

# TRPV1 drugs alter core body temperature via central projections of primary afferent sensory neurons

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TRPV1, a capsaicin- and heat-sensitive ion channel, is expressed by peripheral nociceptors and has been implicated in various inflammatory and neuropathic pain conditions. Although pharmacological modulation of TRPV1 has attracted therapeutic interest, their utility is limited because TRPV1 agonists and antagonists thus far examined show thermo-modulatory side effects in animal models and human clinical trials. These on-target effects may result from the perturbation of TRPV1 receptors on nociceptors, which transduce signals to central thermoregulatory circuits and also release pro-inflammatory factors from their peripheral terminals, such as the vasodilative neuropeptide calcitonin gene-related peptide (CGRP). Alternatively, they may originate from the modulation of TRPV1 on vascular smooth muscle cells (vSMCs), where channel activation promotes arteriole constriction. Here, we ask which of these pathways is most responsible for the body temperature perturbations elicited by TRPV1 drugs *in vivo*. We address this question by selectively eliminating TRPV1 expression in sensory neurons or vSMCs and show that only the former abrogates agonist-induced hypothermia and antagonist-induced hyperthermia. Furthermore, lesioning the central projections of TRPV1-positive sensory nerve fibers also abrogates drug-mediated thermo-modulation, whereas eliminating CGRP has no effect. Thus, TRPV1 drugs alter core body temperature by modulating sensory input to the central nervous system, rather than through peripheral actions on the vasculature. These findings suggest how mechanically distinct TRPV1 antagonists may diminish inflammatory pain without affecting core body temperature.

TRPV1 | thermoregulation | sensory neurons | CGRP

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## Introduction

The detection of painful stimuli begins with the activation of peripheral sensory neurons known as nociceptors. The capsaicin (vanilloid) receptor, TRPV1, is a non-selective cation channel expressed by a subset of small diameter unmyelinated (C) and medium diameter myelinated (A $\delta$ ) nociceptors (1). TRPV1 is activated by heat (2, 3) and is modulated by inflammatory mediators, including extracellular protons (4, 5) and bioactive lipids (2), making it one of the key polymodal receptors for detecting noxious stimuli. Many studies have found that TRPV1 contributes not only to acute pain, but also to persistent pain conditions, particularly those associated with inflammation (6). As such, TRPV1 has become an actively pursued drug target for analgesic therapy. Unfortunately, this pursuit has been

hamstrung partly by the fact that many TRPV1 modulators produce core body temperature changes.

Injection or consumption of capsaicin, the active component of chili peppers and a specific TRPV1 agonist, drives a range of thermoregulatory processes, including vasodilation, sweating and panting, all of which promote a decrease in core body temperature (7, 8). Conversely, many TRPV1 antagonists, including drug candidates, induce hyperthermia both in animal models and in human clinical trials. For example, oral administration of AMG517 increases body temperature of some participants to 39-40 °C for 1-4 days (9). Other TRPV1 antagonists, e.g., ABT-102 (10, 11), AZD1386 (12, 13) and JNJ-39439335 (aka. Mavatriptan) (14), also produced varying degree of hyperthermia at therapeutic dosages.

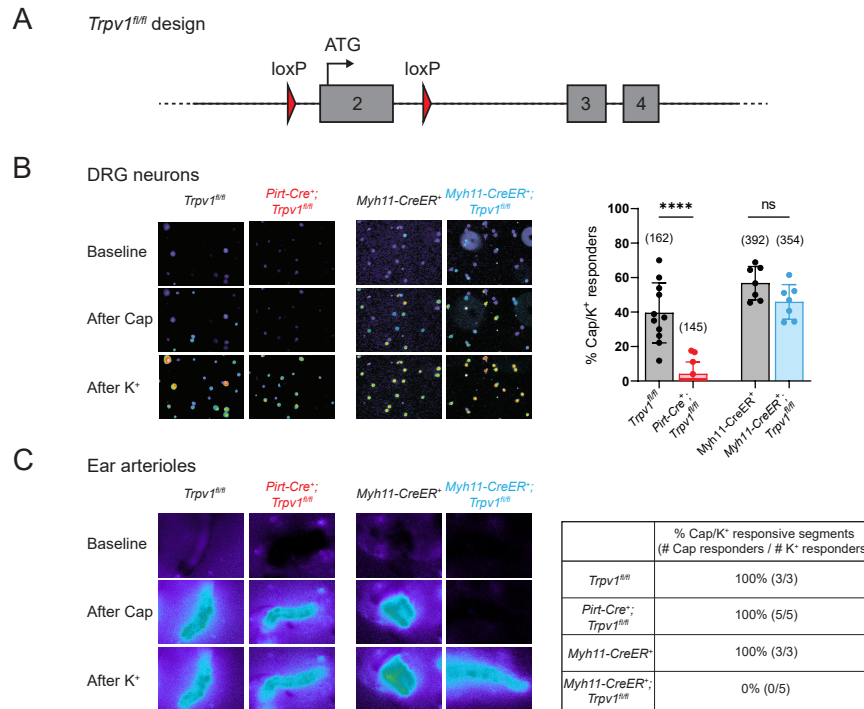
It is unclear what site and mechanism of action underlie these thermoregulatory effects of TRPV1-selective drugs. While nociceptors are the main sites of TRPV1 expression, functional channels are also found on vascular smooth muscle cells (vSMCs) within a subset of terminal arterioles within peripheral (but not central) vasculature (15, 16). The activation of vascular TRPV1 leads to vasoconstriction (15–17) which, depending on the site of action, may shunt blood flow into or away from thermoregulatory tissues, thereby regulating heat dissipation. Unknown, however, is the relative contribution of neuronal and vascular TRPV1 to drug-mediated body temperature modulation. Also at issue is the relative contribution of peripheral versus central processes that are initiated by sensory nerve fiber activation, such as peripheral release of vasodilatory neuropeptides and modulation of thermoregulatory centers in the brain, respectively.

Here, we address these questions by genetically or chemically ablating individual pathways in mice and examining the consequence to agonist/antagonist-induced body temperature responses. We find that TRPV1 expression on sensory neurons and their input to the central nervous system (CNS) (rather than peripheral release of the vasodilative peptide CGRP) predominates in mediating drug-dependent thermo-modulatory effects.

## Results

### Neuronal TRPV1 is essential for drug-mediated changes in core body temperature.

To determine whether drug-evoked body temperature change is mediated by TRPV1 in peripheral nociceptors



**Figure 1. Tissue-specific knockout selectively abolishes functional expression of TRPV1 in sensory neurons or vascular smooth muscle cells.** (A) Design of the *Trpv1*-floxed mouse line. Numbered gray boxes indicate exons. (B) Calcium responses of Fura2-AM-loaded DRG neurons from sensory neuron-specific knockouts (*Pirt-Cre<sup>+</sup>;Trpv1<sup>fl/fl</sup>*), smooth muscle-specific knockouts (*Myh11-CreER<sup>+</sup>;Trpv1<sup>fl/fl</sup>*) and their respective genotype controls following capsaicin (5  $\mu$ M) application. In these and all following experiments, control animals received same tamoxifen treatment as knockouts. High (~50 mM) extracellular K<sup>+</sup> was used to reveal all neurons in the imaging field. Left panels show representative images of 340/380 emission ratio in rainbow scale; summary data are in bar graph on right. Each datapoint represents one experimental preparation. Total numbers of K<sup>+</sup> responders are indicated in brackets. Statistical analyses by unpaired, two-tailed Welch's t-test. (C) Calcium responses of Fura2-AM-loaded ear arterioles of the indicated genotypes to capsaicin (10  $\mu$ M) and high extracellular K<sup>+</sup> application. Table on right show the percentage of capsaicin-responsive segments among K<sup>+</sup>-responsive arteriole segments, with respective number of segments given in brackets.

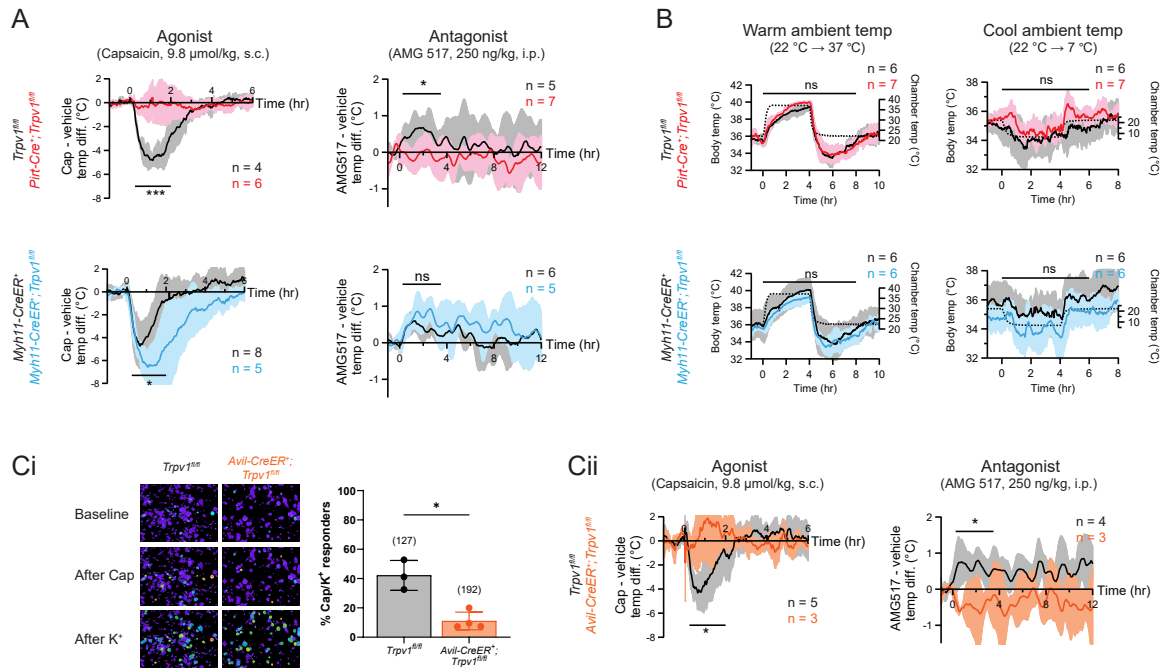
versus vSMCs, we generated mice lacking TRPV1 in each of these locales. First, *Trpv1<sup>fl/fl</sup>* mice were produced by inserting a pair of loxP sites across the second exon of the *Trpv1* gene, which contains the initiator ATG codon (Figure 1A). Crossing this line with *Pirt-Cre<sup>+</sup>* (18) or *Myh11-CreER<sup>+</sup>* (19) mice allowed for specific elimination of TRPV1 expression in primary sensory neurons or vSMCs, respectively. We confirmed the selectivity of these genetic manipulations by using calcium imaging to demonstrate loss of capsaicin sensitivity of dorsal root ganglion (DRG) neurons or of ear skin arteriole segments harvested from these animals (Figure 1B and C). Both cell type-specific knockout lines exhibited normal basal core body temperature and showed no noticeable peculiarities in the circadian pattern of body temperature (Figure 1-figure supplement 1). These phenotypes resemble those observed in global TRPV1 knockout mice (20–22), likely reflecting redundant or compensatory actions of other thermosensitive receptors in these animals (23, 24).

To examine acute effects of TRPV1 modulation in wild type or mutant mice, we administered an agonist (capsaicin, 9.8  $\mu$ mol/kg, single dose, s.c.) and recorded core body temperature changes with telemetric probes implanted in the abdomen. Consistent with previous observations (25), capsaicin induced robust hypothermia of ~5 °C

at peak (~1 hr post-injection) in control (*Trpv1<sup>fl/fl</sup>* or *Myh11-CreER<sup>+</sup>*) mice. Strikingly, this capsaicin-evoked hypothermic response was completely abolished in sensory neuron-specific knockouts (*Pirt-Cre<sup>+</sup>;Trpv1<sup>fl/fl</sup>*), recapitulating the phenotype observed in global TRPV1 knockouts (25). Unexpectedly, the smooth muscle-specific knockout (*Myh11-CreER<sup>+</sup>;Trpv1<sup>fl/fl</sup>*) (Figure 2A, left column) showed a robust hypothermic response that was even more pronounced in both amplitude and duration compared to controls. Together, these observations show that agonist-evoked hypothermia requires TRPV1 expressed by sensory nerve fibers, but activation of channels on the vasculature has an opposing effect, likely related to vasoconstriction that limits radiant heat loss.

We next recorded core body temperature responses following administration of AMG517 (250 ng/kg, 3 doses, i.p.), which we selected as a representative TRPV1 antagonist because its hyperthermic effect has been verified to be on-target (i.e., absent in *Trpv1<sup>-/-</sup>* mice) and its dose-response relationship has been documented previously (26). Indeed, AMG517 elicited a characteristic hyperthermia in control animals (as in human clinical trials), reaching a peak of ~0.5°C within 2 hr after injection (c.f. Garami et al., 2010). This response was abolished in *Pirt-Cre<sup>+</sup>;Trpv1<sup>fl/fl</sup>* mice but did not change significantly in





**Figure 2. Neuronal, rather than vascular, TRPV1 mediates drug-evoked thermo-modulatory effects.** (A) Telemetric recordings of core body temperatures of *Pirt-Cre<sup>+</sup>;Trpv1<sup>fl/fl</sup>* mice (red), *Myh11-CreER<sup>+</sup>;Trpv1<sup>fl/fl</sup>* mice (blue) and their respective genotype controls (black). Traces show responses to subcutaneous injection of capsaicin (9.8  $\mu\text{mol/kg}$ ) and intraperitoneal injection of AMG517 (250 nmol/kg) administered at time 0, after subtracting measurements from vehicle-alone trials. Baselines (mean temperature across the 1-hr window before drug administration) have been offset to zero for comparison. Responses to AMG517 were smoothed by taking running averages to better display trends. All traces are mean  $\pm$  SD. Black bars above traces indicate the time windows over which statistical comparisons (mixed effects analyses) between genotypes were made. (B) Body temperature response of the above genotypes to changes in ambient temperature (dotted line, right axis). Statistical comparisons by mixed effects analyses. (Ci) Representative images (left) and quantification (right) of DRG neurons from *Avil-CreER<sup>+</sup>;Trpv1<sup>fl/fl</sup>* and control mice that are responsive to capsaicin (5  $\mu\text{M}$ ) and high extracellular K<sup>+</sup>. (Cii) Body temperature recordings as in (A) but from *Avil-CreER<sup>+</sup>;Trpv1<sup>fl/fl</sup>* mice (orange) and controls (black).

*Myh11-CreER<sup>+</sup>;Trpv1<sup>fl/fl</sup>* animals (Figure 2A, right column). Loss of TRPV1 in either cell type did not impact body temperature regulation in response to changes in ambient temperature (Figure 2B), suggesting that relevant thermosensory mechanisms (e.g., contribution from other thermosensitive TRP channels) and thermoeffector pathways (24) function normally in these animals and are unlikely to explain the differential pharmacological responses that we observed across genotypes.

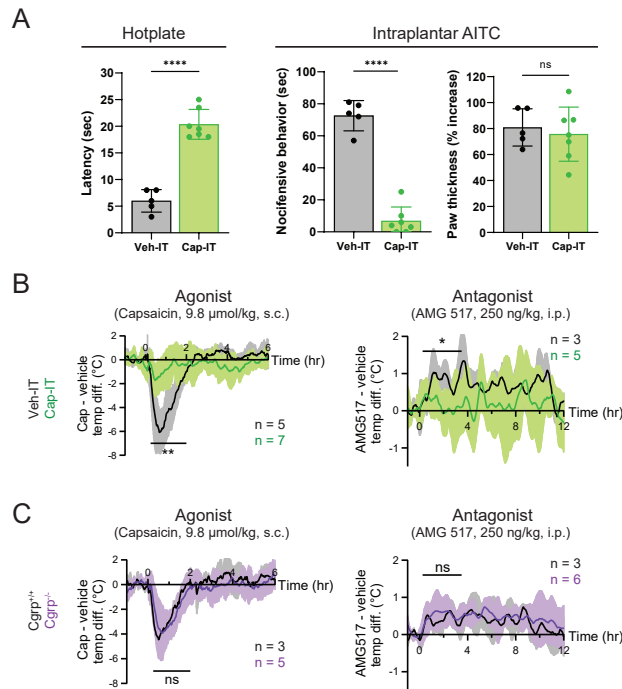
To rule out developmental factors that could account for the observed lack of capsaicin/AMG517-evoked responses in our neuronal TRPV1 knockout, we also studied an *Avil-CreER<sup>+</sup>* (27) line to ablate TRPV1 in primary sensory neurons after 8 weeks of age. Functional ablation was less effective and more variable in *Avil-CreER<sup>+</sup>;Trpv1<sup>fl/fl</sup>* mice compared to *Pirt-Cre<sup>+</sup>;Trpv1<sup>fl/fl</sup>* animals (~25% of capsaicin-responsive neurons remained after tamoxifen treatment) (Figure 2Ci). Nonetheless, capsaicin/AMG517-induced body temperature changes were similarly abrogated in these inducible knockouts (Figure 2Cii). These data further support the conclusion that sensory TRPV1 predominates in mediating drug-evoked thermo-modulatory responses.

### Body temperature perturbations require central transmission, but not peripheral CGRP release.

When activated, primary afferent nociceptors release

transmitters from their central terminals, which signals to the spinal cord and brain. However, these neurons are unique in that they also release transmitters peripherally to initiate neurogenic inflammatory processes, including CGRP-evoked vasodilation. Importantly, such peripheral release occurs independently of central transmission.

We, therefore, asked whether peripheral or central transmission from TRPV1<sup>+</sup> nociceptors is required for the capsaicin/AMG517-evoked responses. To address this question, we selectively ablated the central projections of TRPV1<sup>+</sup> DRG neurons by intrathecal injection of capsaicin (10  $\mu\text{g}/\text{mouse}$ ; control mice received vehicle). Successful ablation of central TRPV1<sup>+</sup> terminals was validated by decreased nocifensive behavior on a hot plate (Figure 3A), as previously described (28). This procedure leaves peripheral projections of TRPV1<sup>+</sup> fibers intact and capable of releasing pro-inflammatory agents (29). Figure 3A shows that in this setting, namely intraplantar injection of the TRPA1 agonist, allyl isothiocyanate (AITC), which activates a subset of TRPV1<sup>+</sup> nociceptors (30), failed to induce nocifensive behaviors in animals treated intrathecally with capsaicin, but still elicited robust local edema, equivalent to that seen in vehicle treated controls. Interestingly, capsaicin/AMG517-evoked body temperature responses were significantly reduced in mice with ablated central projections (Figure 3B), indicating that central transmission from TRPV1<sup>+</sup> DRG fibers is required for



**Figure 3. Thermo-modulatory effects of TRPV1-selective drugs require central transmission, but not peripheral CGRP release.** (A) Comparison of nocifensive behaviors of mice injected intrathecally with capsaicin (Cap-IT, green, 10 μg/mouse, n = 7) versus vehicle controls (black, n = 5). Cap-IT mice were significantly slower (i.e., had higher latency) in showing the first nocifensive response in a hot-plate test (left). Cap-IT mice also spent significantly less time in nocifensive behaviors (middle) after intraplantar AITC injection despite similar levels of local edema (right), suggesting successful selective ablation of TRPV1<sup>+</sup> nociceptors' central projections. Unpaired, two-tailed Welch's t-test in all panels. (B) Core body temperature recordings showing diminished hypo- and hyper-thermic responses to capsaicin and AMG517, respectively, in Cap-IT mice. (C) Normal hypo- and hyper-thermic responses to capsaicin and AMG517, respectively, in *Cgrp*<sup>-/-</sup> mice. All traces are plotted and statistical tests carried out as in **Figure 2**.

drug actions. A small residual effect of these drugs was observed (Figure 3B), which could reflect contributions from incompletely ablated central projections, vagal or trigeminal afferents, or sustained peripheral CGRP release. To address this last possibility, we examined mice lacking CGRP (31)(Figure 3–figure supplement 1) and found that they showed normal body temperature responses to capsaicin or AMG517 (Figure 3C), suggesting that peripheral CGRP release from TRPV1<sup>+</sup> nociceptors is largely dispensable in this drug-mediated thermo-modulatory process, consistent with a centrally-mediated mechanism.

## Discussion

In the past two decades, TRPV1 antagonists have been actively pursued by the pharmaceutical industry to treat diverse persistent pain conditions. Despite analgesic efficacy in some indications, notably osteoarthritic knee pain, two main ‘on-target’ adverse side effects have limited further development of these drugs for clinical use: diminished sensitivity to potentially injurious heat, and increased core body temperature (12, 32, 33). Our current work addresses the mechanism underlying the latter adverse effect. Our findings show that the thermo-modulatory responses of mice to acute systemic activation/inhibition of TRPV1 are mediated predominantly by the central projections of TRPV1-expressing nociceptors and thus likely involve output from thermoregulatory centers in the

hypothalamus. On the other hand, the concurrent and robust agonist-evoked hypothermia in smooth muscle-specific knockouts reveals an opposing action by vascular TRPV1 that may help constrain hypothermic responses within a tolerable temperature or temporal window. Incidentally, Sharif-Naieni et al. (34) has implicated supraoptic hypothalamic expression of an N-terminal variant of TRPV1 in anticipatory vasopressin release during hyperthermia. However, whether or not the neurons are more generally involved in thermoregulation is unclear. Most importantly, our finding that selective deletion of TRPV1 from sensory neurons prevents the thermo-modulatory side effects of pharmacological manipulation of TRPV1 argues that any hypothalamic expression is not sufficient to sustain these effects *in vivo*.

In addition, our results suggest a rationale for developing TRPV1 antagonists that would selectively or preferentially inhibit peripheral release of pro-inflammatory peptides and transmitters, without compromising the central pathway and thus thermoregulation. At least for another TRP channel, TRPA1, we have recently discovered a scorpion toxin that stabilizes this channel in a unique open state with a low Ca<sup>2+</sup> permeability. Thus, application of the toxin to nociceptors *in vivo* promotes neuronal transmission, but not the release of CGRP and likely other transmitters, hence eliciting pain without neurogenic inflammation (29). A similar mode-specific (but inhibitory) modulator for TRPV1 could,

in principle, produce analgesia by reducing neuropeptide release from peripheral terminals to diminish neurogenic inflammation. Interestingly, the  $\text{Ca}^{2+}$  fraction of TRPV1 current is apparently smaller when the channel is activated by low pH, rather than by capsaicin (35). Moreover, recent generation of TRPV1 antagonists that do not block proton-evoked channel activation may be less likely to alter core body temperature (26). It remains to be seen whether these drugs indeed preferentially inhibit peripheral over central signaling, and whether this propensity can be harnessed further to treat inflammatory pain while sparing acute heat sensitivity and thermoneutrality.

## Materials and Methods

**Animals.** All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee at UCSF. *Pirt-Cre<sup>+</sup>* mice were kindly provided by Dr. Xinzhong Dong at Johns Hopkins University. *Myh11-CreER<sup>+</sup>*, *Avil-CreER<sup>+</sup>* and *Cgrp-CreER<sup>+</sup>* mice were obtained from the Jackson Laboratory. *Cgrp-CreER<sup>+</sup>* mice, in which the ATG translational initiator of the *Calca* gene is replaced by a CreER sequence, were crossed to homozygosity to produce *Cgrp<sup>-/-</sup>*. For Myh11-CreER induction, regular food was replaced with chow containing 400 mg/kg tamoxifen citrate (Harlan Teklad, TAM400) for one week when mice were at 5 weeks of age. For Avil-CreER induction, mice were injected with 2 mg tamoxifen/day (Sigma-Aldrich, T5648, 20 mg/ml in corn oil) for 5 consecutive days at 8 weeks of age and 5 mg tamoxifen/day for another 5 consecutive days at 10 weeks of age to ensure effective knockout. Mice were raised under 12:12 light-dark cycles with ad libitum access to food and water. In all experiments, we used male mice that were 2-5 months old. In most cases, littermates were used as genotype controls and were subjected to the same tamoxifen treatment.

**Generation of *Trpv1<sup>fl/fl</sup>* mice.** Two loxP sites were inserted by the CRISPR/Cas system across the second exon of the *Trpv1* gene, where the ATG codon is located. Briefly, two crRNAs (5'-UCA AGG UGU CCU GAU UAA CGG UUU UAG AGC UAU GCU-3' and 5'-CCA CAC UCU UGC GAU CUU GCG UUU UAG AGC UAU GCU-3') were separately annealed to tracrRNA (IDT, 1072532) at 1:1 molar ratio in a thermocycler at 95 °C for 5 min and then ramped down to 25 °C at 5 °C /min. The annealed oligos were mixed and incubated with Cas9 nuclease (IDT, 1081058) at a final concentration of 20 ng/μl (for both oligos and nuclease) at room temperature for 10-15 min to allow the formation of ribonucleoprotein (RNP) complexes. A synthesized DNA gene fragment spanning a homology region from 500 bp upstream to 500 bp downstream of the two loxP insert sites was cloned into pBluescript II KS(+) vector (Genscript). The gene fragment was released by EcoRI and KpnI digestion and was added as a repair template to the RNP complexes at a final concentration of 10 ng/μl. The mixture was filtered through a 0.1-μm pore centrifugal filter (Millipore, UFC30VV25) to remove any

solid particles and was microinjected into mouse embryos. Animals carrying the targeted loxP insertions were identified by PCR on tail DNA and confirmed by sequencing. A set of three primers were used to genotype the *Trpv1* allele: *Trpv1* forward, 5'-GTG TCA GCT CCC TCT CAA GG-3'; *Trpv1*WT reverse, 5'-GCC AGA CCA CCT CTG AAG GCT T-3'; *Trpv1*FL reverse, 5'-GCC AGA CCA CCT CTG AAA TAA C-3'. The primer pair of *Trpv1* forward and *Trpv1*WT reverse gave a 1,185-bp band for the WT allele, whereas the pair of *Trpv1* forward and *Trpv1*FL reverse gave a 1,253-bp band for the floxed allele.

**Calcium imaging.** Mice were euthanized by CO<sub>2</sub> asphyxiation followed by decapitation according to American Veterinary Medical Association's guidelines. For calcium imaging of sensory neurons, DRGs were harvested from a mouse into ice-cold L-15 medium (Thermo Fisher Scientific, 11415064). The tissues were digested with papain (Worthington Biochemical, LS003126, 60 unit) for 10 min, followed by collagenase (Sigma-Aldrich, C9407, 12 mg/ml) and dispase I (Sigma-Aldrich, D4818, 15 mg/ml) for another 10 min, both at 37 °C. The digested ganglia were then mechanically triturated in L-15 medium containing 10% fetal bovine serum (FBS) to dissociate the neurons and were plated onto coverslips pre-coated with poly-D-lysine (Sigma-Aldrich, A-003-E) and mouse laminin (Thermo Fisher Scientific, 23017015). Neurons were cultured in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose (UCSF Media Production, DME-H21) and 10% horse serum overnight at 37 °C under 5% CO<sub>2</sub>. When used for calcium imaging, neurons were washed with Ringer's solution (140 mM NaCl, 5 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM HEPES, and 10 mM Glucose, pH 7.4 with NaOH; 290-300 mOsm kg<sup>-1</sup>) and loaded with 10 μg/ml Fura2-AM (Thermo Fisher Scientific, F1201, stock: 1 mg/ml in DMSO) in Ringer's solution containing 0.02% Pluronic F-127 (Thermo Fisher Scientific, P3000MP) at room temperature in the dark for 1 hr. After Ringer's washes, neurons were bathed in Ringer's solution and imaged under 340 nm and 380 nm excitation (Sutter, Lambda LS illuminator) at a time interval of 2 sec with a FLIR Grasshopper3 camera. Images were acquired and analyzed with the MetaFluor software (Molecular Devices). Capsaicin (diluted to 10 μM from a 10 mM stock in DMSO) was delivered by manual pipetting to achieve a final concentration of 5 μM in the bath. An equal volume of high-K<sup>+</sup> Ringer's solution (same as Ringer's except for 5 mM NaCl and 140 mM KCl) was added to reveal neuronal identities. Multiple coverslips from at least two animals were examined for each genotype.

For calcium imaging of arterioles, third order arterioles were isolated from ears in the following solution: 137 mM NaCl, 5.6 mM KCl, 1 mM MgCl<sub>2</sub>, 0.42 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM NaH<sub>2</sub>PO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 10 mM HEPES, and 1 mg/ml BSA, pH 7.4 with NaOH (15). Arterioles were cut in pieces and laid on Cell-Tak-coated coverslips. Fura loading and calcium imaging were done in Ringer's solution as above, except that a 20 μM stock of capsaicin was used to achieve a final concentration of 10 μM in the bath.

**Telemetry.** Mice were anesthetized and implanted each with a wireless temperature probe (Starr Life Sciences, G2 E-Mitter) in its abdominal cavity. Mice were allowed to recover for at least 1 week before their home cages were transferred onto Energizers/Receivers (Starr Life Sciences, ER4000) in a rodent incubator (Power Scientific, RIS33SD or RIS70SD) maintained at 22 °C. Core body temperature was recorded with the VitalView software at a sampling rate of once per min. Capsaicin/AMG517 injections and chamber temperature changes began after at least 1 day of habituation in the incubator.

**Injections.** For each mouse, a single dose of 9.8  $\mu\text{mol/kg}$  capsaicin (Tocris, 0462, stock: 0.98  $\mu\text{mol/ml}$  in 0.72% saline with 10% Tween 80 and 10% ethanol) was administered by subcutaneous injection. Vehicle was injected to the same animal 3 days before/after capsaicin injection. Since the hyperthermic effect of AMG517 is mild, in order to reveal the response amid random body temperature fluctuations, mice were subjected to 3 alternating sets of intraperitoneal injections of 250 ng/kg of AMG517 (Cayman Chemical Company, 26191, stock: 50 ng/ml in 100% ethanol) and vehicle, with any two injections spaced 3 days apart. Averages across the 3 trials were used. To avoid any circadian effect, all capsaicin and AMG517 injections were done at 10 AM. Some mice experienced both capsaicin and AMG517 challenges; no obvious difference in TRPV1 drug-evoked responses was seen in animals receiving one type vs two types of challenges, so data were pooled for analyses. To ablate the central projections of TRPV1<sup>+</sup> sensory neurons, mice were anesthetized and intrathecally injected with 10  $\mu\text{g}$  capsaicin (stock: 1 mg/ml in 0.72% saline with 10% Tween 80 and 10% ethanol). Cap-IT mice were allowed to recover for at least 1 week before telemetric recording.

**Nocifensive behavioral tests.** Two sets of behavioral tests were used to confirm successful ablation of the central projections of sensory neurons. In the first, we measured the time latency to first nocifensive response (e.g. licking or flicking paws, jumping) when a mouse was placed on a 55 °C hotplate. Mice were removed from the hotplate once nocifensive behaviors were observed or at a cutoff of 30 sec, whichever was the shortest, to minimize tissue damage. Two sets of measurements were taken for each mouse on different days. In the second test, we injected 0.75% (v/v) allyl isothiocyanate (AITC; Sigma-Aldrich, 377430) in mineral oil intraplantar to the hindpaw after light isoflurane anesthesia. After recovery, we videorecorded the individually-housed mice in transparent cylinders for 20 min and quantified the amount of time in which nocifensive behaviors were exhibited. Paw thickness was measured by digital calipers before and after AITC injection to assess the degree of local edema. Only mice that has passed the cutoff in at least one of the two hot plate trials and showed at least 50 sec of nocifensive behaviors after AITC injection were used for telemetric measurements. The time latency for the hotplate experiment reported in Figure 3A is the average of the two measurements.

**Data analysis and statistics.** Unless otherwise specified, collective data herein are reported as mean  $\pm$  SD. Statistical analyses were done with the Prism software (Graphpad). In calcium imaging assays and nocifensive behavioral tests, comparisons across genotypes were made by using unpaired, two-tailed Welch's t-test, assuming that parametric approach is applicable based on previous similar experiments. For telemetric measurements, mixed effects analyses were performed without assuming sphericity over the duration of body temperature change (indicated in plots). Mice that showed considerable body temperature fluctuations at baseline were excluded from analyses. For responses to AMG517, measurements were averaged across the 3 injection trials before comparisons. Significance was determined by the computed p value across genotypes. For all tests,  $\alpha = 0.05$ . \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; \*\*\*\*:  $p \leq 0.0001$ ; n.s.: not significant. For display in figures, circadian body temperature fluctuations and responses to AMG517 were smoothed by taking running averages over a 30-min window before computing the mean and SD. This processing was done only for improving visualization (dampening random, fast fluctuations) and did not dramatically affect the kinetics of the slow AMG517 effect; statistical comparisons were done on raw data, not the smoothed data.

**Immunohistochemistry.** DRGs were acutely harvested into ice-cold L-15 medium and transferred into 4% paraformaldehyde for overnight fixation. After washes with phosphate-buffered saline (PBS, Quality Biological, 119-069-491), the ganglia were allowed to settle in 30% sucrose (Sigma-Aldrich, S7903) at 4 °C and were then cryopreserved in Tissue-Tek O.C.T. Compound (Sakura Finetek USA) for sectioning at 10- $\mu\text{m}$  thickness. Sections were washed with PBST (i.e., PBS containing 0.1% Triton X-100 (Sigma-Aldrich, T8787)) and incubated with blocking buffer (PBST containing 10% normal goat serum from Thermo Fisher Scientific, 16-210-064) at room temperature for 1 hr. Subsequently, sections were incubated with rabbit polyclonal anti-CGRP (Abcam, ab36001, 1:400) and guinea pig polyclonal anti-NeuN (Synaptic Systems, 266004, 1:400) in blocking buffer at 4C overnight. Following several washes, the sections were incubated with goat anti-rabbit IgG Alexa Fluor-488 (Thermo Fisher Scientific, A-11034, 1:500) and goat anti-guinea pig IgG Alexa Fluor-597 (Thermo Fisher Scientific, A-11076, 1:500) in blocking buffer at room temperature for 1 hr. Finally, the sections were washed and mounted with Fluoromount-G (SouthernBiotech, 0100-01). Z-stack images were taken with a Nikon CSU-W1 spinning disk confocal microscope. Maximal intensity projections were generated by Fiji.

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