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Selective Small Molecule Activators of TREK-2 Channels Stimulate Dorsal Root Ganglion c-Fiber Nociceptor Two-Pore-Domain Potassium Channel Currents and Limit Calcium Influx

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Abstract

The two-pore-domain potassium (K2P) channel TREK-2 serves to modulate plasma membrane potential in dorsal root ganglia c-fiber nociceptors, which tunes electrical excitability and nociception. Thus, TREK-2 channels are considered a potential therapeutic target for treating pain; however, there are currently no selective pharmacological tools for TREK-2 channels. Here we report the identification of the first TREK-2 selective activators using a high-throughput fluorescence-based thallium (Tl⁺) flux screen (HTS). An initial pilot screen with a bioactive lipid library identified 11-deoxy prostaglandin F2 α as a potent activator of TREK-2 channels (EC₅₀ $\approx$ 0.294 μ M), which was utilized to optimize the TREK-2 Tl⁺ flux assay (Z' = 0.752). A HTS was then performed with 76 575 structurally diverse small molecules. Many small molecules that selectively activate TREK-2 were discovered. As these molecules were able to activate single TREK-2 channels in excised membrane patches, they are likely direct TREK-2 activators. Furthermore, TREK-2 activators reduced primary dorsal root ganglion (DRG) c-fiber Ca²⁺ influx.

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ASSOCIATED CONTENT

Supporting Information

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Data showing that T2A3 and T2A9 activation of TREK-2 is not temperature dependent and that the external pH sensor of TREK-2 is not required for activation by 2.5 μ M 11-deoxy prostaglandin F2 (PDF)

Author Contributions

Dadi, Vierra, Days, Vinson, Weaver and Jacobson participated in research design. Dadi, Vierra, Days and Jacobson conducted experiments. Dadi, Vierra, Days and Jacobson performed data analysis. Dadi, Vierra, Days, Vinson, Weaver and Jacobson wrote or contributed to the writing of the manuscript.

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Interestingly, some of the selective TREK-2 activators such as 11-deoxy prostaglandin F₂ α were found to inhibit the K₂P channel TREK-1. Utilizing chimeric channels containing portions of TREK-1 and TREK-2, the region of the TREK channels that allows for either small molecule activation or inhibition was identified. This region lies within the second pore domain containing extracellular loop and is predicted to play an important role in modulating TREK channel activity. Moreover, the selective TREK-2 activators identified in this HTS provide important tools for assessing human TREK-2 channel function and investigating their therapeutic potential for treating chronic pain.

Graphical abstract

Keywords

TREK-2; TREK-1; DRG neuron; thallium flux; two-pore-domain potassium channel; pain

Neuronal K₂P channels play an important role in controlling the membrane potential from where action potentials (APs) fire, which controls AP firing frequency and resulting neuronal activity. Nociception is modulated by changes in dorsal root ganglion (DRG) neuron membrane potential that is controlled in part by TREK-2 channels. TREK-2 channels are expressed in small DRG neurons (i.e., IB₄⁺ C-neurons), where they play a significant role in setting the resting membrane potential.^{1–4} The importance of TREK-2 conductance in setting the resting membrane potential was demonstrated by siRNA knockdown of DRG neuron TREK-2, which caused a 10 mV depolarization of the membrane potential.³ As DRG c-fiber hyperpolarization limits electrical excitability, when TREK-2 channels are inactive or removed the DRG neurons show enhanced electrical excitability and increased pain signaling in response to nonaversive stimuli.³ TREK-2 channels are mechanosensitive and also activated by heat; therefore, these channels are responsible for limiting allodynia in response to nonaversive mechanical stimulation as well as heat.^{4–6} Moreover, TREK-2 channels limit spontaneous pain, neuropathic pain, and hyperalgesia.^{3,4} Consequently, ablation of TREK-2 leads to enhanced allodynia in response to mechanical and heat stimulation.⁴ Interestingly, TREK-2 transcript levels are reduced under conditions of DRG damage that leads to chronic pain, which may result from DRG c-fiber electrical hyperexcitability.³ Thus, TREK-2 channels provide a rheostat for pain sensation.

As TREK-2 serves such an important role in limiting pain sensation, it has been proposed that TREK-2 activators could have beneficial therapeutic effects in reducing neuropathic pain.^{3,4,7,8} Indeed, molecules that activate TREK-2 channels have been found to reduce pain.

⁸ For example, aristolochic acid from plant extracts is a traditional medicine for treating pain and activates TREK-2.^{8,9} TREK-2 is also activated by arachidonic acid, lysophosphatidic acid, halothane, chloroform, riluzole, chalcones, wagonin, baicalein, 2-aminoethoxydiphenyl borate (2ABP), and ML67-33.^{10–17} However, while molecules such as aristolochic acid and riluzole have both been shown to reduce peripheral pain signaling, there are currently no selective TREK-2 activators.^{8,18,19} Thus, it is not known whether any of these compounds exhibit their antinociceptive effects via activation of TREK-2 channels. The lack of selective TREK-2 channel activators has impeded characterization of the physiological relevance of TREK-2 in human pain sensation, as well as assessment of the therapeutic potential of activating these channels to reduce postoperative or neuropathic pain. Importantly, TREK-2 expression either has not been detectable or has been shown to be minimally expressed in human cardiac tissue, therefore activation of this channel would not be predicted to cause significant cardiac side effects.^{10,20} Furthermore, the potential exists for developing peripherally restricted TREK-2 activators that do not have addictive properties associated with opioid based pain medications, which stimulate the CNS reward circuitry.

Here we detail a Ti^+ flux-based screen optimized for a TREK-2 inducible cell line that was used to identify the first selective TREK-2 small molecule activators as well as TREK-2 inhibitors. These small molecules are structurally diverse and have EC_{50} 's for TREK-2 activation or inhibition in the low micromolar range. We also identified an important region in the C-terminal half of the second pore domain of TREK-2 that is necessary to allow activation of K^+ efflux by these small molecules. Finally we show that TREK-2 activators reduce DRG nociceptor Ca^{2+} influx, which suggests that with further optimization these activators may be therapeutically useful for treating pain.

RESULTS AND DISCUSSION

A Ti^+ Based HTS Identifies Potent Small Molecule Modulators of TREK-2 Channels

To identify small molecule activators of the TREK-2 channel, we designed a tetracycline inducible TREK-2 HEK cell line (T-REx-TREK-2). Following 24 h of induction with 1 μM tetracycline, the T-REx-TREK-2 cells show robust TREK-2 expression (Figure 1A, inset). A fluorescence-based Ti^+ flux assay was used to monitor TREK-2 channel activity in a 384-well format.^{21,22} Induction of TREK-2 expression in T-REx-TREK-2 cells resulted in robust Ti^+ flux when compared to control cells not induced with tetracycline (Figure 1A). Furthermore, tetracycline does not result in any change in Ti^+ flux in T-REx cells alone (data not shown). With a bioactive lipid library of 928 compounds (Cayman Chemical), a pilot screen was run to confirm the T-REx-TREK-2 Ti^+ assay was capable of identifying lipids similar to those that have previously been shown to activate TREK-2.^{10,23} Indeed the pilot screen identified a group of charged lipids that activate TREK-2 including long chain fatty acids such as 1-oleoyl lysophosphatidic acid, 1-palmitoyl lysophosphatidic acid, and 1-hexadecyl lysophosphatidic acid (Figure 1B). As long chain fatty acids have previously been shown to activate TREK-2, these hits confirmed that the Ti^+ flux based screen can identify TREK-2 activators.^{6,10} The pilot screen also identified a novel and potent lipid activator of TREK-2, 11-deoxy prostaglandin $\text{F}_2\alpha$ (Figure 1B–D). 11-Deoxy prostaglandin $\text{F}_2\alpha$ was found to activate TREK-2 with an EC_{50} of $\sim 0.294 \mu\text{M}$ (Figure 1D). The TREK-2 screen was

further optimized for sensitivity to detect 11-deoxy prostaglandin F2 α activation (Figure 1E) and fluoxetine inhibition, which led to a Z' = 0.752 for TREK-2 activators and a Z' = 0.579 for inhibitors.

Next we used the TREK-2 TI⁺ flux assay to screen 76 575 structurally diverse small molecules in singlicate from NIH's Molecular Libraries Probe Production Centers Network (MLPCN) library. The TREK-2 HTS hit rate was 1.58%, and the average Z' activator window was 0.63 ± 0.09 , and Z' inhibitor window was 0.62 ± 0.08 . All hits were further tested in duplicate on T-REx-TREK-2 cells with and without tetracycline induction, and all compounds that either activated or inhibited TI⁺ flux in the TREK-2 expressing cells within the hit threshold criteria were further assessed for selectivity and potency using a concentration–response curve in the TI⁺ flux assay.

Secondary Screens Identify Selective Small Molecule Activators of TREK-2 Channels

To confirm the selectivity of the TREK-2 activators, we tested their regulation of another K2P channel, TREK-1 (transcript variant 1),^{24,25} which shares 64% amino acid sequence homology with TREK-2 (transcript variant 3).^{6,26} The secondary TI⁺ flux based screen was performed on a stable HEK cell with tetracycline inducible expression of TREK-1, T-REx-TREK-1 (Figure 2A). The monoclonal T-REx-TREK-1 cell line showed robust and reproducible increases in TI⁺ flux following tetracycline induction, which did not modify TI⁺ flux in T-REx-HEK cells. We first assessed whether 11-deoxy prostaglandin F2 α also activated TREK-1 in the T-REx-TREK-1 TI⁺ assay. Interestingly, we observed that 11-deoxy prostaglandin F2 α inhibited TREK-1 channels (Figure 2B). Moreover, the T-REx-TREK-1 TI⁺ assay determined that seven of the identified TREK-2 activators also caused inhibition of TREK-1 channels, whereas eight of the TREK-2 activators activated TREK-1 (Figure 3). Importantly, 23 molecules were identified that selectively activate TREK-2 channels and minimally influence TREK-1 channel activity (Figure 3). Many inhibitors of TREK-2 were also identified; however, all of the TREK-2 inhibitors also inhibited TREK-1 channels (Figure 4). To reduce variability, the HTS was run at room temperature. However, TREK-2 channels have increased activity at 37 °C, and previous pharmacology of TREK-2 has also been shown to be temperature-sensitive. Therefore, we determined CRCs for some of the potent and selective TREK-2 activators at both 25 and 37 °C (Figure S1). Importantly, we find that the most selective and potent TREK-2 activators (T2A3, T2A8, and T2A9) show similar CRCs at 25 and 37 °C (Figure S1).

We next assessed the regulation of TREK-2 currents by the most potent and selective activators utilizing whole cell voltage clamp electrophysiology. A voltage ramp (–120 to +60 mV) elicited robust TREK-2 currents from tetracycline induced T-REx-TREK-2 cells. The TREK-2 currents were significantly activated in response to 10 min of treatment with three of the most potent selective small molecule activators at 10 μ M (Figure 5A,B). Two of the activators (T2A8 and T2A9) primarily increased outward K⁺ flux through TREK-2 channels. However, one of the molecules (T2A3) caused an increase of both inward and outward K⁺ flux through the TREK-2 channels. Thus, differences in the biophysical modulation or mechanism of action of TREK-2 activation may play a role in these divergent influences on TREK-2 activation. Electrophysiology confirmed that the outward K⁺ flux

induced by TREK-2 activators shows similar estimated EC₅₀ values as compared to the CRCs run with inward TI⁺ flux through TREK-2 channels (data not shown). We also performed voltage clamp recordings to test the selectivity of the TREK-2 activators. Six different K⁺ channels were screened for their regulation by selective TREK-2 activators (Figure 5C); these K⁺ channels included TREK-1, TALK-1, TASK-3, K_{ATP}, GIRK1/2, and Kir4.1.^{22,27,28} At 10 μM concentrations, the activators showed only modest regulation of the six K⁺ channels tested during a voltage ramp when assessed at 0 mV (Figure 5C). Therefore, at physiological voltages, these TREK-2 activators are selective for TREK-2 channels.

Small Molecule Activators Act Directly on TREK-2 Channel Complexes

We next utilized inside out patch clamp recording of TREK-2 currents to determine whether small molecule TREK-2 activators acted directly on the channel. Perfusion of the small molecule activators across the cytoplasmic side of a T-REx-TREK-2 membrane patch significantly increased the open probability of TREK-2 channels (Figure 6A–D). Furthermore, upon washout of these molecules TREK-2 channel open probability reduced to its baseline levels (Figure 6A). This indicates that the identified TREK-2 activators likely act directly on TREK-2 channels. Future studies with radioligand binding assays, crystallography, or a TREK-2 liposome flux assay will enable confirmation of a direct interaction of the TREK-2 activators with the channel.^{29,30}

A Region in the Second Extracellular Domain of TREK Channels Allows for Certain Small Molecules To Activate TREK-2 and Inhibit TREK-1

Since some of the identified TREK-2 activators cause inhibition of TREK-1, we used chimeric TREK-2/TREK-1 channels to identify potential regulatory domains responsible for this small molecule mode switching. Four chimeric channels were tested in a TI⁺ flux assay with 11-deoxy prostaglandin F2α and one of the small molecule activators (T2A3, Figure 7A–C). A short amino acid sequence in the second extracellular domain of TREK-2 (GNAGINYREWYKP) was found to be required for activation of these channels; this region is significantly divergent between TREK-1 and TREK-2 channels. Importantly, the chimeric channels containing the TREK-1 s extracellular loop showed inhibition with 11-deoxy prostaglandin F2α and no activation with T2A3 (Figure 7A–C). We went on to examine how the chimeric channel currents respond to 11-deoxy prostaglandin F2α. Similar to the TI⁺ flux experiments, the chimeric channels containing the second extracellular domain of TREK-1 (GNAGINYREWYKP) show inhibition with 11-deoxy prostaglandin F2α (2.5 μM, Figure 7D–F), whereas the channels with the second extracellular domain of TREK-2 show activation with 11-deoxy prostaglandin F2α (2.5 μM, Figure 7G–I). As the C-terminal half of the second extracellular region of the TREK channels allows closure of TREK-1 channels and activation of TREK-2 channels by acidic pH, we also tested whether pH insensitive TREK-2 channels could be activated with 11-deoxy prostaglandin F2α (2.5 μM, Figure S2).³¹ The pH insensitive TREK-2 channel (histidine to alanine substitution in the first extracellular loop; H156A in TREK-2 transcript variant 3) could also be activated by 11-deoxy prostaglandin F2α (2.5 μM, Figure S2), which indicates that 11-deoxy prostaglandin F2α does not activate TREK-2 by preventing alkaline pH inhibition of the channel. Future studies will determine how this region influences the regulation of TREK channel activity in response to pharmacological modulation.^{29,32,33}

Selective TREK-2 Activators Increase DRG c-Fiber K2P Current Amplitude Reducing Pain Signaling

TREK-2 channels play an important role in pain perception; thus, one important question is whether TREK-2 activators can be utilized to reduce postoperative or neuropathic pain. To provide some initial insight into this possibility, DRG c-fiber neurons were utilized to assess how the TREK-2 activators modulate K2P currents and Ca^{2+} influx. K2P currents were recorded in response to a voltage ramp, and the TREK-2 activator T2A3 was found to cause an increase in K2P current amplitude in a subpopulation of IB4⁺ neurons (7 out of 14, 50%, Figure 8). To test whether TREK-2 activation in DRG c-fibers influences DRG neuron signaling, we used a Ca^{2+} assay that measured DRG neuron cytosolic Ca^{2+} dynamics. Pretreatment of DRG neurons with the TREK-2 activator T2A3 caused a significant reduction in Ca^{2+} levels in the neurons (Figure 9A,B). Moreover, the increase in Ca^{2+} observed in DRG c-fibers in response to aversive temperature (40 °C) was significantly blunted in neurons treated with T2A3 (Figure 9A,B). This data only suggests the possibility that TREK-2 activators may reduce pain signaling in DRG c-fibers. However, future studies are required to determine the exact contribution of TREK-2 to the influence of T2A3 and other activators on DRG nociception.

CONCLUSIONS

TREK-2 channel activation serves important physiological roles in modulating DRG neuron excitability and pain signaling.^{3,4} While TREK-2 channels limit rodent neuropathic pain, the roles of TREK-2 channels in human pain signaling have not been determined due in part to a lack of selective pharmacology for these channels. To overcome this obstacle we optimized a fluorescence based Ti^{+} flux screen assay using a Ti^{+} ion as a surrogate to K^{+} for measuring the activity of an inducible human TREK-2 channel. This Ti^{+} flux HTS enabled us to identify the first selective activators of this channel. These TREK-2 activators and their optimized derivatives will be useful in determining the therapeutic applicability of activating TREK-2 for reducing postoperative or neuropathic pain.

Although some of the TREK-2 activators identified in this HTS were selective and do not modulate the activity of any other proteins tested to date (e.g., T2A8), many also influenced the activity of TREK-1 channels. These molecules will still provide useful tools in understanding TREK channel function as well as potential therapeutic modalities that target both TREK-1 and TREK-2 for treating conditions such as pain. Some of the TREK-2 activators caused inhibition of TREK-1 channels, and these molecules will provide structural insights into what controls TREK channel biophysical activity and outward rectification.³⁴ For example, T2A3 activates TREK-2 and at higher concentrations slightly inhibits TREK-1 as well as causing less TREK-2 outward rectification. As TREK-2 rectification is also reduced by a number of other activators such as arachidonic acid, T2A3 may activate TREK-2 channels like these activators and differentially from T2A8 or T2A9 that only modestly influence rectification.³⁴ Interestingly, T2A3 has also been shown to inhibit KCNQ1 as well as TMEM16A in other MLPCN based bioassays (pubchem bioassays 588353 and 588511, respectively). Furthermore, as predicted based on arachidonic acid activation of both TREK-1 and TREK-2, some of the small molecules from this TREK-2

HTS were found to activate both TREK-1 and TREK-2.^{6,10} These molecules may prove useful for treating chronic pain because both TREK-1 and TREK-2 are involved in limiting DRG neuron pain signaling.^{4,35} Importantly, this HTS also identified molecules that show TREK-2 selective activation in both the Ti^+ flux assay and with electrophysiological K^+ current recordings (e.g., T2A8). Although activators were identified that selectively activate TREK-2, there were no selective inhibitors identified. Future studies will determine whether the TREK-2 activators that inhibit TREK-1 can be chemically optimized into selective TREK channel inhibitors.

One of the most potent bioactive lipid activators of TREK-2 was 11-deoxy prostaglandin $\text{F2}\alpha$, which was found to activate TREK-2 and inhibit TREK-1. Interestingly, long-chain unsaturated free fatty acids have previously been shown to activate both TREK-2 and TREK-1.^{6,23} However, certain physiological stimuli such as extracellular acidification cause activation of TREK-2 and inhibition of TREK-1. In an elegant study by Sandoz et al.,³¹ a small region in the C-terminal half of pore domain 2 (P2) was identified that is necessary for extracellular acidic conditions activating TREK-2 and inhibiting TREK-1. A similar region of the TREK channels is also responsible for the divergent influences of 11-deoxy prostaglandin $\text{F2}\alpha$ on TREK channel activity. We identified a short region in P2 of TREK-2 on the C-terminal side of the selectivity filter sequence, which is necessary for channel activation by 11-deoxy prostaglandin $\text{F2}\alpha$ as well as T2A3. When this region of TREK-2 is replaced with the TREK-1 sequence, the resulting chimeric channel shows inhibition with 11-deoxy prostaglandin $\text{F2}\alpha$. Therefore, the extracellular P2 loop of TREK channels is an important mediator of pharmacological regulation of channel activity. Interestingly, the N-terminal region of transmembrane domain 4 (TMD4), which is adjacent to the extracellular P2 loop, serves as an element necessary for c-type gating of TREK-1 and TREK-2 channels.^{36–38} Thus, the C-terminal half of P2 may help dictate whether the N-terminal TMD4 causes opening or closing of the c-type gate in response to small molecule binding. Future crystallographic studies will determine how these TREK-2 activators (molecules that activate both TREK-1 and TREK-2, as well as molecules that activate TREK-2 and inhibit TREK-1) differentially influence movement of the C-terminal half of TREK channel P2 and how this controls c-type gating of TREK channels.

While TREK-2 primarily modulates pain signaling, TREK-2 also serves a role protecting epithelial cells against pressure induced apoptosis.³⁹ TREK-2 is a stretch-activated K^+ channel found in renal epithelial cells.^{26,39} When activated, TREK-2 hyperpolarizes the renal epithelial cell membrane potential, which helps prevent epithelial cells from undergoing an apoptotic signaling cascade.³⁹ Therefore, TREK-2 activation may serve an important role in preventing kidney damage under conditions of increased blood pressure.³⁹ The TREK-2 activators identified in this manuscript will allow characterization of TREK-2 modulation of human epithelial cell mechanoprotection. Moreover, small molecule activators of TREK-2 will help determine whether TREK-2 activation can preserve kidney function in the context of high blood pressure.

Pharmacological activators of TREK-2 may also be a useful therapeutic strategy for treating postoperative or neuropathic pain.³ TREK-2 channels limit allodynia by reducing DRG excitability and serve as a brake to pain signaling such as in conditions of inflammation and

neuropathy.^{3,4} Similarly TREK-2 activators show the capability of limiting DRG neuron Ca^{2+} influx, which would be predicted to reduce pain signaling. While this suggests that TREK-2 activators may be useful in reducing pain signaling, future studies are required to confirm that the TREK-2 activators identified in this study are only working through activation of TREK-2. Interestingly, the TREK-2 activator T2A3 does not activate DRG neuron K2P currents at hyperpolarized membrane potentials as it does with heterologously expressed human TREK-2 channels. This may result from DRG c-fiber TREK-2 channel complexes that have subtle differences in their biophysical activity in response to pharmacological activation. For example, TREK-2 can heterodimerize with TREK-1 or TRAAK; thus, it will be important for future studies to determine how TREK-2 modulators influence TREK-1/TREK-2 or TRAAK/TREK-2 heterodimers.^{40–42} While TREK-2 selective activators will be useful for uncovering the roles of TREK-2 in pain signaling, they may also be useful in identifying new strategies for reducing postoperative pain or neuropathic pain without the potential for addiction. Currently pain strategies are being developed to reduce CNS addictive properties such as μ - δ opioid receptor heteromer-biased agonists.⁴³ TREK-2 KO mice have minimal defects in a wide range of behavioral assays; thus, targeting TREK-2 for treating pain may have less addictive potential than opioid based therapy.⁴⁴ Moreover, as TREK-2 activators are potentially amenable for reducing peripheral pain signaling in DRG neurons, pharmacokinetic and pharmacodynamic studies may also be able to identify TREK-2 activators that are peripherally restricted and reduce pain without CNS side effects.

METHODS

DNA Constructs

Tetracycline inducible K2P channel vectors were created that contain human K2P cDNA in the pcDNA5/TO vector (Invitrogen); these include TREK-2 and TREK-1. The TREK-2/TREK-1 chimera constructs were placed into the pcDNA3.1 vector. The pH insensitive TREK-2 mutation was generated in transcript variant 3 TREK-2 pcDNA3.1 using site directed mutagenesis to convert histidine codon 156 into alanine, which corresponds to histidine 151 in TREK-2 transcript variant 1.³¹ The percent of TREK-1 versus TREK-2 CDS contained in these constructs is detailed in the Results section.

Cell Lines

T-REx-HEK293 cells were transfected with pcDNA5/TO vectors containing K2P channels. Two days post-transfection, the media was supplemented with hygromycin and changed every other day (250 $\mu\text{g}/\text{mL}$). Upon colony generation, the cells were dislodged with trypsin, counted, and seeded to a 384-well plate at a density of 0.5 cells/well. Each well that had cell growth underwent further analysis, and six clones that showed robust tetracycline inducible Ti^+ flux were selected for expansion and cryopreservation. A single monoclonal T-REx-HEK293-TREK-2 and T-REx-HEK293-TREK-1 were employed for these studies.

HEK Cell Culture

Stable T-REx-293 cell lines containing the pcDNA5/TO ion channel plasmids were cultured at 37 °C with 5% CO_2 in DMEM high glucose media with 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$

streptomycin, 250 µg/mL hygromycin, 5 µg/mL blasticidin S, and 10% FBS. T-REx-293 cells were cultured in the same media without hygromycin.

DRG Isolation and Culture

Adult mouse DRG neurons were prepared as previously described.⁴⁵ Briefly, DRG neurons were dissected from 8–10 week old C57Bl6 mice. The DRG neurons were dissociated in collagenase and DNase for 1 h at 37 °C (1250 units of DNase + 1 mg of collagenase A + 0.1 × pen–strep/1 mL/mouse). The dispersed DRG neurons were then plated in 35 mm dishes with coverslip bottoms pretreated with laminin. DRG neurons were cultured at 37 °C with 5% CO₂ in 48% neural basal media (Gibco 21103-049), 48% Ultra Culture media (Lonza, 12-725F), 1% N-2 supplement (Gibco, 17502-048), 1% B27 supplement (Gibco, 17504-044), 2 mM L-glutamine, and 1% pen/strep.

Western Blot Analysis

HEK cell lysates from an equal number of cells with or without tetracycline induction (1 µg/mL) of the TREK-2 channel were run on 4–12% Bis-Tris polyacrylamide gels (Invitrogen). The protein was then transferred to nitrocellulose filter paper (Bio-Rad). The filters were probed with TREK-2 and TREK-1 antibodies (Santa Cruz) as previously described.^{46,47}

Tl⁺ Flux Assay

T-REx-293-TREK-2 cells were plated with 30 000 cells/well in cell culture medium (20 µL/well) with tetracycline at 1 µg/mL in 384-well black-wall, clear-bottom, amine-coated plates (BD Biosciences). These plates were then incubated overnight in a 5% CO₂ incubator at 37 °C. The cells were washed with assay buffer leaving 20 µL/well (assay buffer: HBSS + 20 mM HEPES, pH 7.3). Cells were loaded with 20 µL of Thallo Ion Channel reagent (AM) (TEFLabs, Austin, TX) dye loading solution for 45 min at ambient temperature for the HTS (dye solution: 50 µg of Thallo-AM in 20 µL of pure, dry DMSO with 10 µL of 20% pluronic F-127, diluted to 2 µM in assay buffer) and for 1 h for subsequent experiments. Each additional 384-well plate was washed and dye loaded in 10 min intervals. After the first plate was incubated for 45 min or 1 h, the plate was washed with assay buffer using an ELx405CW plate washer (Bio-Tek Instruments, Inc.), leaving 20 µL remaining. The plate was incubated 2 min at ambient temperature, then loaded into a whole-plate kinetic-imaging functional drug screening system (FDSS 6000, Hamamatsu, Bridgewater NJ), and fluorescence was recorded at 1 Hz (excitation, 470 ± 20 nm; emission, 540 ± 30 nm) for 10 s followed by addition of compounds to a final concentration of 10 µM followed 5 min later with addition of Tl⁺-containing buffer to a final Tl⁺ concentration of 2.4 mM for TREK-2 and 3 mM for TREK-1 for 5 min (Tl⁺ buffer: sodium gluconate 125 mM, MgSO₄ 1 mM, CaSO₄·2H₂O 1.8 mM, D-glucose 5 mM, Tl⁺ sulfate 12 mM, HEPES 20 mM, pH 7.3; used 50% Tl⁺ buffer for TREK-2 and 25% Tl⁺ buffer for TREK-1). The experiments following the full HTS were run slightly differently in that following thallo loading the plates were washed, incubated for 10 min, and monitored for thallo fluorescence with an FDSS for 5 min following small molecule treatment followed by Tl⁺ addition. Four samples of compound were prepared as fresh aliquots from 10 mM compound collection in DMSO stocks by dispensing 80 nL using an ECHO OMICS (Labcyte, Sunnyvale CA) and then

diluting with assay buffer to 20 μ M using a multidrop combi (ThermoFisher Scientific). Subsequent testing of compound hits in duplicate cherry-picked wells or dose-response of compound from 37 μ M to 310 nM in DMSO backfilled samples at 0.37% DMSO final concentrations were also prepared as fresh samples using the OMICS.

Electrophysiology

Whole Cell Voltage Clamp—Patch pipettes (World Precision Instruments) were pulled to a fine diameter with a resistance of 3–5 M Ω . The pipettes were backfilled with intracellular solution containing (in mM) 150 KCl, 3 MgCl₂·6H₂O, 5 ethylene glycol tetraacetic acid (EGTA), 10 HEPES (adjusted to pH 7.25 with KOH). The cells were recorded as previously described with constant perfusion (2 mL/min) of extracellular solution containing (in mM) 150 NaCl, 5 KCl, 3 MgCl₂·6H₂O, 1 CaCl₂, 10 HEPES, 10 glucose (adjusted to pH 7.4 with NaOH).⁴⁸ Where indicated small molecules at the concentrations listed were added to the extracellular solution. For DRG recordings, neurons were incubated with Alexa Fluor 488 conjugated isolectin GS-IB4 (Invitrogen) as previously reported, and green cells were recorded.³

Inside-out Voltage Clamp—Patch pipettes (World Precision Instruments) were pulled to a fine diameter with a resistance of 7–10 M Ω . The recording pipette solution contained extracellular solution (in mM): 120 NaCl, 5 KCl, 3 MgCl₂·6H₂O, and 1 CaCl₂ (pH 7.35 with NaOH). Upon obtaining gigaohm resistance, the extracellular solution was adjusted to (in mM) 140 KCl, 5 EGTA, and 10 HEPES (adjusted to pH 7.2 with KOH). A small piece of membrane was quickly pulled from the cell, and solution was constantly perfused across the inside of the membrane with and without the indicated concentrations of small molecules or lipid.

Calcium Imaging

Adult mouse DRG neurons were plated on 35 mm dishes with glass coverslip bottoms pretreated with laminin (10 μ g/mL). The neurons were loaded with 2 μ M Fura2-AM for 25 min in culture medium at 37 °C in a CO₂ TC incubator. The neurons were then incubated in KREB Ringer buffer and the specified treatment for 20 min at 37 °C. The plates were transferred to a Ca²⁺ imaging system (Nikon TE2000, Nikon elements software, Sutter filter wheel and lambda 300 W xenon arc lamp, Roper Coolsnap CCD camera) and perfused (2 mL/min) with the indicated buffers and treatment.⁴⁹ Relative intracellular Ca²⁺ (reported as F340/F380) was measured every 5 s at 25 °C for 5 min. The imaging continued, and the temperature was ramped up to 40 °C and held at 40 °C for 10 min. The time-lapse images were analyzed with Nikon elements and Excel software.

Statistical Analysis

Experimental data are presented as average values $\pm$ SEM. Unpaired two-tailed *t* tests and paired two-tailed *t* tests were used to determine statistical significance (*P* < 0.05) between test groups as appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CRC	concentration–response curve
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
DRG	dorsal root ganglion
EGTA	ethylene glycol tetraacetic acid
FCS	fetal calf serum
FDSS	functional drug screening system
HBSS	Hanks' balanced salt solution
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTS	high-throughput screen
T-Rex-HEK293	tetracycline inducible HEK-293 cells
K2P	two-pore-domain potassium channel
TREK-2	K2P10.1
TREK-1	K2P2.1
TASK-3	K2P9.1

TRAAK	K2P4.1
CDS	coding sequence

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Figure 1.

Identification of TREK-2 bioactive lipid activators with a thallium flux assay. (A) Thallos fluorescence monitored before and after addition of Tl^+ (arrow) to T-Rex-TREK-2 cells induced with tetracycline (1 μ g/mL, blue traces) or not induced (red traces). Inset is a Western blot run with T-Rex-TREK-2 cell lysates with (+) or without (–) tetracycline induction and probed with a TREK-2 antibody. (B) Bioactive lipids that activate TREK-2 channels when preincubated with T-Rex-TREK-2 cells for 5 min at a 10 μ M concentration. (C) Tl^+ flux (arrow) into thallos loaded TREK-2 expressing cells with (blue) or without (red) pretreatment with 11-deoxy prostaglandin $F2\alpha \pm$ SEM ($p < 0.001$ after 300 s). (D) Dose–

response curve for TREK-2 activation with 11-deoxy prostaglandin F2 α $\pm$ S.E.M.; fitting determined an EC₅₀ value of 0.294 μ M. (E) Calculation of the Z' using the slope of TI⁺ influx for each well of a 384 well plate containing TREK-2 expressing cells treated with 11-deoxy prostaglandin F2 α (top) or vehicle (bottom, Z' of 0.752).

Figure 2.

Optimization of the secondary TREK-1 TI⁺ flux assay. (A) Thallos fluorescence (F488) monitored before and after addition of TI⁺ (arrow) to T-Rex-TREK-1 cells induced with tetracycline (1 μg/mL, blue) or not induced (red) ± SEM. (B) TI⁺ flux (arrow) into thallos loaded T-Rex-TREK-1 expressing cells with (red) or without (blue) 10 min pretreatment with 1.8 μM 11-deoxy prostaglandin F₂α ± SEM.

Figure 3.

Top 10 TREK-2 activators. Concentration–response curves of compounds identified to activate TREK-2 channels measured as an increase in TI^+ flux and compared to TREK-1. Shown are the top 10 activators of TREK-2 with structures and estimated EC_{50} s ranked by potency (red traces) and compared to TREK-1 channels (blue traces). Each dose represented by a triplicate data point is expressed as normalized to a maximally effective dose of 11-deoxy prostaglandin $F2\alpha$ treatment (TREK-2) or TI^+ concentration (TREK-1). When displayed the EC_{50} was estimated using a nonlinear fit model with variable slope and normalized response in GraphPad Prism.

Figure 4.

Top 10 TREK-2 inhibitors. Concentration–response curves of compounds identified to inhibit TREK-2 channels measured as decrease in TI^+ flux and compared to TREK-1. Shown are the top 10 compounds with structures ranked by potency for inhibition of TREK-2 channels (red traces) compared to TREK-1 channels (blue traces). Each dose represented by a triplicate data point is expressed as normalized to a maximally inhibitory dose of fluoxetine. When displayed, potency is an IC_{50} estimated using a nonlinear fit model with variable slope and normalized response in GraphPad Prism.

Figure 5.

TREK-2 activators are selective for TREK-2. (A) Representative TREK-2 channel activity during the indicated voltage ramp 5 min after addition of vehicle or 10 μ M of the indicated TREK-2 activator. (B) Average change in TREK-2 current at 0 mV following 5 min after indicated treatment $\pm$ SEM (* $p < 0.05$). (C) The same voltage ramp experiments as in panels A and B were run on six other K⁺ channels (listed below the bar graph) before and after 10 μ M of each TREK-2 activator; the resulting average change in current at 0 mV following each treatment is plotted $\pm$ SEM.

Figure 6.

TREK-2 activators increase the channel's open probability. (A) Inside-out voltage clamp recording of a TREK-2 channel at $-30\ \text{mV}$ with 11-deoxy prostaglandin $\text{F}_2\alpha$ treatment (black bar). (B) Voltage ramp activation of TREK-2 channels with (blue) or without (red) $2\ \mu\text{M}$ 11-deoxy prostaglandin $\text{F}_2\alpha$ treatment. (C) Inside-out voltage clamp recording of a TREK-2 channel at the indicated voltages before and 5 min after the indicated treatment with T2A3. (D) Average open probability of TREK-2 at $-30\ \text{mV}$ before or 5 min after T2A3 treatment $\pm$ SEM (* $p < 0.05$).

Figure 7.

A region on pore domain two is responsible for the activation of TREK-2 or inhibition of TREK-1 with 11-deoxy prostaglandin $\text{F}_2\alpha$. (A) Cartoon structures of the TREK-2 (gray)/TREK-1 (black) chimeric channels used to identify the region responsible for either activation or inhibition of TREK-2 or TREK-1, respectively. (B) Average change in Ti^+ flux for the chimeric channels 5 min after addition of 2.5 μ M 11-deoxy prostaglandin $\text{F}_2\alpha$ $\pm$ SEM. (C) Ti^+ flux change of the chimeric channels 5 min after addition of 10 μ M T2A3 $\pm$ SEM. (D) Representative chimeric-1 TREK channel activity during the indicated voltage ramp 10 min after addition of vehicle (black trace) or 2.5 μ M 11-deoxy prostaglandin $\text{F}_2\alpha$ (red trace). (E) Representative chimeric-2 TREK channel activity during the indicated voltage ramp 10 min after addition of vehicle (black trace) or 2.5 μ M 11-deoxy prostaglandin $\text{F}_2\alpha$ (red trace). (F) Average change in chimeric channel current inhibition at -60 , -30 , and 60 mV following 10 min treatment with 2.5 μ M 11-deoxy prostaglandin $\text{F}_2\alpha$ $\pm$ SEM ($n = 6$ chimeric 1, $n = 4$ chimeric 2, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). (G) Representative chimeric-3 TREK channel activity during the indicated voltage ramp 10 min after addition of vehicle (black trace) or 2.5 μ M 11-deoxy prostaglandin $\text{F}_2\alpha$ (red trace). (H) Representative chimeric-4 TREK channel activity during the indicated voltage ramp 10 min after addition of vehicle (black trace) or 2.5 μ M 11-deoxy prostaglandin $\text{F}_2\alpha$ (red trace). (I) Average change in chimeric current activation at -60 , -30 , and 60 mV following 10 min

treatment with 2.5 μ M 11-deoxy prostaglandin F2 α $\pm$ SEM (n = 5 chimeric 3, n = 5 chimeric 4, n = 7 chimeric 5, * p < 0.05, ** p < 0.01, *** p < 0.001).

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Figure 8.

Mouse DRG c-fiber TREK-2-like currents are activated by T2A3. (A) Mouse DRG currents recorded during a voltage ramp (–120 to 60 mV) before (red) and 5 min after vehicle (blue) treatment. (B) Mouse DRG currents recorded during a voltage ramp (–120 to 60 mV) before (red) and 5 min after T2A3 (blue) treatment. (C) Normalized increases in DRG K2P currents 5 min following vehicle or T2A3 treatments $\pm$ SEM (** $p < 0.005$).

Figure 9.

TREK-2 activator T2A3 reduces DRG neuron Ca^{2+} influx. (A) Representative Ca^{2+} responses of IB4+ DRG neurons (inset of IB4+ staining) following 20 min pretreatment with DMSO (blue trace) or 10 μM T2A3 (red trace) and in response to the change in temperature (indicated above the traces). (C) The area under the Ca^{2+} curve for all DRG neuron Ca^{2+} responses at 25 and 40 °C $\pm$ SEM ($n > 80$ DRG neurons per group isolated from 4 mice, *** $p < 0.001$).