TRESK background $K^+$ channel deletion selectively uncovers enhanced mechanical and cold sensitivity

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Key points
- TRESK background $K^+$ channel is expressed in sensory neurons and acts as a brake to reduce neuronal activation.
- Deletion of the channel enhances the excitability of nociceptors.
- Skin nociceptive C-fibres show an enhanced activation by cold and mechanical stimulation in TRESK knockout animals.
- Channel deletion selectively enhances mechanical and cold sensitivity in mice, without altering sensitivity to heat.
- These results indicate that the channel regulates the excitability of specific neuronal sub-populations involved in mechanosensitivity and cold-sensing.

Abstract  Background potassium-permeable ion channels play a critical role in tuning the excitability of nociceptors, yet the precise role played by different subsets of channels is not fully understood. Decreases in TRESK (TWIK-related spinal cord $K^+$ channel) expression/function enhance excitability of sensory neurons, but its role in somatosensory perception and nociception is poorly understood. Here, we used a TRESK knockout (KO) mouse to address these questions. We show that TRESK regulates the sensitivity of sensory neurons in a modality-specific manner, contributing to mechanical and cold sensitivity but without any effect on heat sensitivity. Nociceptive neurons isolated from TRESK KO mice show a decreased threshold for activation and skin nociceptive C-fibres show an enhanced activation by cold and mechanical stimulation that

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was also observed in behavioural tests *in vivo*. TRESK is also involved in osmotic pain and in early phases of formalin-induced inflammatory pain, but not in the development of mechanical and heat hyperalgesia during chronic pain. In contrast, mice lacking TRESK present cold allodynia that is not further enhanced by oxaliplatin. In summary, genetic removal of TRESK uncovers enhanced mechanical and cold sensitivity, indicating that the channel regulates the excitability of specific neuronal subpopulations involved in mechanosensitivity and cold-sensing, acting as a brake to prevent activation by innocuous stimuli.

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**Introduction**

The combined activation of depolarizing and hyperpolarizing ion channels determines the likelihood of excitation and generation of action potentials (APs) in specific subtypes of sensory neurons such as thermoreceptors, mechanoreceptors or nociceptors. Each subtype presents a characteristic pattern of expression of ion channels that define their intrinsic physiological properties such as firing pattern and AP characteristics (Zheng *et al.* 2019). Two-pore domain potassium channels (K_{2P}) are expressed in different subpopulations of sensory neurons, including nociceptors where they carry most of the ‘leak’ or background hyperpolarizing current (Enyedi & Czirják, 2010). Their electrophysiological properties allow them to carry K⁺ currents over a wide range of membrane potentials and hence they are key determinants of neuronal excitability, decreasing the probability of depolarizing stimuli to reach AP threshold, as well as shaping the neuron firing response (Enyedi & Czirják, 2010). TRESK (TWIK-related spinal cord K⁺ channel), along with other K_{2P} family members TREK-1, TREK-2 and TRAAK channels, generate the major contribution to leak currents in trigeminal (TG) and dorsal root ganglion (DRG) neurons (Patel *et al.* 1998; Kang & Kim, 2006; Dobler *et al.* 2007; Yamamoto *et al.* 2009; Tulleuda *et al.* 2011). These channels have been implicated in pain perception induced by mechanical, thermal and chemical stimuli, as well as in neuropathic and inflammatory pain (Alloui *et al.* 2006; Noël *et al.* 2009; Tulleuda *et al.* 2011; Marsh *et al.* 2012; Acosta *et al.* 2014; Pereira *et al.* 2014). The modulatory effect that these channels will exert on nociceptors will depend on their expression pattern and their individual properties. Indeed, deletion of one of the K_{2P} channels enhances the specific sensitivity to certain external stimuli but not others, rather than exerting a general brake on neuronal excitability by decreasing the total K⁺ current. In humans, rats and mice, TRESK shows a high expression in DRG and TG neurons and is particularly enriched in sensory ganglia compared to other neural and non-neural tissues (Kang & Kim, 2006; Dobler *et al.* 2007; Marsh *et al.* 2012; Manteniotis *et al.* 2013; Flegel *et al.* 2015; Usoskin *et al.* 2015; Ray *et al.* 2018; Zeisel *et al.* 2018). Single-cell RNA sequencing data have provided further insight into the role of TRESK, showing that it is present in a subpopulation of low-threshold mechanoreceptors involved in touch sensation (expressing TRKB and Piezo2) and, predominantly, in non-peptidergic nociceptors (Usoskin *et al.* 2015; Zeisel *et al.* 2018; Zheng *et al.* 2019). Interestingly, TRESK expression is down-regulated in different pain conditions, comprising sciatic nerve axotomy (Tulleuda *et al.* 2011), spared nerve injury (Zhou *et al.* 2013) and chronic inflammation (Marsh *et al.* 2012), contributing, together with other changes, to enhance neuronal excitability. In contrast, increasing TRESK expression resulted in a decrease in neuronal excitability and amelioration of painful behaviours (Zhou *et al.* 2012, 2013; Guo & Cao, 2014). Similarly, mice with a loss-of-function mutation in TRESK [G339R] showed a significant reduction of outward K⁺ current, increased excitability and reduced rheobase (Dobler *et al.* 2007). Furthermore, a frameshift mutation leading to the truncation of the channel has been associated with familial migraine with aura, thus involving TRESK in the enhanced activation of the trigeminovascular system and the release of inflammatory neuropeptides in the meninges and cerebral vessels triggering migraine pain (Lafrenière *et al.* 2010). This is supported by a recent study demonstrating that mutant TRESK can heteromerize with TREK-1/2 to decrease their function and trigger migraine pain (Royal *et al.* 2019).

Despite the fact that TRESK shares some functional properties with other K_{2P} channels, its high expression in sensory neurons points to a relevant and yet unexplored role in sensory perception and pain. We report that TRESK removal uncovers enhanced cold and mechanical sensitivity without affecting thermal sensitivity to warm or hot conditions.

**Methods**

**Animals**

All behavioural and experimental procedures were carried out in accordance with the recommendations of the

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Definitive Null Allele Design. At 3 weeks of age, WT or KO newborn mice were weaned, separated and identified by ear punching. Genomic DNA was isolated from tail snip cultures, electrophysiology, calcium imaging, nerve fibre recording and behavior] unless differentially indicated. Mice were housed at 22°C with free access to food and water in an alternating 12 h light and dark cycle. TRESK (Kcnk18/K2P18.1) knockout mice (KO) and wild-type (WT) littermates were obtained from the KOMP Repository (Mouse Biology Program, University of California, Davis, CA, USA). The TRESK KO mouse was generated by replacing the complete Kcnk18 gene by a ZEN-UB1 cassette according to VelociGene’s KOMP Definitive Null Allele Design. At 3 weeks of age, WT or KO newborn mice were weaned, separated and identified by ear punching. Genomic DNA was isolated from tail snip samples with a Maxwell Mouse Tail DNA Purification Kit (Promega, Madison, WI, USA). PCR was performed with primers to detect the Kcnk18 gene: forward 5′-ACCAA CACCAAGCTGTCTTGTTTCTC-3′ and reverse 5′-GCAG AGATGGACGGACAGACATAGATG-3′ or the inserted cassette in the KO mice: forward (REG-Neo-F) 5′-GCAG CCTCTGTCCACATACACTTCA-3′ and reverse (gene-specific) 5′-AGACCTCTCCAGGCTAAACAATTCTGC-3′. The PCR mixture contained 1 µl DNA sample, 2.5 μl PCR buffer (10× concentration), 2 µl dNTP mixture (2.5 mM), 0.5 µl (20 μM) forward and reverse primers, 0.2 µl Taq DNA polymerase (5 U µl−1), 1.7 µl MgCl2 (25 mM), 6.5 µl betaine (5 M), 0.325 µl DMSO and 9.7 µl water (final volume of 25 µl). PCR amplifications were carried out with 31 cycles in a programmable thermal cycler (Eppendorf AG, Hamburg, Germany). The programme used was: 94°C for 5 min and cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 40 s, with a final extension at 72°C for 5 min. PCR products were analysed by electrophoresis in 1% agarose gels. Once identified, genotyped animals were used as breeders for colony expansion and their offspring were used in all experimental procedures in which mice were required.

**Behavioural studies**

Female and male WT or TRESK KO mice between 8 and 15 weeks of age were used in all behavioural studies. To avoid stress-induced variability in the results, mice were habituated to the experimental room and the experimental setup prior to testing. Behavioural measurements were done in a quiet room, taking great care to minimize or avoid discomfort of the animals. All experimenters were blinded to the genotype or the drug/vehicle assayed.

**Mouse mechanical sensitivity**

Mechanical sensitivity of WT and TRESK KO mice was assessed using the ‘up and down’ method by the application of calibrated von Frey filaments (North Coast Medical, Inc., Morgan Hill, CA, USA) as previously described (Tulleuda et al. 2011; Castellanos et al. 2018). The von Frey filaments [size: 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17, 4.31 and 4.56; equivalent to (in grams) 0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2 and 4] were applied perpendicularly to the plantar surface of the hind paw and gently pushed to the bending point for 5 s. The 50% withdrawal threshold was determined using the up and down method (Chaplan et al. 1994). A brisk hind paw lift in response to von Frey filament stimulation was regarded as a withdrawal response. A dynamic plantar aesthesiometer (Ugo Basile, Gemonio, Italy) was also used to assess mechanical sensitivity. A von Frey-type 0.5 mm filament was applied with a 10 s ramp (0–7.5 g) and the hind paw withdrawal threshold of mice was recorded.

**Mouse thermal sensitivity**

**Hot plate and cold plate test.** After habituation, the cold/hot plate apparatus (Ugo Basile) was set to 2, 52 or 56°C and animals were individually placed in the centre of the plate. Latency time to elicit a nociceptive behaviour (a jump or a paw lick/lift) was counted with the apparatus stopwatch and the average of three separate trials was used as a measurement. Since the temperatures tested are in the noxious range, a cut-off time of 25 s was established to avoid tissue damage.

**Dynamic hot plate test.** In contrast to the conventional hot plate, the dynamic hot plate allows the testing of a wide range of temperatures. Before testing, animals were habituated and later placed individually within the hot/cold plate apparatus where the plate temperature increased from 30 to 50°C at 1°C min−1. To determine the temperature that is perceived as noxious for mice and to quantify pain-related behaviours, the number of jumps at each temperature was scored.

**Radiant heat test.** The heat sensitivity of mice was assessed by measuring hind paw withdrawal latency from a radiant infrared source (Hargreaves’ method) using the Ugo Basile Model 37370 Plantar test. Each measurement was the mean of three trials spaced 15 min apart. For all experiments, infrared intensity was set to 30% and a cut-off time of 20 s was established to avoid skin burn damage.

**Thermal place preference test.** The thermal place preference test is a test of better comfort temperature...
rather than an indicator of temperature aversion. The hot/cold plate apparatus was placed side by side with a complementary plate and a small divider platform was situated between them to connect the two devices (Ugo Basile). The reference plate was always set at 30°C and the test plate was set at 50, 40, 30, 20, 15 or 10°C. Animals were habituated at the experience room for 30 min before testing and then they were allowed to investigate the testing setup for 10 min or until animals crossed consistently from one plate to the other. Mice were then placed individually onto the centre of the platform and once they crossed to a plate the cumulative time they spent on each plate for a total of 5 min was then counted. Only animals that performed properly when both plates were set to 30°C were used for the study (50% of the time at each plate and more than two crossings). To exclude the possibility that animals could be learning which was the reference plate, the position of the reference and the test plate was switched randomly between trials.

Cold plantar assay

To complement the cold plate test, we studied noxious cold sensitivity of TRESK KO mice using the cold plantar assay (Brenner et al. 2012). This assay produces an unambiguous nocifensive response that is easily identified when compared to the cold plate test. Animals were placed on top of a 1/8-inch thick glass plate and were enclosed in transparent boxes separated by opaque dividers to prevent animals seeing each other. The cold probe consisted of a modified 3 ml syringe filled with freshly powdered dry ice. This powder was then packed into a pellet and its surface was flattened. Using a mirror to target the mouse hind paw, we applied the dry ice pellet below the glass, making sure that the paw was completely in contact with it. This delivers a cooling ramp to the mice paw and a few seconds later withdrawal responses occur. The withdrawal latency time was measured with a stopwatch and the final withdrawal latency time for each animal was the average of three trials, which were tested at intervals of at least 15 min. A cut-off time of 20 s was used to prevent tissue damage.

Evaluation of nocifensive behaviour

Using a 30 g needle, 10 µl of a solution containing 100 µM allyl isothiocyanate (AITC; 10%), capsaicin (1 µg/10 µl) or their vehicle solutions were administered intradermally into the plantar surface of the hind paw. Mouse behaviour was observed and the number of flinches and lickings of the paw were counted for a 5-min period starting immediately after the injection. On the day previous to testing, animals were habituated to the testing room and to the handling procedure. The flinching and licking test was also used to examine the painful response of mice to different osmolality solutions, both in naive and in sensitized conditions. Then, 10 µl of the following solutions were administered in the hind paw to different groups of male mice: NaCl 33% (hypotonic, 100 mOsm kg⁻¹), NaCl 2% (hypertonic, 622 mOsm kg⁻¹), NaCl 10% (hypertonic, 3157 mOsm kg⁻¹) and PBS (isotonic, 298 mOsm kg⁻¹). A different group of animals was used to study osmotic pain under inflammatory conditions. A 5 µl injection of prostaglandin E₂ (PGE₂; 10 µM) was injected into the hind paw of each mouse and 30 later, 10 µl of NaCl 33% or NaCl 2% were injected into the sensitized paw. After the injection of different osmolality solutions, nocifensive behaviours of paw licking and shaking were manually counted for 5 min.

CFA model of inflammatory pain

After baseline measurements and under brief isoflurane anaesthesia, complete Freund’s adjuvant (CFA, Sigma-Aldrich, St Louis, MO, USA; 20 µl; 1 mg ml⁻¹) was injected subcutaneously (glabrous skin) in the hind paw of mice to induce a local inflammation. Mechanical von Frey threshold and heat withdrawal latency (radiant heat test) was examined at different time points after the injection (1 h, 5 h, 1 day, 3 days, 7 days and 16 days).

Formalin test

After habituation, mouse hind paw was subcutaneously injected with formalin (10 µl of a 5% formaldehyde solution) and nocifensive behaviours were measured for 50 min after the injection. The cumulative time spent shaking and licking the injected paw was counted with a stopwatch in 5 min periods.

Oxaliplatin-induced cold hypersensitivity

Prior to oxaliplatin injection, naive thermal preference was measured with the thermal place preference test set at 20°C vs. 30°C. Thirty minutes later, paw withdrawal latency to cold stimuli was determined using the cold plantar assay. A single intraperitoneal injection of oxaliplatin (6 mg kg⁻¹ in PBS with 5% glucose) was delivered to mice. Cold preference and cold sensitivity were re-evaluated 90 h after oxaliplatin injection, a time point that is known to correlate with the peak of oxaliplatin-induced cold hyperalgesia (Descoeur et al. 2011).

Cuff-induced neuropathic pain model

The sciatic nerve cuffing model was used to induce mechanical and thermal hyperalgesia in the hind paw of mice, as described by Yalcin et al. (2014). Mice were
housed individually to avoid stress derived from the surgery and to prevent injury. Cardboard rolls were placed into their home cages to provide shelter and additional stimulation after the surgery. All surgeries were done under aseptic conditions using intraperitoneal ketamine/xylazine anaesthesia. The left leg of mice was shaved from the hip to the knee and the surgical field was disinfected. A 0.5 cm incision parallel to the femur was made to expose the common branch of the sciatic nerve, which appeared after separating the muscles close to the femur. A drop of sterile physiological saline was applied to prevent the nerve from dehydrating and this concluded the procedure for the sham group. The ‘cuff’ consisted of a 2-mm-long piece of polyethylene tubing (PE-20) with an inner diameter of 0.38 mm and an outer diameter of 1.09 mm, opened by one of its sides. For the cuff group, the sciatic nerve was gently straightened with two sterile sticks and the ‘cuff’ was inserted around the main branch of the nerve and closed by applying moderate pressure with surgical forceps. To ensure that the ‘cuff’ was correctly positioned and closed, it was turned gently around the nerve. Both sham and cuff surgeries ended by suturing the incision with surgical knots. After the procedure, animals were placed in their respective home cages lying on their right side and were under constant monitoring until they were completely awakened. Mechanical and heat sensitivity of mice were determined with the von Frey and the radiant heat tests as previously described, before surgery and 5, 7, 14 and 21 days after surgery.

Skin-nerve preparation and single fibre recordings

The isolated skin–saphenous nerve preparation for single C-fibre recording was used as previously described (Noel et al. 2009). The hind paw skin of male mice 10–20 weeks of age was isolated with the saphenous nerve. The skin was pinned corium side up in a perfusion chamber with the nerve being pulled in a recording chamber filled with paraffin oil. The skin was perfused with warm (∼30–31°C) synthetic interstitial fluid (SIF; in mM): 120 NaCl, 3.5 KCl, 5 NaHCO₃, 1.7 NaH₂PO₄, 2 CaCl₂, 0.7 MgSO₄, 9.5 sodium gluconate, 5.5 glucose, 7.5 sucrose and 10 Hepes, pH 7.4.

Culture of DRG neurons

Mice were killed by decapitation under anaesthesia (isoflurane) and thoracic, lumbar and cervical DRG were removed for neuronal culture as previously described (Tulleuda et al. 2011; Callejo et al. 2013). Briefly, DRGs were collected and maintained in cold (4–5°C)
Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS supplemented with 10 mM glucose, 10 mM Heps, 100 UI ml\(^{-1}\) penicillin and 100 µg ml\(^{-1}\) streptomycin until dissociation. Subsequently, ganglia were incubated in 2 ml HAM F-12 with collagenase CLS I (1 mg ml\(^{-1}\); Biochrome AG, Berlin, Germany) and bovine serum albumin (BSA, 1 mg ml\(^{-1}\)) for 1 h 45 min at 37°C followed by 15 min trypsin treatment (0.25%). Ganglia were then resuspended in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (100 µg ml\(^{-1}\)) and L-glutamine (100 mg ml\(^{-1}\)) and mechanical dissociation was conducted with fire-polished glass Pasteur pipettes of decreasing diameters. Neurons were centrifuged at 100 g for 5 min and re-suspended in culture medium (DMEM + 10% FBS, 100 µg ml\(^{-1}\) penicillin/streptomycin, 100 mg ml\(^{-1}\) L-glutamine). Cell suspensions were transferred to 12-mm-diameter glass coverslips pre-treated with poly-L-lysine/laminin and incubated at 37°C in a humidified 5% CO\(_2\) atmosphere for up to 1 day, before being used for patch-clamp electrophysiological recordings or calcium imaging experiments. Nerve growth factor or other growth factors were not added.

**Calcium imaging**

Cultured DRG neurons from wild-type and TRESK KO mice were loaded with 5 µM fura-2/AM (Invitrogen) for 45–60 min at 37°C in culture medium. Coverslips with fura-2-loaded cells were transferred into an open flow chamber (0.5 ml) mounted on the stage of an inverted Olympus IX70 microscope equipped with a TILL monochromator as a source of illumination. Pictures were acquired with an attached cooled CCD camera (Orca II-ER, Hamamatsu Photonics, Shizuoka, Japan) and stored and analysed on a PC computer using Aquacosmos software (Hamamatsu Photonics). After a stabilization period, pairs of images were obtained every 4 s at excitation wavelengths of 340 nm (λ1) or 380 nm (λ2; 10 nm bandwidth filters) in order to excite the Ca\(^{2+}\)-bound or Ca\(^{2+}\)-free forms of the fura-2 dye, respectively. The emission wavelength was 510 nm (12-nm bandwidth filter). Typically, 20–40 cells were present in the microscope field. [Ca\(^{2+}\)]\(_i\) values were calculated and analysed individually for each single cell from the 340- to 380-nm fluorescence ratios at each time point. Only neurons that produced a response >10% of the baseline value and that, at the end of the experiment, produced a Ca\(^{2+}\) response to KCl-induced depolarization (50 mM) were included in the analysis. Several experiments with cells from different primary cultures and different animals were used in all the groups assayed. The extracellular (bath) solution used was 140 mM NaCl, 4.3 mM KCl, 1.3 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose and 10 mM Heps (pH 7.4 with NaOH). Experiments were performed at room temperature.

**Electrophysiological recording**

Electrophysiological recordings in DRG sensory neurons were performed as previously described (Callejo et al. 2013, 2015; Castellanos et al. 2018). Briefly, recordings were performed with a patch-clamp amplifier (Axopatch 200B, Molecular Devices) and restricted to small DRG neurons (<30 µm soma diameter), which largely correspond to nociceptive neurons (Le Pichon & Chesler, 2014). Patch electrodes were fabricated in a Flaming/Brown micropipette puller P-97 (Sutter instruments, Novato, CA, USA). Electrodes had a resistance of 2–4 MΩ when filled with intracellular solution (in mM): 140 KCl, 2.1 CaCl\(_2\), 2.5 MgCl\(_2\), 5 EGTA, 10 Hepes and 2 ATP (pH 7.3). Bath solution was (in mM): 145 NaCl, 5 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 10 Hepes and 5 glucose (pH 7.4). The osmolality of the isotonic solution was 310.6 ± 1.8 mOsm kg\(^{-1}\). Membrane currents were recorded in the whole-cell patch-clamp configuration, filtered at 2 kHz, digitized at 10 kHz and acquired with pClamp 10 software. Data were analysed with Clampfit 10 (Molecular Devices) and Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Series resistance was always kept below 15 MΩ and compensated at 70–80%. All recordings were made at room temperature (22–23°C), 18–24 h after dissociation. To study sensory neuron excitability, after achieving the whole-cell configuration in the patch clamp technique, the amplifier was switched to current-clamp bridge mode. Only neurons with a resting membrane voltage below −50 mV were considered for the study. To study neuronal excitability, we examined the resting membrane potential (RMP); AP rheobase (minimum current to elicit an AP; obtained with 400 ms depolarizing current pulses in 10 pA increments); whole-cell input resistance (R\(_{in}\)) was calculated on the basis of the steady-state I–V relationship during a series of 400 ms hyperpolarizing currents delivered in steps of 10 pA from −50 to −10 pA; AP amplitude was measured from RMP to AP peak, AP duration was measured at 50% of AP amplitude and hyperpolarizing afterpotential (HAP) was measured as the voltage difference between the firing level of the spike (beginning of the ascending phase of the AP) and the potential of the HAP. The interspike interval was measured from peak to peak of two succeeding APs. The average of the initial three interspike intervals from spikes elicited by a 1 s current ramp from 0 to 500 pA was used (Fig. 2C).

**Drugs**

All reagents and culture media were obtained from Sigma-Aldrich (Madrid, Spain) unless otherwise
indicated. Menthol (100 µM), AITC (100 µM) and capsaicin (1 µM) were also purchased from Sigma.

**Data analysis**

Data are presented as mean ± SD and, in some specific analyses, the median, coefficient of variation or geometric mean are also provided. Statistical differences between different sets of data were assessed by performing paired or unpaired Student’s t tests, Mann–Whitney test, Wilcoxon matched pairs test, one-way or two-way ANOVA plus the Holm–Sidak correction for multiple comparisons, Chi-square test or Fisher’s exact test, as indicated. The significance level was set at \( P < 0.05 \) in all statistical analyses. Data analysis was performed using GraphPad Prism 8 software and GraphPad QuickCalcs online tools (GraphPad Software).

**Results**

**TRESK deletion reduces total background current and enhances neuronal excitability**

TRESK channels have been detected in small and medium-sized sensory neurons, together with other members of the K\(_{2P}\) family of background K\(^+\) channels (Kang & Kim, 2006; Usoskin et al. 2015; Zeisel et al. 2018). To confirm the loss of TRESK expression in homozygous knockout animals and to assess possible compensatory effects on the expression of other K\(_{2P}\) channels, we examined the mRNA expression in DRGs by real-time qPCR. TRESK mRNA was undetected in KO mice while a significant expression was found in WT animals (Fig. 1A).

We next examined the expression of TREK-1, TREK-2 and TRAAK (the most well-expressed K\(_{2P}\) members in sensory neurons). Analogously to what has been reported in a previous study (Chae et al. 2010), mRNA for these channels were present in WT and KO mice at similar levels, thus excluding any compensation effect. Furthermore, the expression of TRPA1 and TRPV1, two channels highly expressed in nociceptors, was also unchanged.

To assess whether removal of TRESK modifies K\(^+\) background currents in nociceptors, we recorded total K\(^+\) current from small-diameter DRG neurons (soma size <30 µm; capacitance <45 pF) that largely correspond to nociceptive neurons with C-fibre conduction velocities (Ma et al. 2003). Putative nociceptors isolated from KO mice displayed a significantly lower K\(^+\) current density than nociceptors of WT mice, both at \(-25\) mV (15.3 ± 8.5; \( n = 31; \) vs. 9.7 ± 7.8 pA pF\(^{-1}\); \( n = 50; \) for WT and KO mice, respectively; unpaired \( t \) test \( P = 0.004)\), and at \(-135\) mV (\(-11.1 ± 8.9\) pA pF\(^{-1}\) vs. \(-7.3 ± 5.6\) pA pF\(^{-1}\); for WT and KO mice, respectively; unpaired \( t \) test

![Figure 1. Nociceptive sensory neurons lacking TRESK have a decreased standing outward current](https://example.com/figure1.png)
indicating that the absence of TRESK has a significant functional consequence on the membrane current (Fig. 1B). In a second set of nociceptor recordings, we investigated the functional consequence of TRESK deletion on neuronal excitability by measuring their resting membrane potential and AP firing properties (Fig. 2A and B). WT and TRESK KO nociceptors did not present significant changes in RMP (58.3 ± 13.6 vs. −56.2 ± 7.7 mV, respectively; unpaired t test \( P = 0.540 \); Fig. 2B), suggesting that TRESK might have a minor role in setting the RMP and other \( K^+ \) channels are possibly more important in setting the RMP in the cell body. This is in agreement with previous data showing that RMP was not significantly modified after TRESK down-regulation or deletion (Dobler et al. 2007; Tulleuda et al. 2011). Interestingly, current-clamp recordings indicated that rheobase (minimum current injected to elicit an AP) was significantly lower in TRESK KO nociceptors (Fig. 2B, \( P = 0.0013 \), unpaired t test) compared to nociceptors from WT littermates. This effect is probably a consequence of the increased membrane resistance found in TRESK KO neurons (1262.8 ± 655.3 MΩ) compared to controls (WT: 792.7 ± 661.7 MΩ; \( P = 0.045 \), unpaired t test; Fig. 2B). As expected, AP amplitude was not significantly altered (\( P = 0.406 \), unpaired t test) because this parameter is more dependent on the activity of voltage-dependent sodium

![Figure 2. Nociceptive sensory neurons lacking TRESK present a higher excitability](image-url)

A, representative whole-cell current-clamp recordings from wild-type (WT) and TRESK knockout (KO) nociceptive sensory neurons elicited by hyperpolarizing or depolarizing 400 ms current pulses in 10 pA increments from −50 pA. Right: mean membrane capacitance (\( C_m \)) from neurons studied. B, quantification of the electrophysiological parameters analysed in WT (black bars, \( n = 13 \)) and TRESK KO (red bars, \( n = 27 \)) sensory neurons. RMP: resting membrane potential. Rheobase was measured using 400 ms depolarizing current pulses in 10 pA increments; \( R_{in} \): whole-cell input resistance was calculated on the basis of the steady-state \( I–V \) relationship during a series of 400 ms hyperpolarizing currents delivered in steps of 10 pA from −50 to −10 pA. Action potential (AP) amplitude was measured from the RMP to the AP peak. AP duration/width was measured at 50% of AP amplitude. Data are presented as mean ± SD. Statistical differences between groups are shown (∗\( P < 0.05 \), ∗∗\( P < 0.01 \) unpaired t test). C, examples and quantification of neuronal excitability as the number of APs fired in response to a depolarizing current ramp (0–500 pA, 1 s) from a holding voltage of −60 mV (black, WT \( n = 10 \); red, TRESK KO \( n = 18 \)). The peak to peak interspike interval value between the four initial APs was measured and averaged. Statistical differences between groups are shown (∗\( P < 0.05 \), ∗∗\( P < 0.01 \) unpaired t test). [Colour figure can be viewed at wileyonlinelibrary.com]
channels (Na\textsuperscript{+}). However, APs were significantly wider in nociceptors from TRESK KO animals, probably reflecting the consequence of the decrease in total K\textsuperscript{+} current (width at 50% AP: TRESK KO 4.03 ± 2.4 ms; n = 27 neurons; WT: 2.38 ± 1.9 ms; n = 13; P = 0.041; Fig. 2B). In a similar fashion, HAP measured from the firing level of the spike was also reduced in KO neurons (TRESK KO 36.5 ± 7.7 mV; n = 27 neurons; WT: 46.4 ± 8.1 mV; n = 13; P = 0.001). To investigate if these changes affect the excitability of nociceptors, we injected a depolarizing current ramp (0–500 pA, 1 s) and counted the number of APs generated for each genotype. As shown in Fig. 2C, TRESK KO neurons fired on average more spikes (13.6 ± 5.4 spikes; n = 18) than WT neurons (8.3 ± 5.8 spikes; n = 10; P = 0.023), and showed a reduced interspike interval (KO: 70.6 ± 23.9 ms vs. WT: 111.2 ± 54.7 ms; P = 0.006), indicating that removal of TRESK increased the excitability of sensory neurons.

To further characterize the responsiveness of sensory neurons in TRESK KO mice, we measured the intracellular Ca\textsuperscript{2+} signals ([Ca\textsuperscript{2+}]) of cultured DRG neurons from WT and KO mice in response to capsaicin (1 μM), a TRPV1 agonist and AITC (100 μM; a TRPA1 agonist), two markers of nociceptive neurons, and menthol (100 μM; a TRPM8 agonist), a marker of cold sensory neurons. Among WT sensory neurons (total number of neurons analysed = 1124), 49.2% responded to capsaicin, 40.4% to AITC and 7.0% to menthol. Responses to both capsaicin and AITC were seen in 17.9% of the neurons (Fig. 3A and B). A percentage of neurons did not respond to any of the agonists tested (246 neurons, 21.9%). Responses to capsaicin (45.7%) and menthol (9.1%) were not different in neurons from TRESK KO mice (n = 1228), but the percentage of neurons activated by AITC was significantly lower (24.7%, P = 0.009). Neurons not responding to any agonists totalled 446 (36.3%). The diameters of sensory neurons responding to the different agonists between WT and KO animals were not different, indicating that they represent similar populations of DRG neurons (mean soma diameter WT: 20.2 ± 5.9 μm; KO: 20.3 ± 5.6 μm). In parallel experiments, the response of cultured trigeminal neurons was assessed. These showed no significant differences for the response to the different agonists (data not shown) between genotypes. Therefore, the diminished response to AITC is restricted to DRG neurons.

We next assessed if the reduced AITC response of DRG neurons from TRESK KO mice had a behavioural correlate by injecting AITC (100 μM) into the mouse hind paw. In agreement with the decreased response to AITC obtained in calcium recordings, nocifensive behaviour measured as the time spent licking or shaking the paw significantly diminished in KO compared to WT mice (TRESK KO: 9.0 ± 7.3 s; WT: 18.1 ± 13.0 s; P = 0.039; n = 13 for each group; Fig. 3C). In contrast, painful behavioural responses to capsaicin injection did not differ between KO and WT mice (40.0 ± 22.7 s vs. 28.8 ± 13.6 s, respectively; P = 0.236; Fig. 3C). Injection of vehicles for each compound did not produce significant behavioural effects (data not shown). To further investigate to what extent chemical nociception was altered by the absence of TRESK, we used the formalin test (Tjølsen et al. 1992; Abbott et al. 1995), which is characterized by an initial phase (phase I; 0–5 min) due to direct nociceptor activation (McNamara et al. 2007) and a second phase (phase II; 15–50 min) that is attributed to a combined nociceptive input together with central spinal sensitization (Tjølsen et al. 1992). The phase I nocifensive response has been directly linked to the activation of TRPA1 (McNamara et al. 2007), although high concentrations of formalin are still able to induce some pain in TRPA1 KO mice (Fischer et al. 2013). Interestingly, animals lacking TRESK showed a diminished response to a 5% formalin injection in phase I (30.8 ± 15.0 s; P = 0.002; Fig. 3D) compared to control WT animals (69.9 ± 17.6 s). The decreased response in phase I of TRESK KO animals is in agreement with the decreased nocifensive response observed after AITC injection (Fig. 3C) which corroborates the decreased fraction of AITC-sensitive DRG neurons from TRESK KO animals in culture. All these observations confirm that TRPA1 activation is decreased in TRESK KO animals. Phase II of the formalin test can be further split into phase IIa (15–25 min) and IIb (25–50 min), where IIa has a higher nociceptive input than IIb. TRESK KO animals showed a decreased licking time during phase IIa (107.7 ± 39.9 s) compared to WT animals (154.8 ± 42.2 s; P = 0.026); this probably reflects decreased nociceptor activation as found in phase I. In contrast, phase IIb of the formalin test did not show significant differences and licking behaviour was similar between groups (P = 0.814, Fig. 3D). Injection of hypertonic saline stimulates primary afferent nociceptors and produces pain in humans (Alessandri-Haber et al. 2003, 2005). This response can be further enhanced by sensitization of nociceptors with PGE\textsubscript{2}. Injection of hypertonic stimuli did not induce significant nocifensive behaviour in resting conditions (P = 0.146, Fig. 3E) but, as previously described (Alessandri-Haber et al. 2003; Alloui et al. 2006), nocifensive responses were enhanced after sensitization with PGE\textsubscript{2} in WT mice but not in TRESK KO mice (P = 0.019, unpaired t test WT vs. KO). Nocifensive responses to mild hypertonic saline were diminished in TRESK KO animals, both in resting conditions (2% or 10% NaCl, P = 0.049 and P = 0.002) and after sensitization with PGE\textsubscript{2} (P = 0.002; Fig. 3E). Again, these effects are similar to that reported after knocking out TREK-1 and TREK-2, but not in the single TRAAK KO mice (Alloui et al. 2006; Noël et al. 2009; Pereira et al. 2014), thus implying that TREK-1, TREK-2 and TRESK are involved in the sensitivity to hypertonic stimuli and their absence prevents sensitization by PGE\textsubscript{2}. © 2020 The Authors. The Journal of Physiology © 2020 The Physiological Society
Figure 3. TRESK KO mice present diminished responses to osmotic stimuli and TRPA1 activation

A, representative recordings of intracellular calcium (Fura-2 ratiometric imaging) of wild-type (WT) or TRESK knockout (KO) DRG sensory neurons exposed to menthol (100 µM), allyl isothiocyanate (AITC, 100 µM) and capsaicin (1 µM). Venn diagrams show the relative size and overlap between the populations of neurons activated by each agonist. The number of total cells analysed was 1124 (WT) and 1228 (KO). The number of responding cells in each subgroup is shown in parentheses. Non-responding neurons to any of the agonists assayed were 246 (WT) and 446 (KO).

B, quantification of the percentage of neurons responding to each agonist in intracellular calcium recordings. Statistical differences between groups are shown (**P < 0.01 unpaired t test).

C, nocifensive behaviour: quantification of the time the animals spent licking and shaking the paw after intradermal injection of AITC (10%) or capsaicin (1 µg/10 µl) in the hind paw for WT and TRESK KO mice. The mean time spent showing nocifensive behaviours over a period of 5 min is shown (WT n = 13; KO n = 13 animals).

D, formalin-induced pain. Top: time course of licking/shaking behaviour directed to the formalin-injected hind paw. Bottom: quantification of cumulative time spent in phase I (0–10 min), IIa (11–30 min) and IIb (31–50 min); n = 5–6 animals per group.

E, osmotic pain.
Mice lacking TRESK present mechanical allodynia and normal heat perception

Because TRESK KO enhances nociceptor excitability, we next analysed the sensitivity of nerve fibres from TRESK KO mice to different types of stimuli and whether the activation of sensory fibres was correlated to behavioural responses to these stimuli. Saphenous nerve C-fibres from WT (n = 14) and TRESK KO mice (n = 22) were recorded with the nerve-skin preparation (Zimmermann et al. 2009). Mechanical thresholds were determined with calibrated von Frey filaments applied on the receptive fields of C-fibres. TRESK KO mice presented an enhanced sensitivity to mechanical stimuli. The mean threshold value for the whole population was not significantly different in KO animals (mean ± SD: 27.6 ± 24.6 mN; median: 22 mN) compared to WT mice (mean ± SD: 33.2 ± 23.3 mN; median: 22 mN; P = 0.268, Mann–Whitney test), but threshold values from KO mice showed a wider distribution and a significant shift towards lower values (Fig. 4A). This wider spread might mean a subset of C-fibres have lost the inhibitory effect of TRESK, and can now activate with lower-intensity mechanical stimuli. To examine such a possibility, we compared the percentage of fibres that presented a lower threshold for activation by mechanical stimuli. Our results indicate that KO mice presented a significant percentage of fibres with lower thresholds (<12 mN; 40.9%) compared to fibres from WT animals (0%; P = 0.006; Fisher’s exact test). This wider distribution of mechanical thresholds towards lower values in the fibres from KO animals can be also shown by the larger coefficient of variation (WT: 70.2% vs. KO: 89.1%) and by the geometric mean (WT: 27.8 mN vs. KO: 17.6 mN; Fig. 4A, blue lines), which normalizes the ranges being averaged, and thus no range dominates the weighting. This suggests that TRESK function prevents C-fibre activation by low-intensity mechanical stimuli and therefore deletion of the channel reveals mechanical sensitivity with low threshold values in a fraction of C-fibres (Fig. 4A). We then compared the mechanical sensitivity of TRESK KO to WT mice with the von Frey up and down method. Hind paw mechanical threshold was significantly lower in TRESK KO mice compared to WT mice (P < 0.0001 for males and females; Fig. 4B), indicating that the subset of C-fibres with lower activation thresholds by mechanical stimuli have an important functional consequence because they precipitate mechanical allodynia in awake, freely moving mice. Measures of mechanical sensitivity with the dynamic plantar aesthesiometer also confirmed significant differences to pressure application between WT (5.06 ± 0.61 g; n = 27) and KO animals (4.66 ± 0.81 g; n = 22; t test P = 0.027; Fig. 4B).

Although other members of the K_2P family have been shown to be involved in thermosensation and pain perception in response to hot or cold stimuli (Alloui et al. 2006; Noël et al. 2009; Pereira et al. 2014), TRESK activity is not modulated by changes in temperature in the physiological range (Kang & Kim, 2006). Despite this lack of thermal sensitivity of the channel, it remains possible that the channel modulates the excitability of C-fibres involved in heat sensitivity. We addressed this question by recording responses of C-fibres activated by a heat-ramp applied on their receptive fields in the skin. TRESK KO and WT mice did not show significant differences in heat thresholds for the activation of C-fibres (37.6 ± 2.8°C, n = 35 C-fibres for WT; 38.1 ± 3.9°C, n = 37 C-fibres for KO; P = 0.502 t test; Fig. 4C), nor the distribution of these thresholds over a range of temperatures, nor the number of spikes fired by heat-sensitive C-fibres during heat-ramps (32.6 ± 28.2 spikes WT; 34.8 ± 28.3 spikes TRESK KO; P = 0.720 t test; Fig. 4C), indicating that TRESK does not seem to have a major role in the detection of warm or hot conditions. We then tested heat sensitivity in vivo using the Hargreaves and hot plate tests. Heat sensitivity was unaltered in TRESK KO mice compared to WT in the radiant heat Hargreaves test (P = 0.08 males; P = 0.603 females, Fig. 4D), thus confirming the observations made in C-fibres recordings. To further evaluate a possible implication of TRESK in heat pain, we evaluated the sensitivity to more extreme temperatures in the hot plate test at 52 and 56°C, but no significant differences were detected (Fig. 4D). This indicates that the extreme heat sensitivity is conserved in TRESK KO mice. The dynamic hot plate with a ramp of temperature has been proposed as a valuable method to differentiate between thermal allodynia and hyperalgesia (Yalcin et al. 2009). The number of jumps of the mice on a hot plate when the plate temperature is increased from 39 to 50°C did not show any difference between TRESK KO and WT, neither for the total number of jumps nor the temperature at which animals displayed their first jump (Fig. 4E). These behaviour and skin-nerve preparation results indicate that there is no significant contribution of TRESK to heat sensitivity.
Figure 4. TRESK-lacking mice present mechanical allodynia and normal heat sensitivity

A, mechanical threshold obtained with von Frey hairs from saphenous nerve C-fibres. Left: distribution of von Frey thresholds. Mean ± SD is shown (black lines). The geometric mean is indicated as a blue line. Wild-type (WT) n = 14, knock-out (KO) n = 22. Right: magnification of 0–25 mN range of von Frey thresholds to highlight the larger distribution of fibres from KO mice to lower threshold values. B, mechanical sensitivity. Left: von Frey response thresholds obtained with the up and down method in male and female WT and TRESK KO animals (male WT n = 58, KO n = 33; female WT n = 18, KO n = 19). Right: latency to hind paw withdrawal in the dynamic plantar test (male WT n = 27; KO n = 22). Statistical differences are shown as *P < 0.05, ***P < 0.001 (Student’s unpaired t test) between WT and KO mice. C, left: distribution of heat thresholds from C-fibres recorded from WT (n = 35) and TRESK KO mice (n = 37), measured in skin-nerve experiments. Right: mean number of spikes fired by heat-sensitive C-fibres during a heat-ramp from 30 to 50°C. No significant differences were found. D, heat sensitivity. Left: radiant heat (Hargreaves test) in male (WT n = 39; KO n = 38) and female animals (WT n = 18, KO n = 15). Right: noxious heat sensitivity to 52 and 56°C (hot plate test) in male (WT n = 8/9; KO n = 13/11) and female animals (WT n = 9/9; KO n = 12/8). E, dynamic hot plate: in contrast to the conventional hot plate,
Elevated perception of low temperatures in the absence of TRESK channel

Thermosensitivity to low temperatures is governed by different mechanisms and by distinct neuronal sub-populations than warm/hot perception (Lolignier et al. 2016). We evaluated the role of TRESK in the cold sensitivity of C-fibres by recording fibre activity with the saphenous nerve preparation upon cooling their receptive fields in the skin from 30 to 10°C over 90 s (Fig. 5A–D). C-fibre recordings from TRESK KO mice showed that although the total number of cold-sensitive fibres did not differ significantly (58% in WT and 66% in KO mice), there were significant changes in the fractions of mechano-cold and mechano-heat and cold-sensitive polymodal C-fibres compared to WT mice. Specifically, we observed in KO mice an increase in the fraction of mechano-cold C-fibres compared to WT mice (37% vs. 20% C-MC, respectively; Fig. 5A). This was accompanied by a reduction of the percentage of mechano-heat-cold C-fibres (C-MHC) in KO mice (29%) compared to WT (38%; P < 0.01; chi-square test; Fig. 5A). The distribution of C-fibre activation thresholds by cold stimuli indicated a clear shift toward higher temperature in the KO (between 26 and 28°C) than in the WT mice (Fig. 5B and C). Despite the mean or median cold thresholds not being different (P = 0.565, Mann–Whitney test; Fig. 5C), we observed a tendency to have more fibres activated at temperatures above 25°C in KO animals (58%) compared to WT (42%), although this difference did not reach statistical significance (Fisher’s exact test; P = 0.099). The total response of TRESK KO fibres to a 90 s cooling ramp (33.1 ± 29.5 spikes) was similar to that of WT fibres (34.1 ± 25.1 spikes; P = 0.90 unpaired t test). However, the distribution of the activity upon cooling was higher at temperatures between 25 and 19°C in TRESK KO fibres (Fig 5D). The baseline activity between 30 and 31°C was also higher in TRESK KO than WT fibres (0.3 spikes s\(^{-1}\) for TRESK KO and 0.1 spikes s\(^{-1}\) for WT; n = 36 and n = 31 respectively; P < 0.001, t test). These experiments indicate that the temperature for activation of cold-sensitive C-fibres was higher in TRESK KO mice, but also that the overall fibre activity in response to cooling did not depend drastically on the presence of TRESK.

In agreement with observations in C-fibres, behavioural responses to cold were also altered in animals lacking TRESK. Sensitivity to noxious cold (2°C; cold plate test) was enhanced in knockout animals from both sexes, with shorter latency times to elicit a nocifensive behaviour (P = 0.006 males; P = 0.041 females, Fig. 5E). In addition, cold sensitivity to more moderate temperatures assessed with the cold plantar assay was significantly higher in TRESK KO mice (P < 0.0001 males, P = 0.008 females, Fig. 5F). Finally, we assessed whether changes in cold sensitivity modified the preference the mice displayed between the two different temperatures. TRESK KO animals spent more time on the reference plate (at 30°C) than on the experimental plate at 20°C (P = 0.015, one-way ANOVA plus Holm–Sidak correction for multiple comparisons, Fig. 5G) compared to WT animals, further supporting previous evidence from cold-sensitive C-fibre recordings and cold sensitivity assays. In contrast, place preference assays at other temperatures (10, 15, 30, 40, 50°C) did not show significant alterations between mice genotypes, corroborating the non-involvement of TRESK in the perception of cool, warm or hot conditions (Fig. 4).

Mice lacking TRESK show selective changes in persistent inflammatory and neuropathic pain

To assess the contribution of TRESK in chronic pain conditions, the behaviour of TRESK KO and WT mice was measured after intraplantar CFA injection, a model of persistent inflammatory pain. Both genotypes developed mechanical and thermal hypersensitivity in the injected hind paw beginning 1 h after injection and lasting at least 16 days (Fig. 6A). Despite the initial difference in basal mechanical thresholds (P = 0.047), the extent of mechanical allodynia was similar between WT and KO animals 1 h after CFA injection and during the entire 16-day observation period (P > 0.05; two-way ANOVA plus Holm–Sidak correction for multiple comparisons). The development of thermal hyperalgesia was similar between the two genotypes, thus showing that TRESK does not contribute significantly to peripheral sensitization of nociceptors due to inflammation. The sciatic nerve cuff-model was used to evaluate TRESK contribution in persistent neuropathic pain. The mechanical hyper-sensitivity developed in KO animals was significantly higher to begin with (P = 0.0006; Fig. 6B) but no significant differences were found after sciatic nerve cuffing compared to WT animals. Sham surgery did not show significant effects on this parameter in any

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The dynamic hot plate allows the testing of a wide range of temperatures. Plate temperature was ramped from 30 to 50°C at 1°C min\(^{-1}\). To determine the temperature that is perceived as noxious for mice and to quantify pain-related behaviours, the number of jumps at each temperature was scored. Left: number of jumps elicited at each temperature in male and female WT and TRESK KO mice (n = 11–15 animals per group). No significant differences were obtained between WT and TRESK animals (one-way ANOVA with Holm–Sidak correction). Right: mean temperature at which animals made the first jump (threshold) and total number of jumps for male and female mice in the whole range of temperatures. Values for temperatures between 30 and 38°C are not shown in the plots because they were probably detected as non-noxious and did not produce any observable response. (Colour figure can be viewed at wileyonlinelibrary.com)
Figure 5. Cold alldynia in TRESK-deleted mice

A. Cold-sensitive C-fibres

B. Fraction of cold-sensitive C-fibres (%)

C. Cold thresholds (°C)

D. C-fibre response to cold stimulation

E. Cold sensitivity
   (Cold plate test at 2°C)

F. Cold sensitivity
   (Cold plantar assay)

G. Cold avoidance
   (Thermal place preference test)
genotype compared to baseline values (Fig. 6B). Also, when the percentage change in threshold (difference vs. basal value) was compared between WT and KO animals, this showed similar values (at 5 days, KO: −45.1 ± 42.7%; WT: −37.9 ± 29.6%; P = 0.742; at 7 days, KO: −47.3 ± 41.5%; WT: −45.0 ± 47.3%; P = 0.931), indicating that nerve injury exerted a similar effect in both genotypes but, because KO had a lower mechanical threshold to begin with, animals reached lower mechanical thresholds on days 5 and 7. Mechanical hypersensitivity was undistinguishable between groups at later stages, at 14 and 21 days after injury. Heat sensitivity showed a similar level of hyperalgesia after neuropathy, akin to the lack of implication of TRESK in thermal perception. Surprisingly, withdrawal latencies from KO animals recovered to baseline values faster than in the WT group after 21 days, which could indicate an excitotoxic death of some of the primary afferents due to excessive hyperactivity. Finally, we evaluated chemotherapy-induced neuropathic pain with the anti-cancer drug oxaliplatin, which induces cold allodynia in a majority of patients. We assessed whether the development of cold hyperalgesia after oxaliplatin treatment was modified in TRESK KO. As expected, WT mice showed an enhanced cold sensitivity 90 h after the oxaliplatin injection. Both the cold plantar assay (P = 0.0002; paired t test) and thermal place preference test (30/20°C; P = 0.0045; unpaired t test) showed significant differences compared to pre-injection values, indicating a higher sensitivity to cold stimuli in neuropathic mice (Fig. 6C). In contrast, when TRESK KO animals were tested in the cold plantar assay, we did not observe any further decrease in paw withdrawal latency compared to the baseline value (P = 0.912), which was already lower than that of WT animals. Again, thermal place preference between 20 and 30°C did not show significant differences after oxaliplatin treatment (P = 0.770), suggesting that the enhanced cold sensitivity due to TRESK removal cannot be further increased by neuropathy induced by oxaliplatin.

**Discussion**

TRESK is highly expressed in sensory ganglia (Kang & Kim, 2006; Dobler et al. 2007; Bautista et al. 2008; Tulleuda et al. 2011; Manteniotis et al. 2013; Nguyen et al. 2017; LaPaglia et al. 2018; Ray et al. 2018) within specific subtypes of sensory neurons, mainly nociceptors and especially in non-peptidergic neurons (Chiu et al. 2014; Usoskin et al. 2015; Li et al. 2016; Nguyen et al. 2017; Zheng et al. 2019). TRESK’s main role has been attributed to preventing neuronal depolarization (Enyedi & Czirjak, 2010), and reduction of its expression after nerve injury or inflammation contribute to neuronal hyperexcitability (Dobler et al. 2007; Tulleuda et al. 2011; Marsh et al. 2012). Mutations in this channel have been linked to the enhanced nociceptor excitability that occurs during familial migraine with aura (Lafrenière et al. 2010; Royal et al. 2019). Here, we propose that TRESK balances the effects of depolarizing stimuli, acting as a brake to prevent the activation of specific subpopulations of sensory neurons.

Nociceptors lacking TRESK presented electrophysiological changes and an enhanced excitability consistent with its putative role to prevent neuronal depolarization (Kang & Kim, 2006). RMP was similar between TRESK KO and WT neurons, indicating that TRESK does not contribute significantly in setting this parameter, as previously suggested (Dobler et al. 2007). Indeed, other K_{_2p} members and other K^+ channel families have been proposed to control membrane polarization at rest (Du et al. 2018). We found that TRESK, TREK-1 and TREK-2 were similarly expressed in DRGs, while the expression of TRAAK was lower. Others have found a slightly higher expression of TRESK over TREK-1 or TREK-2 while the expression of TRAAK varies in different studies but in general appears less than for TRESK (Dobler et al. 2007; Marsh et al. 2012). Despite unchanged mRNA expression of other leak channels (TREK-1/2, TRAAK) in TRESK KO mice, it is possible that post-translational

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A, fractions of cold-sensitive C-fibres in wild-type (WT) (total number of C-fibres = 35) and TRESK knockout (KO) (n = 37 C-fibres) mice, measured in skin-nerve experiments. Numbers in bars are the percentage of mechano-heat-cold (C-MHC) and mechano-cold (C-MC) fibres. A significant difference in the distribution of cold fibres is shown (**P < 0.01, chi-square test). B, distribution of the C-fibres activated by cold at different temperatures between WT and KO animals. C, distribution of cold thresholds from fibres recorded (WT n = 19; KO n = 38). Mean and SD are shown. D, representative experiments of C-fibres activated by a cold ramp. Top: action potentials (Spikes) fired in response to a temperature decrease are shown for WT and TRESK KO cold C-fibres. The average action potential is presented on the right. A representative cold ramp from 30 to 10°C is shown below. Bottom: histogram of mean responses to cooling, 5-s bin, of C-fibres from WT and TRESK KO mice. E and F, cold sensitivity measured with the cold plate (2°C) and cold plantar assays in male and female WT and TRESK KO animals (n = 11–19 animals per group). G, cold avoidance measured in the thermal place preference test. The percentage of time spent at the reference plate (30°C) at each experimental temperature is shown (n = 8–14 male animals per group). Statistical differences are shown as *P < 0.05, **P < 0.01, ***P < 0.001 with Student's unpaired t test (E, F) or one-way ANOVA plus Holm–Sidak correction for multiple comparisons (G) between WT and KO mice. [Colour figure can be viewed at wileyonlinelibrary.com]
A. CFA-induced inflammatory pain

Mechanical sensitivity (von Frey test)

![Graph showing mechanical sensitivity over time post-CFA for different groups: Wild-type, vehicle Wild-type, TRESK knock-out, and vehicle knock-out.]

Heat sensitivity (Hargreaves test)

![Graph showing heat sensitivity over time post-CFA for different groups.]

B. Cuff-induced neuropathic pain

Mechanical sensitivity (von Frey test)

![Graph showing mechanical sensitivity over days post-surgery for different groups: Wild-type, Sham WT, TRESK KO, and Sham KO.]

Heat sensitivity (Hargreaves test)

![Graph showing heat sensitivity over days post-surgery for different groups.]

C. Oxaliplatin-induced cold alldynia

Cold Plantar assay

![Graph showing paw withdrawal latency over time for baseline and oxaliplatin for different groups: Wild-type and TRESK KO.]

Thermal place preference test (30/20°C)

![Graph showing time at 30°C over time for different groups: Wild-type and TRESK KO.]

Figure 6. Changes in chronic pain in TRESK-deleted mice

A, mechanical and thermal sensitivity in the complete Freund's adjuvant (CFA)-induced inflammatory pain model. Mechanical sensitivity was measured with von Frey filaments (up and down method) and thermal sensitivity was measured with the Hargreaves test (n = 7 animals CFA groups; n = 5 animals vehicle groups). B, mechanical and thermal sensitivity in the cuff-induced neuropathic pain model. Mechanical sensitivity was measured with...
TRESK is present in 73% of IB4
published characterization in TG neurons shows that
in excitability after TRESK deletion. In fact, a recently
(mainly non-peptidergic) might produce larger effects
specific subpopulations of genetically labelled nociceptors
et al.
TRESK shows different levels of expression in different
types of nociceptors (Usoskin et al. 2015), so studying
specific subpopulations of genetically labelled nociceptors
(mainly non-peptidergic) might produce larger effects
in excitability after TRESK deletion. In fact, a recently
published characterization in TG neurons shows that
TRESK is present in 73% of IB4+ non-peptidergic
neurons, while only 29% of CGRP+ neurons (mainly
peptidergic nociceptors) express TRESK (Weir et al. 2019).
In agreement, IB4+ nociceptive TG neurons showed an
enhanced excitability compared to IB4− neurons.

Another study reported similar results in TG neurons but,
surprisingly, lumbar DRG neurons from the same
TRESK KO mice were not found to be hyperexcitable (Guo et al. 2019). Although we did not differentiate
between IB4+ and IB4− neurons in this study, our data
support previous findings of enhanced excitability of
nociceptive sensory neurons (small DRGs) either after
TRESK deletion or after a significant decrease in its
expression (Dobler et al. 2007; Tulleuda et al. 2011; Yang et al. 2018; Weir et al. 2019). These effects have been also
found after a decreased TRESK expression in a functional
TRESK[G339R] knockout mice (Dobler et al. 2007; Kollert et al. 2015), after sciatic nerve axotomy (Tulleuda et al.
2011) or in a model of cancer-associated pain (Yang et al. 2018). This is in agreement with the neuronal
activation observed by compounds blocking TRESK (Bautista et al. 2008; Tulleuda et al. 2011; Castellanos
et al. 2018) or with the decreased excitability when over-
expressing the channel (Guo & Cao, 2014; Yang et al.
2018), indicating that the regulation of TRESK expression
is an important factor to control sensory neuron
excitability.

Here, we found that instead of producing a general
increase in excitability, removal of TRESK has a selective
effect on certain types of fibres/sensory neuron subtypes
affecting specific sensory modalities. In a similar fashion,
genetic deletion of TREK-1 modifies mechanical, heat
and cold pain perception (Alloui et al. 2006) but when
TRAAL is deleted, cold sensitivity remains unaffected
and animals only present an enhanced heat sensitivity.
Interestingly, deleting both channels (TREK-1/TRAAL
double KO mice) potentiates the effects on cold sensitivity
compared to TREK-1 alone (Noël et al. 2009). Besides,
deletion of TREK-2 only enhances thermal sensitivity to
non-aversive warm conditions but not to cold stimuli
(Pereira et al. 2014). Finally, eliminating the BKCa
channel does not modify acute nociceptive or neuropathic
pain, but enhances persistent inflammatory pain (Lu
et al. 2013), highlighting its specific participation during
inflammation-induced hyperalgesia. Our data suggest that
the lack of TREK enhances cold sensitivity by increasing
the percentage of C-fibres activated at moderate cold,
which is translated at the behavioural level by an enhanced
sensitivity to cold stimuli. An increase in cold sensitivity
has also been reported in TRESK KO mice using the
acetone test (Guo et al. 2019). In our study, the fraction
of C-fibres activated by cold (C-MHC and C-MC; 58% in
WT mice) was higher than other reports (35−45%),
which could be due to the smaller number of fibres
sampled compared to other studies (Bautista et al. 2007;
Noël et al. 2009; Pereira et al. 2014; Winter et al. 2017)
but, as occurred after deletion of other K2P channels,
the fraction of cold-sensitive C-fibres was enhanced in
TRESK KO mice compared to WT animals. Interestingly,
the larger fraction of cold-sensitive C-MC fibres (37%) in
KO animals compared to WT animals (20%) suggests that,
in physiological conditions, TREK silences a population
of neurons that would be normally activated at lower
temperatures. This is consistent with a possible role of
TRESK in preventing cold allodynia, acting as a brake
to avoid C-fibre activation at moderate cool conditions.
In fact, knocking out TRESK mimics cold allodynia
and oxaliplatin injection is not able to further increase cold
sensitivity. Oxaliplatin-induced cold allodynia has been
attributed to a combined remodelling of ion channels,
including down-regulation of TREK-1, TREK-2, TRAAK,
Kv1.1 and Kv1.2, coupled with up-regulation of TRPA1,
Na, I.8 and HCN1, while other channels (TRPM8) are not
significantly modified (Descoeur et al. 2011; Pereira et al.
2014). It has been proposed that a reduced activity of K2P
channels when temperature decreases is responsible for

| von Frey filaments (up and down method) and thermal sensitivity was measured with the Hargreaves test (n = 6 animals in each group). C, oxaliplatin-induced cold sensitization model. Left: paw withdrawal latency to the cold plantar assay before (baseline) and 90 h after oxaliplatin injection in WT and TRESK KO animals (n = 9 and 10 animals per group). Right: cold avoidance measured in the thermal place preference test. The percentage of time spent at the reference plate (30°C) versus the experimental temperature (20°C) is shown (n = 6 and 8 animals per group). Statistical differences between WT and KO mice are shown as *P < 0.05, **P < 0.01, ***P < 0.001. Two-way ANOVA plus Holm–Sidak correction for multiple comparisons (data in A and B) or Student’s paired t test (C; baseline vs. oxaliplatin) were used. [Colour figure can be viewed at wileyonlinelibrary.com] |
lower mechanical thresholds and an increased response observed in animals, which present a higher number of C-fibres with as yet unknown. Even if this is the case, it remains to be determined whether TRPA1 expression in the same neurons that contain TRESK is expressed in different subpopulations of sensory neurons (Usoskin et al. 2015; Yarmolinsky et al. 2016; Winter et al. 2017). If TRESK and TRPA1 are partially co-expressed in some neurons, one would expect an increase in the responses triggered by TRPA1 activation. However, we observed the opposite effect despite TRPA1 mRNA expression being unchanged. It is possible that some other type of functional regulation is present, but we did not explore this possibility further. By contrast, it seems unlikely that enhanced cold sensitivity in TRESK KO animals is related to the observed decrease in TRPA1 activity. K_+ channels can blunt cold responses by opposing depolarizing stimuli in TRPA1+ and TRPM8+ neurons (Madrid et al. 2009; Memon et al. 2017). It is possible that TRESK might exert a similar effect, hence silencing cold responses in normal conditions. Therefore, releasing this excitability brake by knocking out or down-regulating TRESK expression enhances cold sensation. We did not find differences in the cellular responses to menthol, which activates TRPM8, a well-known sensor of low temperatures (Bautista et al. 2007). Also, TRESK and TRPM8 seem to be expressed in different subpopulations of sensory neurons (Usoskin et al. 2015; Nguyen et al. 2017) and the transcriptome of TRPM8+ sensory neurons does not show an appreciable TRESK expression (Morenilla-Palao et al. 2014). Therefore, the enhanced cold sensitivity in TRESK KO mice does not seem related to activation of TRPM8+ fibres. Nevertheless, TRPM8+/IB4+ trigeminal neurons have been reported to be more excitable in the TRESK KO compared with WT neurons (Guo et al. 2019), and thus an increase in activation of TRPM8+ fibres after TRESK deletion cannot be completely discarded. Recently, a new cold sensor (GLR-3) has been identified in Caenorhabditis elegans and the kainate receptor GluK2 (mouse GLR-3 homolog) has been proposed to mediate cold sensitivity in mouse DRGs (Gong et al. 2019). Whether this cold sensor is expressed in the same neurons that contain TRESK it is yet unknown. Even if this is the case, it remains to be examined in greater depth.

Mechanical sensitivity is also enhanced in TRESK KO animals, which present a higher number of C-fibres with lower mechanical thresholds and an increased response to application of von Frey hairs. Similar results were previously reported after knocking down TRESK expression or after injecting TRESK blockers, where animals showed mechanical allodynia (Tulleuda et al. 2011; Castellanos et al. 2018; Yang et al. 2018). Likewise, trigeminal expression of a mutated form of TRESK linked to migraine results in facial mechanical allodynia (Royal et al. 2019). Other studies have also reported an increase in innocuous touch sensitivity in mice after TRESK deletion, although they did not find a significant difference in punctate mechanical sensitivity (Weir et al. 2019). A decrease in the withdrawal threshold to application of von Frey filaments in the forehead skin (trigeminal area) has also been reported but, surprisingly, not in the hindpaw (Guo et al. 2019). In our study, mechanical sensitivity is enhanced by shifting the mechanical threshold toward lower values in a population of sensory fibres, and therefore, a higher number of fibres is activated by low-intensity mechanical stimulation. The Piezo2 channel is involved in the detection of both innocuous touch and high-threshold noxious mechanical stimuli due to its expression in low-threshold mechanoreceptors (LTMRs) and nociceptors (Ranade et al. 2014; Dhandapani et al. 2018; Murthy et al. 2018; Szczot et al. 2018), and has been proposed to play a crucial role in the generation of mechanical allodynia during inflammation and neuropathic pain (Murthy et al. 2018). TRESK is also highly expressed in these populations of sensory neurons involved in mechanical hypersensitivity (Usoskin et al. 2015). We can hypothesize that when these neuronal populations lack TRESK, they are more readily activated by mechanically activated currents coming through Piezo2 or other mechanosensitive channels such as the recently identified TACAN (Beaulieu-Laroche et al. 2018), which would depolarize sensory neurons more effectively. We have not evaluated whether LTMRs lacking TRESK are more excitable and whether other touch modalities such as light touch (brush stroke) are also enhanced, but mechanical allodynia present in these animals suggests that they might also be affected. In agreement, a recent report found an enhanced response to innocuous touch stimuli (Weir et al. 2019).

TRESK is notably expressed in MrgrpD+ neurons (Usoskin et al. 2015; Zheng et al. 2019) and deletion of this subpopulation of neurons abolishes mechanical but not thermal pain (Cavanaugh et al. 2009). Here, the mechanical hypersensitivity found after TRESK deletion might suggest an enhanced excitability of MrgrpD+ neurons. In contrast, mechanical sensitization that occurs after inflammation or injury is not further enhanced by the absence of TRESK. Our data suggest that the molecular effect of injury, down-regulating TRESK expression (Tulleuda et al. 2011), has already been achieved by genetic removal of TRESK, and thus any further decrease in mechanical threshold observed is likely to be due to...
other mechanisms sensitizing nociceptors or derived from central effects.

TRESK KO mice did not display relevant differences in heat sensitivity. Only the thermal place preference between 20 and 30°C showed a difference that seems to reflect the enhanced cold sensitivity rather than any effect on warm/heat sensitivity. In agreement, TRESK KO C-fibres did not show any specific effects upon temperature increase compared to WT. No significant alterations on heat sensitivity either observed were after CFA-induced inflammation or in the neuropathic pain model. In contrast to other K$_{ATP}$ and TRP channels (Alloui et al. 2006; Noël et al. 2009; Julius, 2013; Pereira et al. 2014; Vriens et al. 2014; Yarmolinsky et al. 2016), the role of TRESK in neurons detecting warm or hot stimuli seems not to be highly relevant. Chemical sensitivity was altered for some stimuli (AITC, osmosenitivity) but not for others (capsaicin, menthol). Whether TRESK is directly involved in detecting osmotic stimuli is unlikely, although we previously described that hypertonic and hypotonic stimuli modulate TRESK currents (Callejo et al. 2013).

Similar results in osmotic pain were reported in animals. We previously described that hypertonic and hypotonic stimuli in TRESK KO animals. It has been proposed that the negative regulation of TREK-1/2 channels by cAMP/PKA downstream of the G-coupled PGE$_2$ receptor might be involved in the lack of PGE$_2$-mediated nociceptor sensitization. PKA is known to phosphorylate TRESK and keep it in a resting state where the channel is less active (Czirják & Enyedi, 2010) but a direct link between PKA activation, TRESK activity and osmotic sensing is still unknown. In fact, neurons activated by radial stretch and hypotonic stimuli seem to express TRESK, as they are sensitive to hydroxy-α-sanshool, a TRESK blocker (Bhattacharya et al. 2008). These neurons were initially classified as LTMRs or non-peptidergic nociceptors, which is in agreement with the TRESK expression pattern obtained from single-cell mRNA sequencing (Usoskin et al. 2015).

In summary, we describe that genetic removal of TRESK specifically enhances mechanical and cold sensitivity in mice, not affecting the sensitivity to other stimuli. The development of specific TRESK openers/activators would be worthwhile to improve mechanical hypersensitivity and cold allodynia during chronic pain conditions.

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Additional information

Competing interests

The authors have no conflicts of interest to declare.

Author contributions

A.C., A.A.B. and G.C. performed electrophysiological recordings in neurons and calcium imaging. A.C., A.P.C. and A.A.B. performed behavioural experiments. A.C. and A.A.B. carried out primary cell cultures. A.C., G.C., A.P.C. and N.C. performed qPCR experiments. A.N. and J.N. performed skin-nerve recordings. A.C., D.S., J.N., N.C. and X.G. participated in the design of the study and performed the statistical analysis. X.G. conceived the study, oversaw the research and prepared the manuscript with help from all others. All authors read and approved the final manuscript.

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Keywords

cold, ion channels, mechanosensitivity, neuronal excitability, pain, potassium channel, sensory neurons

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document