribution of Ca$_{v3.2}$ channel, Ca$_{v3.2}$ more likely plays key role in mechanically-evoked itch. Although the phenomenon of mechanically-evoked itch had been observed in human$^{58}$, the molecular basis for mechanically-evoked itch (a sub-modality of itch) is unclear. We provided important clues for roles of Ca$_{v3.2}$ in mechanically-evoked itch, at least for NaHS-induced touch-evoked itch. The precise contribution of Ca$_{v3.2}$ to mechanically-evoked itch warrants further investigation.

We finally asked about the roles of endogenous H$_2$S in acute itch caused by compound 48/80. The results showed that increased endogenous production of H$_2$S (i.p. injection of NaHS or L-cystein) is able to enhance compound 48/80-induced scratching behavior in mice (Fig. 8). Local application of the CBS inhibitor AOAA or CSE inhibitor PPG also dose-dependently reduced compound 48/80-induced scratching behavior in mice (Fig. 8). Although the expression changes of CBS or CSE under pathological itch conditions need further investigation, our data indicates that manipulating the level of endogenous production of H$_2$S is able to modulate itch responses.

In summary, our findings showed that H$_2$S donors-induced itch response is largely independent of histamine. H$_2$S may activate Ca$_{v3.2}$ T-type calcium channel possible in capsaicin-insensitive A-fibers, but not TRPV1 and TRPA1 in capsaicin-sensitive C-fibers, to elicit itch response in mice. Although further investigation is needed to reveal the roles of H$_2$S in chronic itch, our findings strongly suggest that H$_2$S is one of non-histaminergic itch mediators, although other gasotransmitter may also be involved in itch. Targeting H$_2$S synthesis or Ca$_{v3.2}$ T-type calcium channel may lead to the development of novel and effective anti-itch treatment.

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Author Contributions
X.L.W., B.T., Y.H., X.Y.P., L.H.C., J.C.L. and T.L. designed the experiments. X.L.W., B.T., Y.H., X.Y.P. and L.H.C. carried out the experiments, collected and analyzed the data. X.L.W., B.T., J.C.L., L.H.C. and T.L. wrote the manuscript.

Additional Information
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**Corrigendum: Hydrogen sulfide-induced itch requires activation of Ca\textsubscript{v}3.2 T-type calcium channel in mice**

Xue-Long Wang, Bin Tian, Ya Huang, Xiao-Yan Peng, Li-Hua Chen, Jun-Cheng Li & Tong Liu

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This Article contains errors in the legend of Figure 5.

“(A) Systemic zinc chloride (ZnCl\textsubscript{2}; i.p. 1 mg/kg) significantly inhibited NaHS-induced scratching. (B) Local application of ZnCl\textsubscript{2} (i.d. 5 nmol) significantly inhibited NaHS-induced scratching in both RTX- and vehicle-treated mice. (C) ZnCl\textsubscript{2} (i.d. 5 nmol) significantly inhibited NaHS-induced both forelimb wiping and hindpaw scratching in cheek model. (D) ZnCl\textsubscript{2} (i.pl. 5 nmol) significantly inhibited NaHS-induced flinching. (E) Systemic ascorbic acid (Asc; i.p. 1 mg/kg) significantly inhibited NaHS-induced scratching. (F) Asc (i.d. 1 nmol) significantly inhibited NaHS-induced scratching in both RTX- and vehicle-treated mice. (G) Asc (i.d. 1 nmol) significantly inhibited NaHS-induced both forelimb wiping and hindpaw scratching in cheek model. (H) Asc (i.pl. 1 nmol) significantly inhibited NaHS-induced scratching in RTX-treated mice. (I) Local application of mibebradil (Mib) (i.d. 5–25 nmol) dose-dependently inhibited NaHS-induced scratching in mice. (J) Mib (i.d. 10 nmol) significantly inhibited NaHS-induced scratching in RTX-treated mice. (K) Mib (i.d. 10 nmol) significantly inhibited NaHS-induced both forelimb wiping and hindpaw scratching in cheek model. (L) Mib (i.d. 10 nmol) significantly inhibited NaHS-induced flinching. All data are expressed by means ± SEM. n = 6–8 mice per group. *P < 0.05; **P < 0.01, ***P < 0.001 vs. vehicle control, Student’s t test”.

should read:

“(A) Local application of mibebradil (Mib) (i.d. 5–25 nmol) dose-dependently inhibited NaHS-induced scratching in mice. (B) Mib (i.d. 10 nmol) significantly inhibited NaHS-induced scratching in RTX-treated mice. (C) Mib (i.d. 10 nmol) significantly inhibited NaHS-induced both forelimb wiping and hindpaw scratching in cheek model. (D) Mib (i.d. 10 nmol) significantly inhibited NaHS-induced flinching. (E) Systemic zinc chloride (ZnCl\textsubscript{2}; i.p. 1 mg/kg) significantly inhibited NaHS-induced scratching. (F) Local application of ZnCl\textsubscript{2} (i.d. 5 nmol) significantly inhibited NaHS-induced scratching in both RTX- and vehicle-treated mice. (G) ZnCl\textsubscript{2} (i.d. 5 nmol) significantly inhibited NaHS-induced both forelimb wiping and hindpaw scratching in cheek model. (H) ZnCl\textsubscript{2} (i.pl. 5 nmol) significantly inhibited NaHS-induced flinching. (I) Systemic ascorbic acid (Asc; i.p. 1 mg/kg) significantly inhibited NaHS-induced scratching. (J) Asc (i.d. 1 nmol) significantly inhibited NaHS-induced scratching in both RTX- and vehicle-treated mice. (K) Asc (i.d. 1 nmol) significantly inhibited NaHS-induced both forelimb wiping and hindpaw scratching in cheek model. (L) Asc (i.pl. 1 nmol) significantly inhibited NaHS-induced flinching. All data are expressed by means ± SEM. n = 6–8 mice per group. *P < 0.05; **P < 0.01, ***P < 0.001 vs. vehicle control, Student’s t test”.

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