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Pungent agents from Szechuan peppers excite sensory neurons by inhibiting two-pore potassium channels

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Pungent agents from Szechuan peppers excite sensory neurons by inhibiting two-pore potassium channels

by

Yaron M. Sigal

DISSERTATION

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Yaron Sigal completed the following:

Chapter 2

Figure 1D

Chapter 3

Figure 1 A through E

Figure 2

Figure 3

Figure 4 A

Chapter 4

Figure 1 C

Figure 2 A

Figure 3 A and C

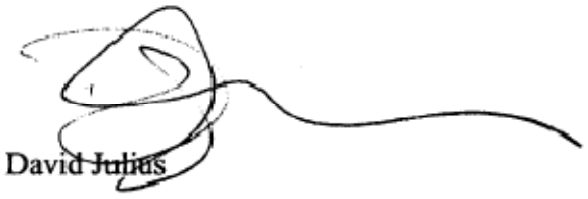
Yaron Sigal completed the following together with Diana Bautista:

Chapter 2

Figure 1 D and F

Figure 3

This constitutes a body of work sufficient to fulfill the requirements for a doctoral degree awarded by the University of California, San Francisco.



Abstract

Pungent agents from Szechuan peppers excite sensory neurons by inhibiting two-pore potassium channels

Yaron Sigal

In traditional folk medicine, Xanthoxylum plants are referred to as 'toothache trees' because their anesthetic or counter-irritant properties render them useful in the treatment of pain. Psychophysical studies have identified hydroxy-α-sanshool as the compound most responsible for the unique tingling and buzzing sensations produced by Szechuan peppercorns or other Xanthoxylum preparations. Although it is generally agreed that sanshool elicits its effects by activating somatosensory neurons, the underlying cellular and molecular mechanisms remain a matter of debate. Here we show that hydroxy-α-sanshool excites two types of sensory neurons, including small-diameter unmyelinated cells that respond to capsaicin (but not mustard oil) as well as large-diameter myelinated neurons that express the neurotrophin receptor TrkC. We found that hydroxy-α-sanshool excites neurons through a unique mechanism involving inhibition of pH- and anesthetic-sensitive two-pore potassium channels (KCNK3, KCNK9 and KCNK18), providing a framework for understanding the unique and complex psychophysical sensations associated with the Szechuan pepper experience.

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CHAPTER 1

Introduction

Introduction

Somatosensation, or the sense of touch, is the process whereby we detect changes in ambient temperature or pressure. This sensory modality is mediated by subsets of primary afferent neurons that detect chemical, thermal or mechanical stimuli over a range of stimulus intensities. Generally speaking, pain-producing (noxious) stimuli are detected by neurons (referred to as nociceptors) that have small- to medium-diameter somata that correspond to unmyelinated C and lightly myelinated Aδ nerve fibers. In contrast, innocuous stimuli, such as light touch, are detected by large-diameter neurons corresponding to more heavily myelinated Aα or Aβ fibers[1]. These main groups of somatosensory neurons can be further subdivided based on their expression of numerous molecular markers or their specific functional (biophysical or pharmacological) characteristics.

A key goal in understanding somatosensation is to elucidate the contribution of sensory neuron subtypes to specific psychophysical sensations. In this regard we, and others, have exploited the power of folk medicine and natural products to probe somatosensory mechanisms and identify functionally and molecularly distinct classes of somatosensory neurons[2-4]. In particular, pungent plant-derived irritants, such as capsaicin, mustard oil and menthol, have been used to define nociceptor subtypes and the receptors that mediate pain-producing thermal or inflammatory responses *in vivo*[5, 6]. This approach has been extremely fruitful in identifying cellular and molecular mechanisms contributing to nociception and the detection of painful stimuli. Thus, it is interesting to ask whether other plant-derived compounds, perhaps those eliciting milder somatosensory percepts,

could be useful in identifying neuronal subtypes that contribute to the detection of non-noxious stimuli. In this regard, we have focused our attention on natural products produced by *Xanthoxylum* plants, such as the Chinese prickly ash, from which Szechuan peppercorns are harvested.

Szechuan peppers or related plants have been exploited for their medicinal and culinary properties in both traditional Asian and Native American cultures[7, 8]. In contrast to the intense, burning pain associated with 'hot' chili peppers of the *Capsicum* family, Szechuan peppers elicit a wholly unique sensation that is best described as a tingling paresthesia or numbing[9, 10], suggestive of an interaction with neurons involved in tactile sensation and innocuous touch[11]. Hydroxy-α-sanshool (sanshool) is the active ingredient in Szechuan peppers, and although there has been some preliminary analysis of its effects on cultured sensory neurons[9, 11, 12], its cellular and molecular site of action remains enigmatic. For example, sanshool was initially proposed to activate subsets of primary afferent fibers that respond to cooling, heat or light touch[11], whereas a more recent study suggests that most, if not all, sensory neurons respond to sanshool. Furthermore, two studies reach different conclusions as to the involvement of specific molecular targets, most notably the capsaicin receptor (TRPV1), in this response[10,12].

Here, we take a multifaceted approach to elucidate the cellular and molecular basis of sanshool action. We show that sanshool activates a constellation of sensory neurons that include specific subpopulations of small- and large-diameter cells, which together represent a unique subset of nociceptors and presumptive light-touch receptors.

Moreover, we find that sanshool excites these neurons by inhibiting background potassium conductances. Specifically, we identify three members of the pH-sensitive two-pore KCNK channel family as being molecular targets for sanshool action. Notably, these channels are also targeted by volatile anesthetics, perhaps accounting for the numbing properties elicited by sanshool compounds and for the use of *Xanthoxylum* extracts in traditional folk medicine for treating toothache and other types of orofacial pain.

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CHAPTER 2

Cellular Actions of Hydroxy-α-sanshool and Related Alkylamides

Cellular Actions of Hydroxy-α-sanshool and Related Alkylamides

The somatosensory system, the way in which we perceive both innocuous and noxious thermal and mechanical stimuli, is a complex system involving aspects of both the peripheral and central nervous systems. Primary afferents in the trigeminal ganglia (TG) or dorsal root ganglia (DRG) directly sense these stimuli in the environment of its nerve terminals, ultimately generating an action potential and transmitting the proper signal to the central nervous system[1]. Historically, these neurons have been characterized based on their anatomy and electrophysiology in a range of model systems. More recently, molecular markers have begun to emerge that can provide a detailed and functional description of different populations of neurons.

Broadly speaking, there are two kinds of neurons within these sensory ganglia: low-threshold mechanosensors that are responsible for detecting normal cutaneous touch and nociceptors that are responsible for detecting painful stimuli both in the skin as well as in deep tissue. Within the sensory ganglia, low-threshold mechanosensors (LTMs) generally have the largest diameter soma (termed Aα and Aβ). The nerve fibers that project both peripherally and centrally from these neurons are heavily wrapped and insulated by glial Schwann cell called myelin. This myelination greatly increases the resistance of the nerve cell membrane and alters the propagation of the electrical signal, thereby allowing for transmission at a fast velocity (14-55 m/s)[2]. The peripheral nerve terminals of these LTMs are often involved in interactions with different specialized cell types within the skin, such as Merkel cells and Pacinian corpuscles, that provide for the

different types of cutaneous touch[3]. Other afferents that respond to innocuous stimuli such as gentle warming or cooling can also be found throughout the skin[4, 5].

Nociceptors can also be further divided based on several properties. Aδ fibers are lightly myelinated, resulting in a fast signal responsible for the first wave of pain from a stimulus[6]. Within this class of nociceptors, some fibers respond to either heat or to mechanical forces, while others are polymodal and can respond to both[6]. Furthermore, these responses can be significantly modulated upon both chemical stimulus and injury, with otherwise mechanically insensitive afferents becoming highly sensitized[7]. Nociceptors known as c-fibers are unmyelinated, therefore conducting currents at a much slower rate (< 1.4m/s), and are responsible for the second wave of pain resulting from a stimulus. Most c-fibers are polymodal and can respond to thermal, mechanical, and chemical stimuli. The majority of these fibers can also be significantly modulated by tissue or nerve injury[8].

Several molecular markers can now be used to visualize and characterize different populations of sensory neurons. Large and medium diameter, Aα, Aβ, and Aδ fibers are myelinated and as such, they can be visualized and identified using antibodies to the myelin protein, neurofilament 200 (NF200)[9]. This population accounts for about 40% percent of neurons found in rat DRGs[10]. The expression of neuropeptides currently defines another population that comprises approximately 40% of DRG neurons[10]. This group is best characterized by the peptide calcitonin gene-related product (CGRP) and the nerve growth factor (NGF) receptor TrkA, both of which are expressed by the majority of

peptidergic neurons[11]. Cells that express other neuropeptides such as Substance P and peripherin also fall into this category, but are found in only subpopulations of this group. CGRP and TrkA expressing neurons are predominantly small c-fibers, however about 10% of total DRG neurons are both peptidergic and express NF200 and are thought to represent Aδ nociceptors[12]. This population of cells that express both NF200 and neuropeptides predominantly also express the neurotrophin 3 receptor TrkC[12]. The remaining population of sensory neurons is mainly non-peptidergic c-fibers and comprises about 30% of the total DRG neuron population. There are a few markers that can be used to identify this population, the most widely used of which is the binding of the lectin *Griffonia simplicifolia* IB4 (IB4+)[13]. While it is useful to use these molecular tools to characterize different populations of sensory neurons, it is equally important to remember that significant overlap exists between different markers and subpopulations of neurons and that the expression of different markers can be drastically altered under physiological conditions such as nerve or tissue injury.

While the neurons have been characterized on a cellular level, due to the nature of the stimuli, the molecular identification of receptors for these somatosensory modalities has proven more difficult. Both temperature and mechanical forces are parameters that cannot be completely dissociated from any experiment, and all biological processes are affected by these parameters to some extent. One method which has been successful in overcoming this difficulty has been the use of chemical natural products whose psychophysical effect mimics the somatosensory stimuli. The first successful example of this was the identification of the capsaicin receptor, TRPV1. In order to identify the

receptor, a cDNA library was constructed from mRNA isolated from mouse DRGs. Pools of several thousand cDNA clones were transfected into HEK293T cells, a nonresponsive cell type. The cells were then loaded with a calcium sensitive dye, and examined for the influx of calcium following the application of capsaicin. Once a positive pool of cDNA was found, it was iteratively subdivided and rescreened until a single clone encoding the receptor was identified. When the receptor was heterologously expressed, it was found to be directly activated not only by vanniloids such as capsaicin, the pungent ingredient in chili peppers, but also by noxious heat (>47°C) and acidic pH[14,15].

TRPV1 is found entirely in small c-fiber nociceptors and is probably the best marker of this population of neurons. As TRPV1 is the only protein in sensory neurons that responds to capsaicin, capsaicin sensitivity can also be used as a marker for c-fiber nociceptors. TRPV1 is expressed in both peptidergic and non-peptidergic c-fibers and is found in the majority of CGRP and IB4+ cells (~60% and 75%, respectively)[16, 17]. In addition, TRPM8 and TRPA1 have been identified and characterized, and demonstrate a conserved role for TRP channels in somatosensation[18, 19]. Furthermore, channels related to the mammalian TRPV family have been found in *Drosophila* and *C. Elegans* and are required for different aspects of somatosensation, clearly showing a role for TRP channels across species in both vertebrates and invertebrates[20].

Despite the work described above that has been done to characterize and identify the molecular mechanisms of somatosensation, many of the proteins involved in the

transduction and modification of noxious and innocuous stimuli have not yet been identified. Even among the best studied example of TRPV1, the mechanism by which the channel is able to respond so precisely to temperature changes in not well understood. In order to gain a better understanding of these complex sensory functions it is necessary to both identify more molecular players involved in the transduction of these signals as well as a better comprehend the cellular networks that allow for both fidelity and cross-talk in the transduction of these diverse stimuli. To this end, we decided to focus on the main pungent compound found in Szechuan peppers, hydroxy-α-sanshool, and the underlying cellular mechanisms that leads to its distinctive psychophysical sensations.

Sanshool activates a unique subset of somatosensory neurons

We first purified sanshool from Szechuan pepper (**Fig. 1a**,**b**) and asked whether it excites a specific subpopulation of sensory neurons. Neurons were cultured from trigeminal or dorsal root ganglia of the mouse and responses assessed using live-cell calcium imaging as a functional readout (**Fig. 1c**–**f**). We found that sanshool (100 μM) serves as an excitatory agent for a specific subgroup (52.3%; n = 2,063) of sensory neurons that could be further categorized into two main classes based on expression of molecular markers[21]. One class corresponds to a subset of small-diameter, unmyelinated neurons that express the capsaicin receptor, TRPV1; the other class corresponds to a subset of large-diameter, myelinated neurons that show NF-200 immunoreactivity and express the neurotrophin (NT-3) receptor TrkC (**Fig. 1c**,**d**). We also examined the pharmacological properties of these sanshool-sensitive classes using three additional natural-product agonists—capsaicin, mustard oil and menthol—that define cells expressing excitatory TRPV1,

TRPA1 and TRPM8 channels, respectively[22, 23]. Consistent with our histological results, we found that the sanshool-sensitive small-diameter neurons were also activated by capsaicin, but not by mustard oil or menthol (**Fig. 1e**,**f**). In contrast, the sanshool-sensitive, large-diameter cells were not activated by any of these irritants, but did respond to osmotic stimuli (**Fig. 1f**). Based on these characteristics, we conclude that sanshool sensitivity is exhibited by both presumptive nociceptors and low-threshold mechanoreceptors. Notably, sanshool sensitivity now provides a functional marker for the subset of capsaicin-sensitive neurons that do not respond to mustard oil (the TRPV1-positive, TRPA1-negative population) and that represent ~50% of capsaicin-excitable cells[24].

It is possible that the selectivity of sanshool in activating a distinct subpopulation of neurons is due to the application of a submaximal dose rather than any inherent signaling specificity. To test this, following the application of 100 μM sanshool, we challenged the neurons with sanshool at concentrations ranging up to 1mM as well as a crude extract of Szechuan pepper **(Fig. 2).** In both cases, there was no new population of neurons which was activated at these higher concentrations, demonstrating that in this assay, 100 μM sanshool represents a saturating dose and is able to activate a distinct constellation of sensory neurons.

Szechuan pepper extracts also contain several other isoforms of sanshool, most prevalently, hydroxy-β-sanshool. However, this isomer plays a minor role in eliciting the tingling psychophysical effects of *Xanthoxylum* plants[25]. Indeed, we found that

application of hydroxy-β-sanshool (100 μM–1 mM) did not excite sensory neurons (**Fig. 3a**). In addition, several independent families of have been found to contain structurally similar alkylamides. Particularly, those found in *Echinacea purpurea* have been suggested as the primary active immunomodulatory of the herb. Two such alkylamides were obtained and applied to sensory neurons (**Fig, 3b)**, and at 100μM, the two compounds were found to activate a similar population of sensory neurons as hydroxy-α-sanshool both in terms of prevalence and pharmacology of the response **(Fig. 3c, 3d).**

Sanshool inhibits a pH-sensitive K^+ leak conductance

We next used whole-cell voltage-clamp recording methods to determine the nature of the ionic current(s) underlying sanshool-evoked depolarization of these neurons. Application of sanshool led to a dose-dependent inhibition of an outwardly rectifying background leak current (**Fig. 4a**). Moreover, the sanshool-evoked block was reversible and showed no sensitization in response to repetitive application. Inhibition was observed in a subset of both small- and large-diameter trigeminal neurons, with a half-maximal inhibitory concentration (IC_{50}) of 69.5 ± 5.3 μM (**Fig. 4b**). The extent of inhibition (at 100 μM sanshool) was equivalent for each size class (59.7 ± 2.6% and 58.0 ± 2.4%, respectively; n = 6–7; **Fig. 4c**). Replacement of extracellular Na^+ or Ca^{2+} with NMDG or EGTA, respectively, had no effect on this sanshool-inhibited current, demonstrating that it is not mediated by influx of these cations. In contrast, replacement of extracellular Na^+ with 135 mM K^+ resulted in a shift of the reversal potential from –78.5 ± 6.2 mV to –3.8 ± 2.9 mV (data not shown). In addition, sanshool-sensitive currents were not observed when K^+

was replaced with Cs^+. Taken together, these data show that sanshool depolarizes sensory neurons by inhibiting a background K^+ channel.

We further found that the sanshool-sensitive current was unaltered by classical K^+ channel blockers, such as tetraethylammonium (TEA) or 4-aminopyridine (4-AP) (data not shown), but could also be inhibited by extracellular protons (**Fig. 4a**; blue). Both KCNQ and KCNK channels fit this pharmacological profile[26-28], suggesting that sanshool targets one or more members of these K^+ channel families. The KCNQ inhibitors linopiridine (50 μM) and XE-991 (50 μM) neither triggered calcium influx nor altered sanshool-evoked calcium responses in cultured sensory neurons (data not shown). Thus, we conclude that KCNK channels are the likely targets of sanshool action. Indeed, members of this two-pore K^+ channel subfamily (KCNK2, KCNK3, KCNK9, KCNK10 and KCNK18) have been proposed to set the resting membrane potential of primary afferent sensory neurons, with KCNK18 (TRESK) having the predominant role[29-31].

Discussion

Here we describe the cellular actions through which pungent agents of *Xanthoxylum* plants mediate their unique psychophysical effects, akin to the experience of touching one's tongue to the terminals of a 9-V battery. This sensation is rather distinct from that elicited by other pungent natural products, such as those derived from chili peppers or wasabi, which produce a more acute and painful irritation that is also associated with local tissue inflammation and pain hypersensitivity. Moreover, our data suggest that sanshool is unique among pungent agents in that its excitatory actions are mediated by

inhibition of pH-sensitive background potassium channels, rather than through the more familiar mechanism involving direct activation of an excitatory TRP channel[32]. Indeed, such functional differences may contribute to perceived differences in the pungency (onset or intensity) of these natural-product irritants. More broadly, the mechanism of sanshool-evoked K^+ channel inhibition differs from that of most chemosensory agents, which involve G protein–coupled receptor signaling pathways[33, 34].

Differences in pungency perception and other neurally mediated effects may also reflect the activation of overlapping, but distinct, subpopulations of primary afferent sensory neurons. For example, capsaicin activates a subgroup of small-diameter sensory neurons, of which approximately 50% are also activated by mustard oil. This latter component of dually responsive neurons also express proinflammatory neuropeptides (CGRP and substance P), accounting for both the acute pain and the neurogenic inflammatory actions commonly associated with exposure to chili peppers, wasabi or garlic. In contrast, we show that sanshool excites only the subset of capsaicin-sensitive neurons that are mustard oil insensitive, thereby excluding most of the peptidergic nociceptors. This is consistent with the fact that exposure to sanshool is not associated with intense pain, neurogenic inflammation or hyperalgesia.

We also found that sanshool activates large-diameter, TrkC-positive, myelinated neurons, which are generally associated with proprioception and the detection of non-noxious mechanical stimuli, such as light touch or vibration[35]. It is therefore notable that the majority of TrkC-positive, osmotically sensitive neurons are also sensitive to sanshool,

consistent with the idea that sanshool elicits its tingling and buzzing sensation by activating a cohort of touch-sensitive fibers. Sanshool therefore serves as the first pungent natural product with which to identify this specific cohort of mechanosensitive primary afferent neurons.

By examining related alkylamides, we can begin to decipher some molecular requirements for the activation of sensory neurons by this class of molecules. A simple switch between hydroxy-α-sanshool and hydroxy-β-sanshool from a *cis* to *trans* double-bond is sufficient to abolish neuronal sensitivity to the molecule. In contrast, the saturation of a similar portion of the alkyl tail, as seen in the Echinacea compound, E2, still retains the activity seen with hydroxy-α-sanshool. These changes may result in a change in the binding affinity of the alkylamide to a sensory receptor. The lack of activity that is seen with hydroxy-β-sanshool could be due either to a decrease in inhibition of the background K^+ current or, conversely, could represent a gain in function through the additional inhibition of voltage-gated calcium currents that are presumably necessary to trigger the detected calcium influx. Further determination of mechanism will require more detailed electrophysiological studies.

Xanthoxylum plants have been exploited for centuries as natural analgesics to alleviate acute and chronic pain. Fruits of these plants have also been use extensively in the kitchen because of their unique pungent qualities. In contrast to the familiar burning or irritating pain elicited by chili peppers or mustard extracts, the sensorial experience produced by Szechuan peppercorns is more generally described as “tingling and

numbing," "mild electric shock" or a "pins and needles" effect[36, 37]. These psychophysical percepts are in many ways consistent with the cellular sites of sanshool action. The tactile component may result from the activation of large-diameter, touch-sensitive fibers, whereas the pungent or irritant qualities may involve excitation of nonpeptidergic, capsaicin-sensitive nociceptors. The identification of sanshool-sensitive sensory neuron subtypes represents an essential first step in understanding mechanisms underlying this unique pungency and its relationship to tactile sensitivity.

Methods

Pharmacological reagents. Hydroxy-α-sanshool (2*E*,6*Z*,8*E*,10*E*)-2′-hydroxyl-*N*-isobutyl-2,6,8,10-dodecatetraenamide) was purified as follows: dried seeds from *Zanthoxylum piperitum* (50 g; San Francisco Herb Company) were ground to a fine powder and extracted twice, each time with 1 liter diethyl ether for 24 h at 4 °C. Extracts were combined and filtered, solvent removed *in vacuo*, and the residue further dried on high vacuum overnight to yield 4.25 g of crude material. This was further purified by flash chromatography (1:2 ethyl acetate/hexanes; 230–400-mesh silica gel, Selecto Scientific) followed by preparative HPLC (30–100% methanol gradient over 40 min; 10 ml min^{-1}; COMBI-A C18 preparatory column, Peeke Scientific; absorbance monitored at 215 and 245 nm) to give 55.2 mg of sanshool (0.1% yield; pale brown solid). As indicated by NMR spectra, this material consists predominantly of hydroxy-α-sanshool. LC-ESI-MS $[MH]^+$ *m*/*z* for hydroxy-α-sanshool ,$C_{16}H_{25}NO_2$: calculated, 264.38; observed, 264.48. Pure hydroxy-α-sanshool was isolated using an Xterra C-18 column

(Waters) on a Parallex Flex (Biotage) preparative HPLC instrument with a solvent gradient of 20–70% acetonitrile/water at a flow rate of 20 ml min^{-1} for 60 min. Injections were monitored at 254 nm and each fraction lyophilized and assessed by ^{1}H NMR.

^{1}H spectra were recorded on a Varian 400 spectrometer at 400 MHz. Chemical shifts were reported as parts per million (p.p.m.) downfield from an internal tetramethylsilane standard (d = 0.0 for ^{1}H NMR) or from solvent references. Low-resolution electrospray ionization mass spectra (EI^{+}-MS) were recorded on a Waters Micromass ZQ 4000 spectrometer. LC-MS (MS: EI^{+}) was performed on a Waters Alliance HT LC-MS with a flow rate of 0.2 ml min^{-1} (monitored at 215 nm and 245 nm) using an Xterra MS C18 column (Waters). Analytical thin-layer chromatography was performed with silica gel 60 F254 glass plates (EM Science). Purified hydroxy-α-sanshool was dissolved in dimethylformamide (DMF at a concentration of 100 mM and stored at –80 °C. 200 μM β-cyclodextrin was added to all solutions containing 1 mM sanshool to increase solubility.

Neuronal cell culture, calcium imaging and electrophysiology. Preparation of mouse trigeminal or dorsal root ganglion neurons and ratiometric calcium imaging were carried out as previously described[24]. Extracellular Ringer's solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM d-glucose and 10 mM sodium HEPES (pH 7.4). Extracellular Ringer's was supplemented with 1 μM tetrodotoxin citrate (Tocris) for voltage-clamp recordings. High-potassium Ringer's solution, used for excised patch recordings, contained 150 mM KCl, 5 mM NaCl, 1 mM $CaCl_2$, 1 mM EGTA, 2 mM

$MgCl_2$ and 10 mM sodium HEPES (pH 7.4). Internal pipette solution containing 150 mM potassium methyl sulfate, 10 mM KCl, 4 mM NaCl, 10 mM HEPES, 0.4 mM tetrasodium GTP and 4 mM dimagnesium ATP, pH 7.25, was used for all recordings. Agonist was applied via a local perfusion barrel system (Automate Scientific). For calcium imaging, cells were loaded with 10 μM Fura-2-AM (Molecular Probes) at 22–25 °C for 60 min in extracellular Ringer's solution. Cells were illuminated using a xenon light source and filter wheel (Lambda LS and Lambda-10, Sutter Instruments) for 300 ms, alternately at 350 nm and 380 nm (band-pass filters from Chroma Technology). Fluorescence emission at >480 nm (long-pass filter from Chroma Technology) was captured with an intensified CCD camera (Hamamatsu) and was digitized, background corrected and analyzed with the MetaFluor imaging system (Molecular Devices) Background-corrected 340/380 ratio images were collected every 3 s. $[Ca^{2+}]_i$ was determined from the relationship $[Ca^{2+}]_i = K^*(R - R_{min})/(R_{max} - R)$, where R is the F340/F380 ratio, R_{min} and R_{max} are the ratios at 0 Ca^{2+} and saturating Ca^{2+} (10 mM), respectively, and K^* is the apparent dissociation constant[38].

Immunohistochemistry. Prior the plating of the neurons onto the coverglass slide, the bottom of the coverglass was carefully scored using a diamond-tipped pen. This marking served as a point of reference with which to realign the imaged field after immunohistological staining. After calcium imaging, neurons were fixed in PBS containing 4% formaldehyde at 4 °C for 10 min, then washed with PBS and permeabilized in PBS containing 0.1% Triton X-100 for 5 min at 22-25 °C. Samples were then incubated with 10% horse serum and 0.1% (vol/vol) Triton X-100 for 1 h,

followed by anti-NF200 mouse monoclonal antibody (1:500 dilution, Sigma) overnight at 4 °C. Samples were washed three times, for 10 min each, with PBS containing 0.3% Triton X-100, and then incubated with Alexa Fluor 488 secondary antibody (Molecular Probes) at room temperature for 30 min, washed three times for 10 min each with PBS, realigned with the reference image and visualized by indirect immunofluorescence.

Mice and behavior. Mice (20–35g) were housed with 12 h/12 h light/dark cycle at 21°C. All experiments were performed according to the policies and recommendations of the International Association for the Study of Pain and approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. TrkC-GFP mice have been described elsewhere[39].

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Figure Legends

Figure 1 Hydroxy-α-sanshool excites a subset of presumptive nociceptors and mechanoreceptors. (**a**) Szechuan peppers are the spicy berries of *Xanthoxylum piperitum*, a species of prickly ash found in China and Japan. (**b**) Structure of hydroxy-α-sanshool, the main pungent compound from *Xanthoxylum* plants. (**c**) Cultured sensory neurons were exposed to sanshool (San; 100 μM) followed by capsaicin (Cap; 1 μM) and subsequently 140 mM potassium chloride (KCl) and were analyzed by calcium imaging. No response to sanshool was observed in the absence of extracellular calcium, demonstrating that the calcium signal is due to influx (data not shown). After calcium imaging, neurons were fixed and probed for NF200 reactivity by immunohistochemistry. Responses to sanshool were observed in NF200-positive, capsaicin-insensitive neurons (cell 1) as well as NF200-negative, capsaicin-sensitive neurons (cell 2). (**d**) Cultured DRG sensory neurons from TrkC-GFP mice were treated and analyzed as in **c**. Sanshool responses were observed in TrkC-positive, capsaicin-insensitive neurons (cell 1) as well as TrkC-negative, capsaicin-sensitive neurons (cell 2). (**e**) Calcium imaging shows that some sanshool-sensitive cells are capsaicin sensitive, whereas others are not, corresponding to small- and large-diameter neurons, respectively (average sizes = 18.0 and 35.7 μm; average response from 15 representative cells). (**f**) Quantitative analysis of concordance between sanshool sensitivity and other histological or pharmacological attributes. Cells showing sensitivity to sanshool (100 μM) were examined for activation by capsaicin (Cap; 1 μM), mustard oil (MO; 100 μM), menthol (ME; 500 μM), or hypo-osmotic (Osmo; 226 mOsm) stimuli, as well as for expression of TrkC or neurofilament (NF200) immunoreactivity. Note the high (>40%) preponderance of capsaicin or hypo-

osmotic sensitivity among sanshool-sensitive neurons, as compared to the relatively low (≤1%) sensitivity to mustard oil or menthol. Moreover, many (≥50%) of sanshool-positive cells were myelinated (NF200 positive) and/or TrkC positive.

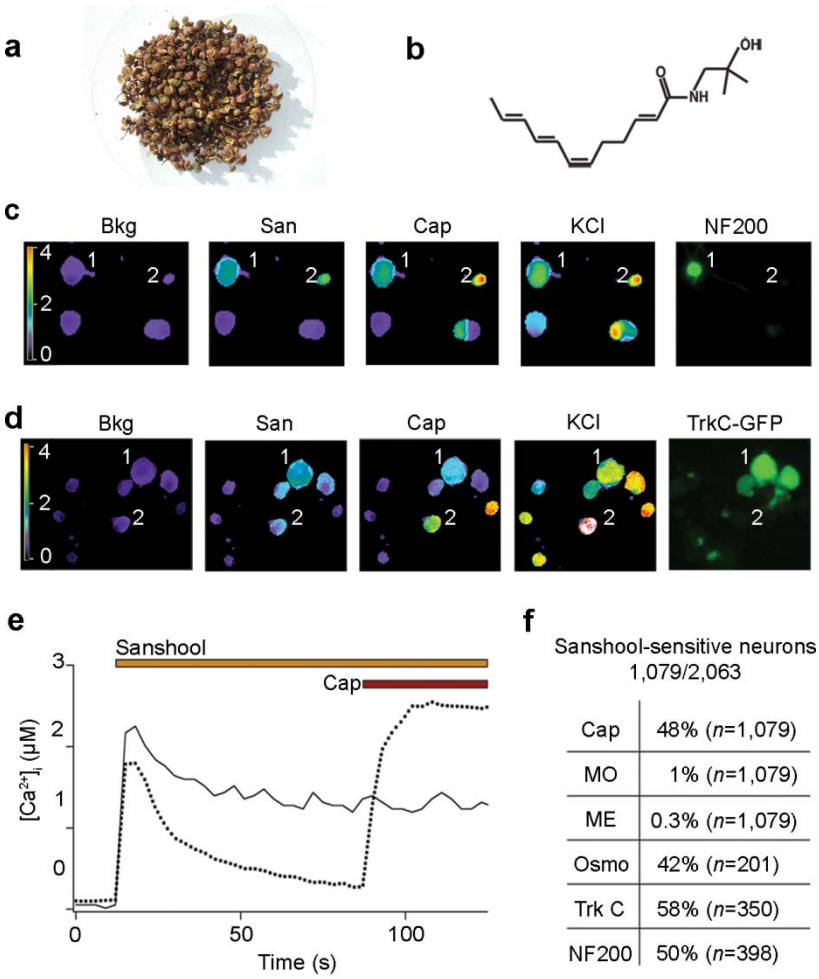
Figure 2 Crude Szechuan pepper extract and purified hydroxy-α-sanshool activate the same population of cultured sensory neurons. A representative calcium response of cultured sensory neurons to application of 100 μM, then 1mM hydroxy-α-sanshool, followed by crude Szechuan pepper extract (3%).

Figure 3. Related alkylamides from *Echinacea purpurea*, but not hydroxy-β-sanshool, can activate a similar population of sensory neurons. **(a)** Representative calcium response of cultured sensory neurons to hydroxy-β-sanshool (1 mM), hydroxy-α-sanshool (100 μM), and capsaicin (1 uM). **(b)** Chemical structures of alkylamides isolated from *Echinacea purpurea*. Structure of Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (E1) (top) and dodeca-2E,4E-dienoic acid isobutylamide (E2)(bottom). **(c)** Representative calcium response of cultured sensory neurons to the alkylamide E1 (100 μM), and capsaicin (1 uM). **(d)** Representative calcium response of cultured sensory neurons to the alkylamide E2 (100 μM), and capsaicin (1 uM).

Figure 4. Sanshool inhibits pH-sensitive background potassium channels in sensory neurons. (**a**) Representative whole-cell voltage-clamp recording from a cultured trigeminal neuron subjected to a voltage ramp (+60 mV to –100 mV; every 2 s. Current-

voltage relationship before (black) or after application of sanshool (100 μM; orange) or low pH (pH 6.5; blue) (left). Average current recorded at –60 mV in response to sanshool or low pH (right). (**b**) Dose-response curve of sanshool-evoked inhibition of background potassium conductance in sensory neurons (holding potential = –60 mV) recorded in extracellular Ringer's solution (IC_{50} = 69.5 ± 5.3 μM; *n* = 3–7 cells per point). (**c**) Summary of sanshool-sensitive currents measured in small- and large-diameter neurons.

Figure 1



Cap	48% (*n*=1,079)
MO	1% (*n*=1,079)
ME	0.3% (*n*=1,079)
Osmo	42% (*n*=201)
Trk C	58% (*n*=350)
NF200	50% (*n*=398)

Figure 2

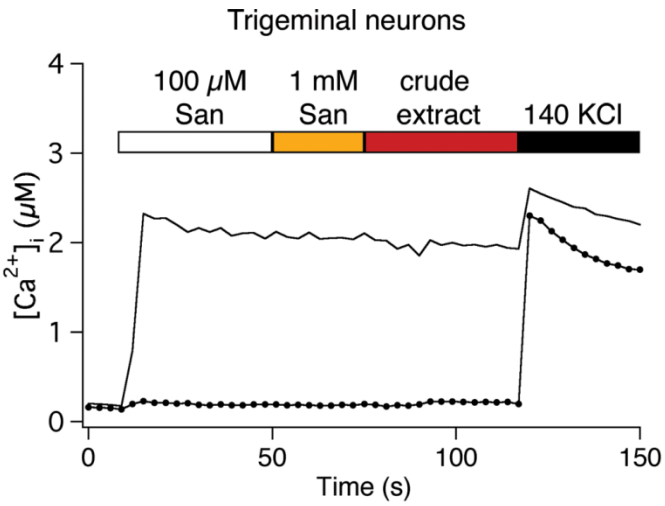

Trigeminal neurons
100 μM San
1 mM San
crude extract
140 KCl
[Ca2+]i (μM)
0
1
2
3
4
0
50
100
150
Time (s)

Figure 3

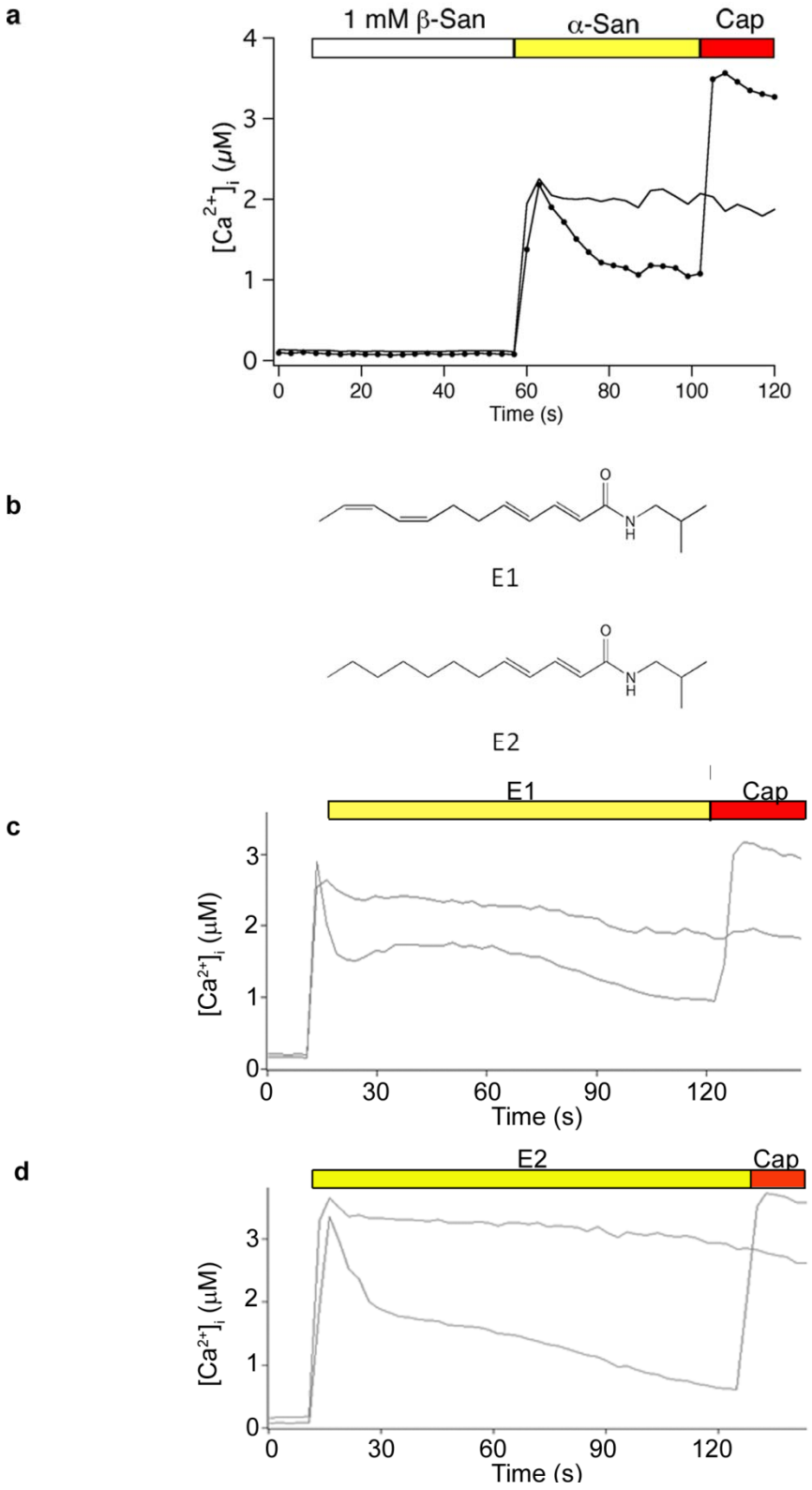
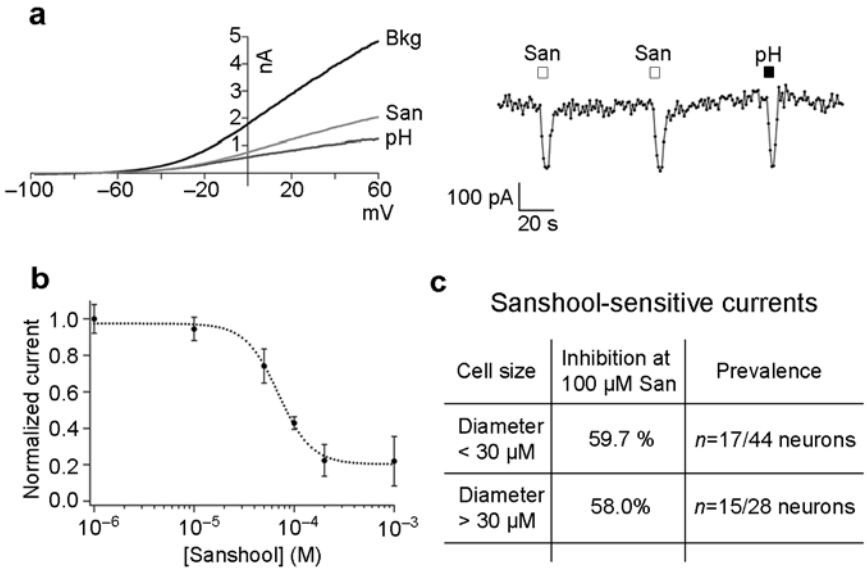

a
1 mM β-San
α-San
Cap
[Ca2+]i (μM)
Time (s)
b
E1
E2
c
E1
Cap
[Ca2+]i (μM)
Time (s)
d
E2
Cap
[Ca2+]i (μM)
Time (s)

Figure 4



c

Sanshool-sensitive currents

Cell size	Inhibition at 100 μM San	Prevalence
Diameter < 30 μM	59.7 %	*n*=17/44 neurons
Diameter > 30 μM	58.0%	*n*=15/28 neurons

CHAPTER 3

Molecular Actions of Hydroxy-α-sanshool and Related Alkylamides

While the exact value may vary from cell to cell and condition to condition, all mammalian neurons maintain a resting membrane potential around -65mV. There are two main classes of proteins that are responsible for the majority of this phenomenon. First, a K^+/Na^+ ATPase exchanges extracellular potassium ions for intracellular sodium ions, thereby resulting in steep gradients of these two ions across the plasma membrane. This active transport of ions requires the input of energy and can account for 20% to 40% of a mammalian brain's energy consumption. A membrane potential that is more negative on the inside of the cell arises due to the high permeability of potassium through open background K^+ channels. This efflux of K^+ down its concentration gradient results in a net negative charge inside the cell. One manner in which a cell can be depolarized is by inhibiting and closing these background K^+ channels, thereby decreasing the membrane permeability to K^+ and by default increasing the relative membrane permeability for Na^+. This type of mechanism has been previously implicated in sensory function and, has been shown to be involved in the detection of both sweet and sour taste modalities.

Based on membrane topology, there are three broad classes of potassium channels. The two most well studied classes each form functional channels as tetramers were each subunit containing one pore domain and either two or six transmembrane segments. The third and most recently discovered class of potassium channels form dimers, contain either four or eight transmembrane passes, and possess two pore domains per subunit. As

a result of this last unique feature, this class has been termed two-pore (2P) potassium channels, or K2P channels.

While all three classes of potassium channels can play a role in setting the resting membrane potential in various cell types, the K2P channels as a whole have very little voltage dependence, and as such, are likely to help set the resting membrane potential in cells in which they are expressed. Based on the current IUPHAR channel nomenclature, these genes encode the KCNK family of which 15 mammalian pore forming subunits have been identified, and 11 have been shown to form functional channels. KCNK channels are a diverse group of proteins, with many human subunits having >45% sequence homology. Currents produced by these channels are insensitive to many classical potassium channel blockers such as tetraethylammonium (TEA) or 4-aminopyridine (4-AP) and most have a reduced sensitivity to block by barium. However, interestingly, many of the KCNK channels are modulated by volatile anesthetics such as isoflurane and halothane and/or local anesthetics such as lidocaine and bupivicaine. It has, in fact, been proposed that the activation of some KCNK channels by volatile anesthetics is a primary mode of action for these drugs.

Functionally, KCNK channels can be further divided into several groups. Among the best characterized are KCNK2, KCNK4, and KCNK10 which form one such group characterized by their inhibition at alkaline pH, and activation by unsaturated fatty acids and the neuroprotectant riluzole. Interestingly, this group of channels has also been

shown to be activated by membrane stretch. Another functional group consists of KCNK3 and KCNK9, both of which are inhibited at acidic pH and by local anesthetics. Based on sequence homology KCNK18 is less closely related to other family members, is also inhibited by acidic pH, and has been shown to be directly regulated by calmodulin binding. Under varying conditions, many of these channels are also regulated by GPCR signaling, PKA and PKC modulation, representing myriad mechanisms for regulation of these channels. In the end, while the many pathways in which these channels can be both up- and down-regulated are still coming to light, they undoubtedly place important roles in the regulation of neuronal excitability and cellular signaling.

Sanshool targets members of the KCNK two-pore K+ channel family

To determine whether KCNK channels show sanshool sensitivity, we expressed each family member in *Xenopus laevis* oocytes and asked whether bath-applied sanshool suppressed basal K^+ currents in these cells (**Fig. 1a**). Only three subtypes showed significant inhibition, including KCNK3, KCNK9 and KCNK18 (also known as TASK-1, TASK-3 and TRESK, respectively; **Fig. 1a**,**b**), all of which are targeted by volatile and local anesthetics[1-8]. The greatest potency was observed with KCNK3 and KCNK18 (IC_{50} = 30.3 ± 4.9 and 50.2 ± 1.9 μM, respectively), whereas KCNK9 was much less sensitive (IC_{50} = 450 ± 30.1 μM) (**Fig. 1c**). KCNK3 and KCNK9 are capable of forming functional heteromeric complexes[9], and we found that such heteromeric channels showed intermediate sanshool sensitivity, with an IC_{50} = 252 ± 31 μM (**Fig. 1c**). As observed in cultured neurons, sanshool-evoked inhibition of cloned KCNK channels was fully

reversible (**Fig. 1d**). Moreover, although KCNK channels are strongly regulated by protons, changes in extracellular pH did not affect the sanshool sensitivity of KCNK3, KCNK9 or KCNK18 (**Fig. 1e**), demonstrating that their effects are not additive. Analysis of inside-out patches excised from KCNK18-expressing HEK293 cells showed that sanshool is fully capable of inhibiting this channel in a membrane-delimited manner, whether applied to the inside or the outside surface of the membrane (**Fig. 1f**). Taken together, these results demonstrate that a subset of pH-sensitive KCNK channels is directly inhibited by hydroxy-α-sanshool.

Among cloned KCNK channels, only KCNK3 showed significant inhibition by hydroxy-β-sanshool (**Fig. 2a**–**d**), suggesting that this subtype does not mediate sanshool sensitivity in primary afferent neurons. Furthermore, these results are consistent with the finding that hydroxy-β-sanshool is not the principal pungent agent in Szechuan peppercorns. In contrast, both alkylamides derived from Echinacea showed a similar pattern of inhibition within the sanshool sensitive KCNK family members as was obtained with hydroxy-α-sanshool **(Fig. 2e)**. This observation is also in agreement with the activity of these compounds on a cellular level when applied to cultured sensory neurons.

Excitatory channels are not targets of sanshool action

The idea that sanshool depolarizes neurons by blocking an outward K^+ current is somewhat unexpected because a recent report has suggested that excitability is achieved through activation of inward currents. Specifically, TRPV1 and TRPA1 have been

proposed to serve as sanshool receptors[10]. In light of our findings, we carried out a more comprehensive analysis of sanshool specificity by examining its effects on a range of other heterologously expressed potassium channels as well as excitatory ion channels known to be present on sensory neurons, including members of the K_v, K_{ir}, TRP, ASIC/MDEG, P_{2X} and 5-HT_3 families (**Fig. 3**). No significant actions were observed with any of these candidates, except for minor and somewhat anomalous effects on TRPV1 and TRPA1 at very high (1 mM) sanshool concentrations. In these cases, sanshool activated an outward current that was blocked by ruthenium red (a nonselective TRP channel inhibitor), but inward currents were extremely small (<1% of maximal response to capsaicin or mustard oil) and therefore unlikely to account for neuronal excitation (**Fig. 4a** and data not shown). One surprising finding was that pluronic acid, which was previously used to enhance sanshool solubility, was itself an activator of TRPA1; this may account for the previous suggestion[10] that sanshool activates cloned or native TRPA1 channels and induces nocifensive behavior (**Fig. 5**). To avoid such nonspecific actions in our experiments, we used DMF and/or β-cyclodextrin to increase sanshool solubility, as these agents have no effect on sensory neurons or cloned channels (**Fig. 5**).

Additional observations further suggest that the physiological actions of sanshool are unlikely to be mediated by activation of TRPV1 or TRPA1. For example, we found that crude Szechuan pepper extracts or purified sanshool (over a wide concentration range: 0.01–1 mM) does not excite TRPA1-positive (mustard oil–sensitive) sensory neurons and activates only a subset of TRPV1-positive (capsaicin-sensitive) cells (**Fig. 1d**, **Ch.2 and Fig. 2, Ch.2**). Furthermore, sanshool-evoked neuronal responses were not affected by

ruthenium red (**Fig. 4b**) or by the TRPV1-selective antagonist capsezapine (data not shown). Perhaps most significantly, sanshool-evoked responses were not altered (in prevalence or magnitude) in sensory neurons cultured from mutant mice lacking both TRPV1 and TRPA1 channels (**Fig. 4c**). Finally, these double-mutant mice showed the same aversion to sanshool-infused water as their wild-type littermates in a timed drinking test: both wild-type and TRPV1 TRPA1 double-mutant mice drank sanshool-containing (1 mM) water for 5–15 s, but then became agitated, moved quickly around the cage and rubbed their faces, reflecting a gradual onset of sanshool-elicited irritancy of the oral cavity. In contrast, when provided with capsaicin- and mustard oil-laced water, wild-type animals showed a characteristically rapid onset of drinking cessation and irritancy[11, 12], to which TRPV1 TRPA1 double-mutant mice were completely insensitive (**Fig. 4d**). Although these findings contrast with the conclusions of one earlier study, they are consistent with other work showing that sanshool and capsaicin sensitivity are non-overlapping at the cellular level[13] and that activation of cloned TRPV1 channels by sanshool is insufficiently robust to account for its pungency[14].

Discussion

At the molecular level, sanshool has been proposed to activate neurons by opening excitatory ion channels[10, 13, 14], and we were therefore surprised to find that it depolarizes neurons by inhibiting background potassium channels. In addition to our direct electrophysiological characterization of sanshool-sensitive membrane currents in sensory neurons, our examination of various cloned channels also rules out a significant

involvement of members of the TRP channel family that have been suggested to play a role in this response, including receptors for capsaicin, mustard oil and menthol (TRPV1, TRPA1 and TRPM8, respectively). Rather, our data demonstrate that a subset of pH-sensitive two-pore K^+ channels, namely KCNK3, KCNK9 and KCNK18, are the molecular targets of sanshool action. Little is known about the role of these channels in the regulation of neuronal excitability and other cellular functions. Even less is known about how these channels are physiologically modulated on a molecular level. One barrier to the study of KCNK channels has been a lack of pharmacology with which to specifically identify and regulate individual members of this family. Based on our studies to date, when used together, both the hydroxy-α and hydroxy-β forms of sanshool will provide a method for identifying and discriminating between currents arising from KCNK3, KCNK9, and KCNK18. Indeed, sanshool now provides a new pharmacological tool for discriminating among two-pore K^+ channel subtypes, one that is more selective in its action compared to ruthenium red, zinc, protons and anesthetics, all of which target multiple KCNK subtypes as well as other ion channels.

Methods

Oocyte Electrophysiology. For oocyte expression, cDNAs were linearized and transcribed with T7 RNA polymerase (mMessage mMachine, Ambion). Two-electrode voltage-clamp analysis was performed 1–5 d after cRNA injection. Currents were recorded in ND96 (96 mM NaCl, 2 mM KCl, 0.3 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4). For recordings at pH 6.5 and pH 8.5, solutions were adjusted using HCl or NaOH, respectively. Similar results were obtained when using 2-(*N*-

morpholino)ethanesulfonic acid (MES) as a high-pH buffer. TRPV1-expressing cells were analyzed in calcium-free solution (120 mM CsCl, 1 mM EGTA, 10 mM HEPES, 2 mM $MgCl_2$, pH 7.4), and $K_{ir}2.1$ and $K_{ir}3.1/3.4$ were analyzed using high-potassium solutions (20 mM NaCl, 78 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4, for $K_{ir}2.1$ and 2 mM NaCl, 96 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4, for $K_{ir}3.1/3.4$). Because heterologously expressed KCNK1 does not form functional homomeric channels, a point mutant (K274E) that shows basal activity[15] was expressed in these experiments. Currents were recorded with a GeneClamp 500B amplifier and Digidata 1322A interface and acquired with pClamp software (Axon Instruments).

Mice and behavior. Mice (20–35g) were housed with 12 h/12 h light/dark cycle at 21°C. All experiments were performed according to the policies and recommendations of the International Association for the Study of Pain and approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. TRPV1 TRPA1 double-mutant mice were generated by crossing *TRPV1*$^{-/-}$ and *TRPA1*$^{-/-}$ animals[11, 16]; resulting *TRPV1*$^{+/-}$ *TRPA1*$^{+/-}$ progeny were then crossed to yield wild-type and double-knockout siblings for analysis.

Sanshool sensitivity was assessed with an aversive drinking test as previously described[11]. On days 1–5 and 7, mice were allowed to drink for 3 h per day from a bottle containing 0.125% saccharine plus vehicle (1:200 DMF) in water. On day 6, this solution was supplemented with sanshool extract (3%, described above) or purified hydroxy-α-

sanshool. 100 μM sanshool was not adequate to elicit robust avoidance responses, which we ascribe to issues such as long-term stability, light sensitivity, solubility of sanshool in drinking containers, and tissue access. We therefore used 1 mM sanshool in these behavioral tests. Notably, 100 μM or 1 mM sanshool, and crude extracts, do not elicit different spectra of sensory neuron responses. On day 8, the drinking solution was supplemented with 1 μM capsaicin and 100 μM mustard oil. All drinking solutions were presented to both wild-type and TRPV1 TRPA1 double-knockout mice (n = 10 per genotype). Volumes consumed were measured each day.

PCR. RNA was isolated from trigeminal ganglia or whole brains of P0 mice with Trizol (Invitrogen). First-strand cDNA was transcribed using murine Moloney leukemia virus reverse transcriptase and poly dT(12–18) primers. After PCR amplification, resulting cDNAs were blunt-cloned into the *Eco*RV site of the pMO vector. All sequences were verified by DNA sequencing. KCNK family members were PCR amplified using specific primer pairs for each gene (**Table 1).**

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Figure Legends

Figure 1 Sanshool inhibits KCNK3, KCNK9 and KCNK18. (**a**) *Xenopus* oocytes expressing a given KCNK family member were subjected to two-electrode voltage-clamp analysis and the percent suppression of leak current was determined after bath application of purified sanshool (100 μM) (*n* = 5–8 cells per channel). (**b**) Representative traces of sanshool-evoked inhibition of KCNK3 (left), KCNK9 (middle) and KCNK18 (right) in oocytes (holding potential = –80 mV). Inhibition by extracellular protons (pH 6.5) and barium (2 mM) is shown for comparison. (**c**) Dose-response curves of sanshool-evoked inhibition at 0 mV of KCNK3, KCNK9, KCNK18 or KCNK3/KCNK9 heteromers, recorded in *Xenopus* oocytes (*n* = 5–8 cells per point). (**d**) Representative current recorded from *Xenopus* oocytes expressing KCNK18 in response to sanshool (100 μM; orange) or low pH (pH 6.5; blue) (holding potential = –60mV, *n* = 5). (**e**) Sanshool-evoked inhibition of KCNK channels at pH 6.5 (black), pH 7.4 (blue) and pH 8.5 (white) (*n* = 4–10 oocytes per condition). (**f**) Inhibition of KCNK18 currents by sanshool (100 μM) applied to an inside-out patch from transfected HEK293 cells (*n* = 7). Seals were obtained with extracellular Ringer's solution in both pipette and bath. After excision of the patch , bath solution was replaced with high-potassium Ringer's (see Methods). Sanshool has no effect on background currents observed in vector-transfected control cells (not shown).

Figure 2 Inhibition of KCNK channel family members by similar alkylamides. **(a-d)** Representative traces of hydroxy-β-sanshool-evoked (100 μM; orange) inhibition of

KCNK18, KCNK3, KCNK9, and KCNK3/9 heteromers (a-d, respectively) expressed in oocytes (holding potential = –80 mV, n=3–5). Inhibition by 2 mM $BaCl_2$ (pH = 6.5; blue). **(e)** Inhibition of KCNK18, KCNK3, KCNK9, and KCNK3/9 heteromers expressed in oocytes by the Echinacea alkylamides E1 and E2.

Figure 3 Sanshool does not affect a variety of ion channels expressed by sensory neurons. **(a)** Representative traces showing lack of sanshool sensitivity of $K_v1.2$ (left) and $K_v2.1$ (right; orange) (holding potential = –80 mV, n=5). Inhibition by TEA is shown for comparison (10 mM; blue). **(b)** Representative traces showing lack of sanshool sensitivity of $K_{ir}2.1$ (left) and $K_{ir}3.1/3.4$ co-expressed with P2Y12 (right) (orange; holding potential = –80 mV, n=5). Inhibition of $K_{ir}2.1$ by 1 mM $BaCl_2$ and activation of $K_{ir}3.1/3.4$ co-expressed with $P2Y_{12}$ by 10 μM ADP are shown for comparison (blue). **(c)** Representative traces showing lack of sanshool-evoked (100 μM; orange) activation of ASIC1 (left) and ASIC2b/3 heteromer (right) (holding potential = –80 mV, n=5). Activation by ND96, pH 5.0 is shown for comparison (blue). **(d)** Summary showing effect of sanshool on a variety of ion channels expressed in *Xenopus* oocytes. Membrane currents were recorded in two-electrode voltage-clamp mode (holding potential = –60 mV for all channels except $K_v1.2$ and $K_v2.1$ where holding potential = +30 mV) and response to bath applied sanshool (100 μM) compared to activation (black) or inhibition (red) by the relevant agonist/antagonist (10 mM TEA for $K_v1.2$ and $K_v2.1$, 1 mM $BaCl_2$ for $K_{ir}2.1$, 10 μM ADP for $K_{ir}3.1/3.4$ co-expressed with $P2Y_{12}R$, 100 μM ATP for P2X channels, pH 5.0 for ASIC channels, 100 μM serotonin for 5-HT3R-A channel, and 1 μM capsaicin, 100 μM mustard oil, or 100 μM menthol for TRPV1, TRPA1, or TRPM8,

respectively). 100 μM sanshool was used for all experiments. Panel at left shows comparison with sanshool effects on KCNK channels; panel at right shows expanded scale view of data in box.

Figure 4 TRPA1 and TRPV1 are not required for sanshool sensitivity. (**a**) Sanshool elicits small, but detectable, currents in TRPV1- (left) or TRPA1-expressing (middle) *Xenopus* oocytes, but only at positive membrane potentials. Bar graph (right) shows summary of sanshool (1 mM)-evoked currents recorded at +60 versus –60 mV holding potential ($n = 5$). (**b**) Sanshool-evoked calcium influx in trigeminal sensory neurons is not blocked by ruthenium red (10 μM), a blocker of TRPV1 and TRPA1 channels ($n = 114$). (**c**) Sanshool-evoked calcium influx is normal in neurons cultured from mice deficient in both TRPV1 and TRPA1 ($n = 108$). (**d**) Consumption of water containing sanshool (1 mM) was significantly decreased in TRPA1 TRPV1–deficient mice (closed circles), as well as in their wild-type littermates (open circles). In contrast, only wild-type animals showed decreased consumption of water containing capsaicin and mustard oil. $^{**}P < 0.01$; one-way ANOVA; $n = 10$ animals per genotype.

Figure 5 Pluronic activates endogenous and heterologous TRPA1**. (a)** Cultured trigeminal neurons show an increase in calcium in response to 0.1% pluronic acid (top; n=178), but not 0.1% dimethylformamide (DMF) or 0.1% DMF and 0.1 % β-cyclodextrin (β-CD)(bottom; n=167). **(b)** Human TRPA1 was transiently transfected into

HEK293 cells. Calcium influx was observed in response 0.1% pluronic acid and mustard oil (100 uM), but not 0.1% DMF and 0.1 % β-CD (n=131).

Table 1

Primers sequences used to clone mouse KCNK gene coding regions		
KCNK1	5′	ATGCTGCAGTCCCTGGCCGGCAGC
	3′	TCAGTGGTCTGCAGAGCCATCCTCATAGGG
KCNK2	5′	ATGCTTGCCAGCGCCTCGCGG
	3′	CTACTTGCCAGCGCCTCGCGG
KCNK3	5′	ATGAAGCGGCAGAATGTGCGCACGTTGG
	3′	TCACACCGAGCTCCTGCGCTTCATGAG
KCNK4	5′	ATGCGCAGCACCACACTCCTGGCTCTG
	3′	CTACACCGGCACGGCCTTGTCTCGGAG
KCNK5	5′	ATGGTGGACCGGGGTCCTTTACTCACC
	3′	TCACGTGCCCCTGGGGTTATCTGCCTT
KCNK9	5′	ATGAAGCGGCAGAACGTGCGTACCC
	3′	TTAGATGGACTTGCGACGGAGGTGCAGCCTATG
KCNK10	5′	ATGAAATTTCCAATCGAGACGCCAAG
	3′	TTAGTTTCTGTCTTCAAGTAAAGAATTGTTCTCC
KCNK13	5′	ATGGCTGGCCGCGGTTGCGG
	3′	CTACCTATCTCCACTGGTCTCTGCCAACCTG
KCNK18	5′	ATGGAGGCTGAGGAGCCACCTGAGGCC
	3′	TTACCAAGGTAGCGAAACTTCCCTTTGGC

Table 1 Specific primers designed to clone all functional KCNK family members. Primer sequences were designed to specific sections of the 5' and 3' ends of the coding regions of all known functional KCNK channels. PCR amplification was performed as described in **Methods.**

Figure 1

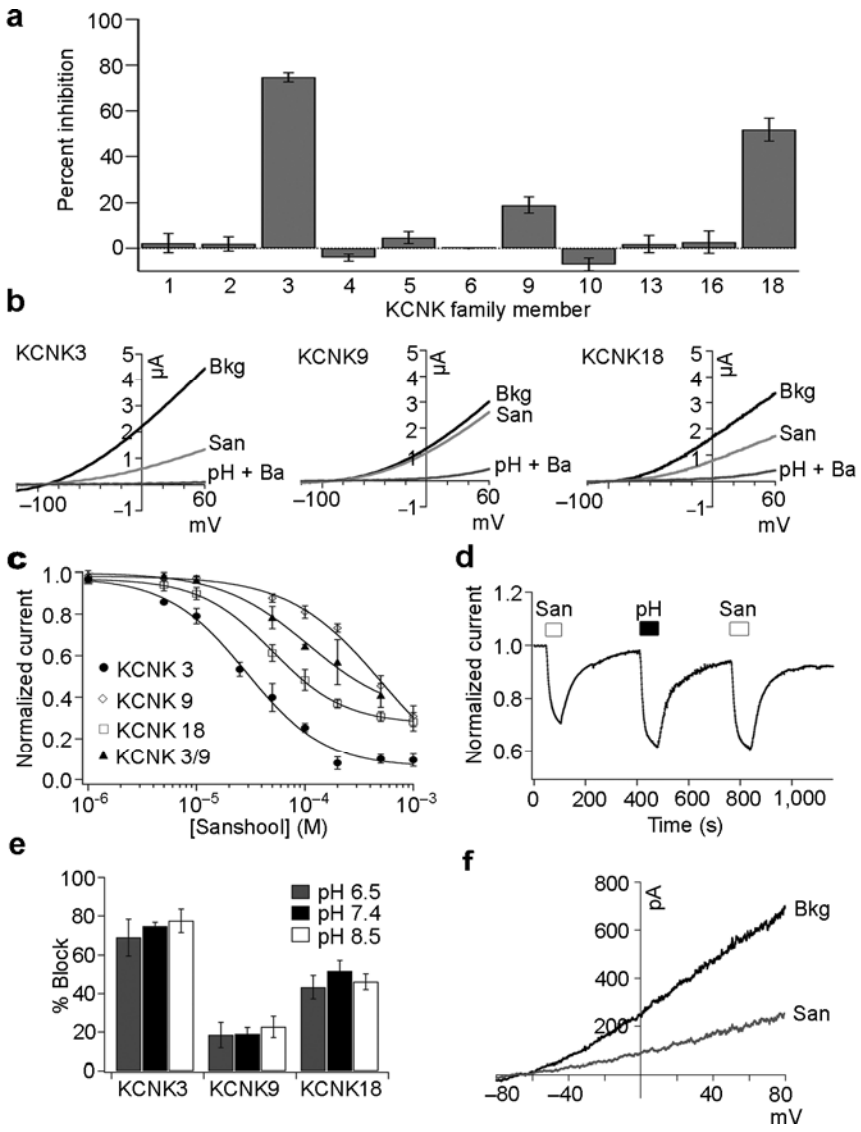
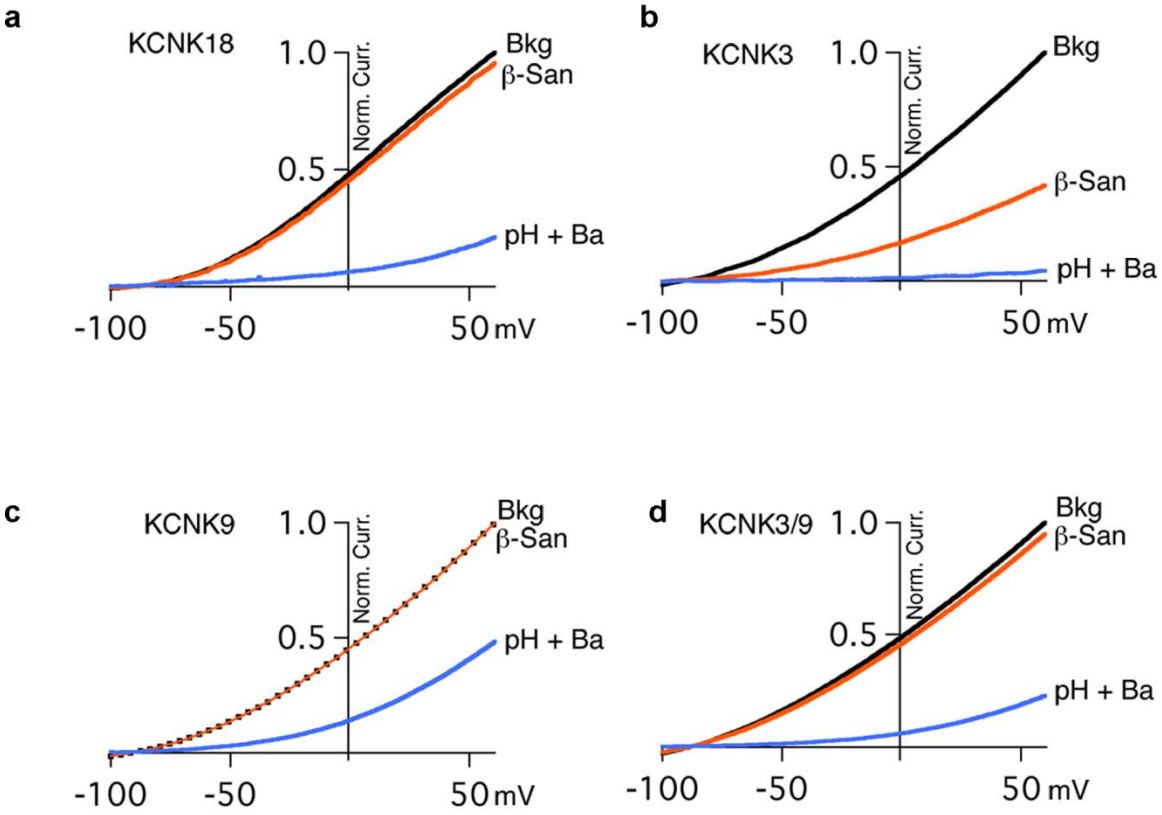

a
Percent inhibition
KCNK family member
b
KCNK3
KCNK9
KCNK18
Bkg
San
pH + Ba
μA
mV
c
Normalized current
KCNK 3
KCNK 9
KCNK 18
KCNK 3/9
[Sanshool] (M)
d
San
pH
Normalized current
Time (s)
e
% Block
pH 6.5
pH 7.4
pH 8.5
f
pA
Bkg
San
mV

Figure 2

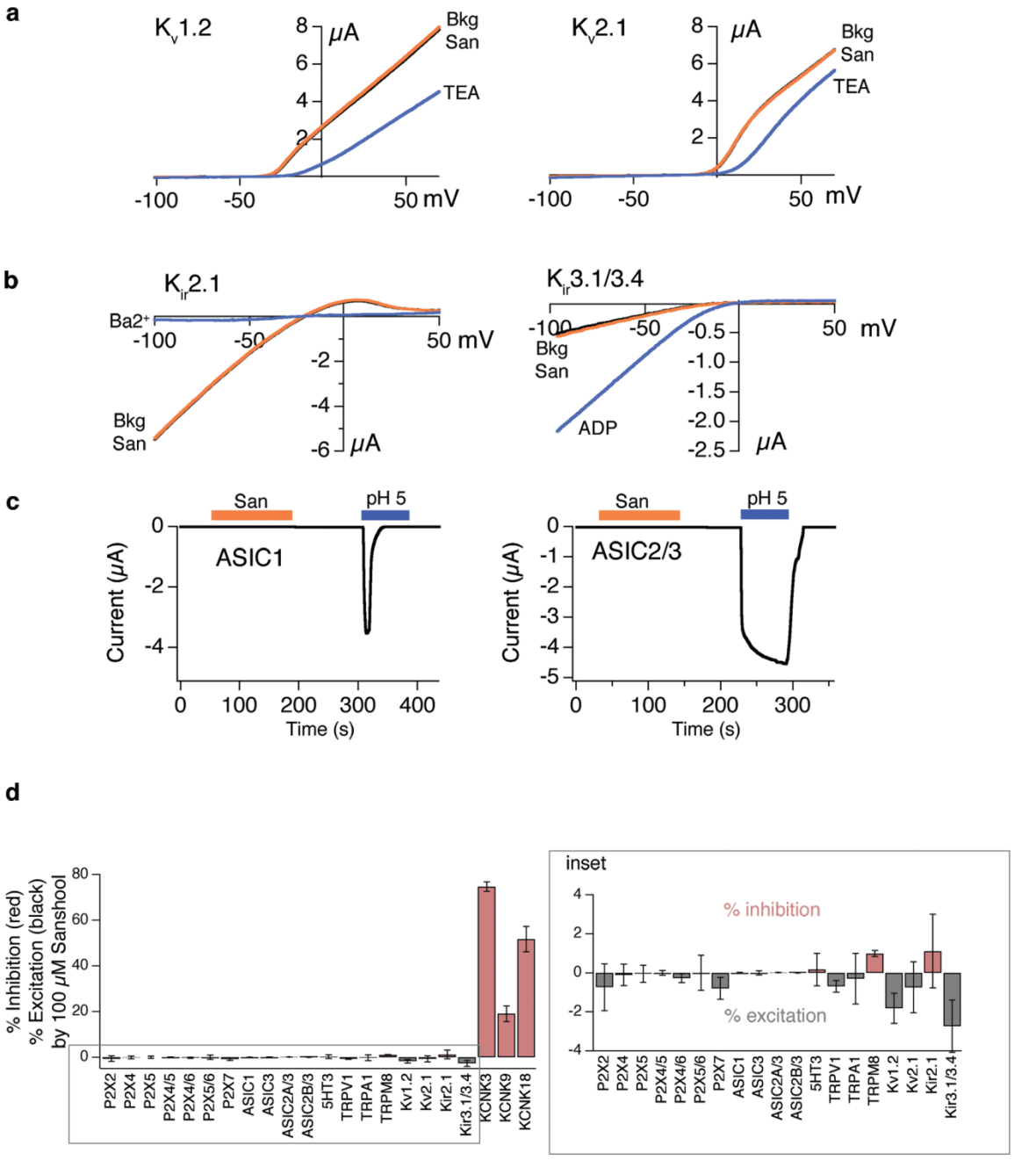


e

Selective Inhibition of KCNK channels by Echinacea Alkylamides

	100μM E1 (%)	100μM E2 (%)
KCNK3	79.9±3.9	51.4±9.9
KCNK3/9	25.7±6.0	11.5±0.4
KCNK9	5.1±1.3	6.0±1.9
KCNK18	55.2±2.1	48.4±3.7

Figure 3


a
$K_v1.2$
8
6
4
2
μA
Bkg
San
TEA
-100
-50
50 mV
$K_v2.1$
8
6
4
2
μA
Bkg
San
TEA
-100
-50
50 mV
b
$K_{ir}2.1$
Ba2+
-100
-50
50 mV
-2
-4
-6
μA
Bkg
San
$K_{ir}3.1/3.4$
-100
-50
-0.5
-1.0
-1.5
-2.0
-2.5
50 mV
Bkg
San
ADP
μA
c
San
pH 5
ASIC1
0
-2
-4
Current (μA)
0
100
200
300
400
Time (s)
San
pH 5
ASIC2/3
0
-1
-2
-3
-4
-5
Current (μA)
0
100
200
300
Time (s)
d
% Inhibition (red)
% Excitation (black)
by 100 μM Sanshool
80
60
40
20
0
P2X2
P2X4
P2X5
P2X4/5
P2X4/6
P2X5/6
P2X7
ASIC1
ASIC3
ASIC2A/3
ASIC2B/3
5HT3
TRPV1
TRPA1
TRPM8
Kv1.2
Kv2.1
Kir2.1
Kir3.1/3.4
KCNK3
KCNK9
KCNK18
inset
4
2
0
-2
-4
% inhibition
% excitation
P2X2
P2X4
P2X5
P2X4/5
P2X4/6
P2X5/6
P2X7
ASIC1
ASIC3
ASIC2A/3
ASIC2B/3
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Kir2.1
Kir3.1/3.4

Figure 4

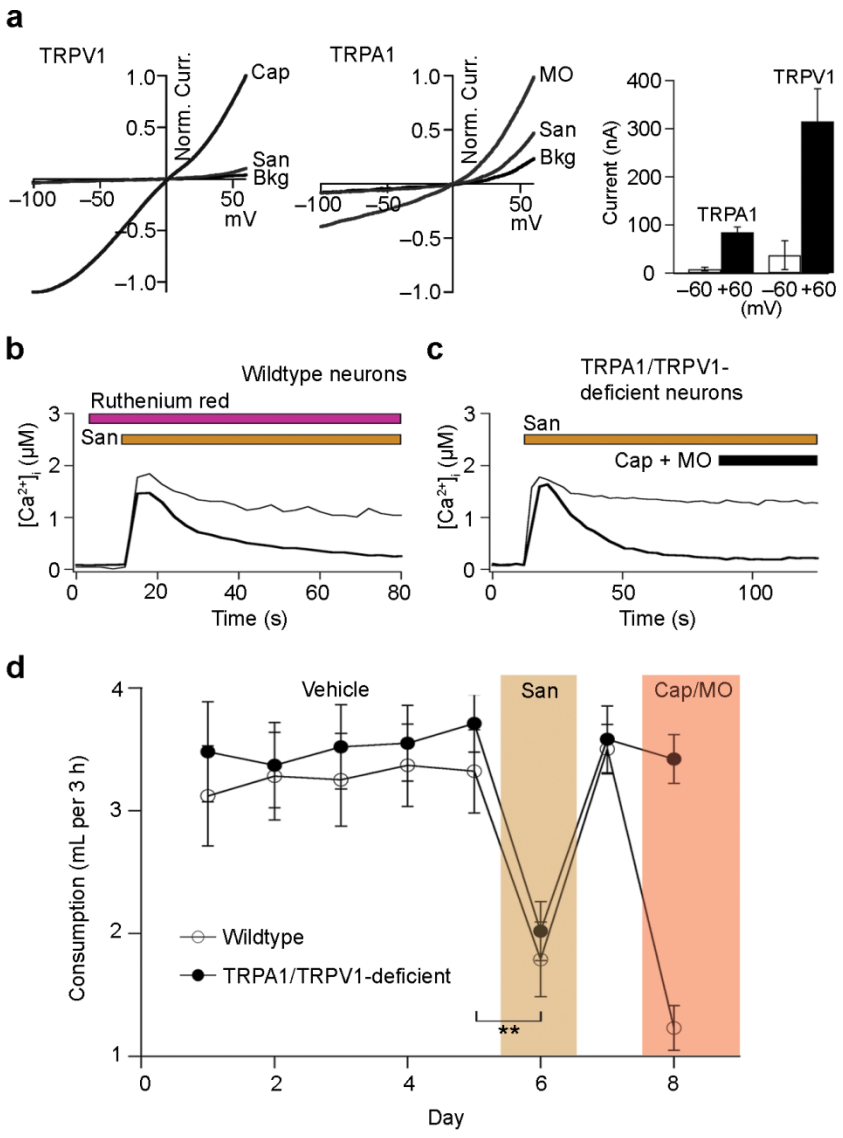
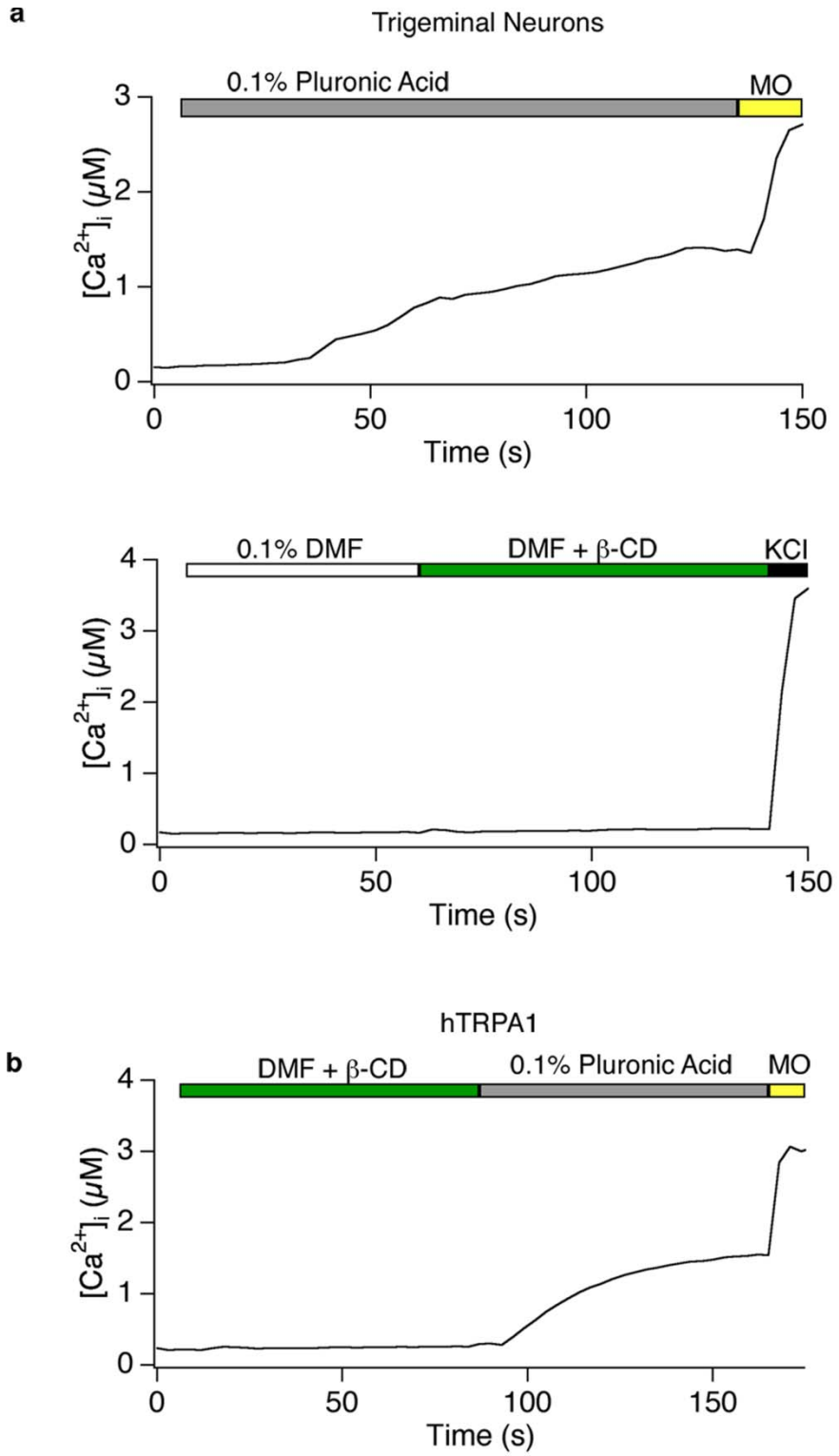

a
TRPV1
Norm. Curr.
1.0
0.5
–0.5
–1.0
–100
–50
50
mV
Cap
San
Bkg
TRPA1
MO
TRPV1
Current (nA)
400
300
200
100
0
–60 +60 –60 +60
(mV)
b
Wildtype neurons
Ruthenium red
San
[Ca2+]i (μM)
Time (s)
c
TRPA1/TRPV1-
deficient neurons
Cap + MO
d
Vehicle
Cap/MO
Consumption (mL per 3 h)
Wildtype
TRPA1/TRPV1-deficient
**
Day

Figure 5


a
Trigeminal Neurons
0.1% Pluronic Acid
MO
[Ca2+]i (μM)
Time (s)
0.1% DMF
DMF + β-CD
KCl
b
hTRPA1
DMF + β-CD
0.1% Pluronic Acid
MO

Chapter 4

Correlation between neuronal KCNK channel expression and hydroxy-α-sanshool activity

Correlation between neuronal KCNK channel expression and hydroxy-α-sanshool activity

Since the discovery of mammalian two pore potassium channels in 1996, members of the KCNK family have been shown to be expressed and play a role in a wide range of both neuronal and non-neuronal cell types. As determined by RT-PCR as well as RNA in situ hybridization experiments using autoradiography, most KCNK channels are expressed in several different cell types, and some, such a KCNK1 are expressed quite broadly. Though all KCNK channels don't play a dominant role in controlling the resting membrane potential in every cell type in which they are expressed, either due to low expression levels or through chronic inhibition of the channel, the presence of many types of background potassium channels creates an additional mechanism for tuning neuronal excitability and adaptation under altered physiological states. A good example of the complexity of KCNK channel biology can be found in cerebellar granule neurons. It has been noted that over the time-course of culture for cerebellar granule cells the resting membrane potential decreases dramatically[3]. Whether this change reflects the physiological development of the neuron or is a result of the culture conditions which contain a depolarizing concentration of K^+ is unknown. Components of the potassium conductance have been identified and molecular correlates have been proposed[3]. During the early stages of culture several conductances contribute to the resting membrane potential, while the two-pore potassium channels KCNK3 and 9 as well as the functional KCNK3/9 heteromer retain the same physiological characteristics of the large background conductance that arises during culture[1]. Indeed, knockouts of KCNK3 and 9

have demonstrated important functional roles for these background channels in cerebellar granule neurons[2,8].

CNS neurons expressing KCNK3 and 9 are sanshool sensitive

Taken together, our data suggest that KCNK channels, and not TRP or other excitatory channels, are the physiological targets of sanshool action. To further test this hypothesis, we asked whether cerebellar granule neurons (CGN), which lack sensory TRP channels but express KCNK channels as their major background K^+ conductance[1-4], are sanshool sensitive. Indeed, we observed marked and robust calcium responses in most, if not all, of these neurons after bath application of sanshool (100 μM) (**Fig. 1a**). In contrast, cultured hippocampal neurons, in which KCNQ rather than KCNK channels account for primary background K^+ conductance[5], were insensitive to sanshool (data not shown).

As observed with sensory neurons, sanshool-evoked responses in CGNs were not altered by classical K^+ channel inhibitors (TEA, 4-AP) or by ruthenium red (data not shown). Moreover, CGNs were insensitive to capsaicin and mustard oil, ruling out expression of functional TRPV1 or TRPA1 channels by these cells (data not shown). These results were corroborated by whole-cell voltage-clamp recordings, in which sanshool inhibited a pH-sensitive background K^+ current (43% at 200 μM) (**Fig. 1a**). Taken together, these results support the idea that KCNK channels serve as physiological targets for sanshool action in both sensory and CNS neurons.

Sanshool sensitivity correlates with KCNK subtype expression

While examining CGNs in culture, we found that these cells are insensitive to sanshool during the first 2 d in culture, but develop sensitivity by day 7 (**Fig. 1b**). In fact, previous studies have shown that background potassium conductances change significantly during the first week of CGN culture[3]. We therefore asked whether the acquisition of sanshool sensitivity corresponds with a temporal change in the expression of one or more KCNK channel subtypes. Quantitative PCR analysis revealed a marked increase in the expression of KCNK3 and KCNK9 transcripts (17.4 ± 4.4 and 8.6 ± 1.3-fold, respectively) from the second to the seventh day of culture (**Fig. 1c, Fig. 2**). In contrast, expression of KCNK18 did not change significantly (1.2 ± 0.5-fold increase) during this time. Thus, we conclude that sanshool sensitivity of CGNs is likely mediated by KCNK3 and/or KCNK9, but not KCNK18. This is consistent with previous studies demonstrating that KCNK3 and KCNK9 account for the bulk of background K^+ current in these cells[1,2,4].

Finally, we assessed the profile of KCNK expression in trigeminal sensory ganglia using quantitative PCR (**Fig. 2**, **Fig. 3a**). When compared to CGNs on day 2 (when they are sanshool insensitive), trigeminal neurons have similar levels of KCNK3 and lower levels of KCNK9, indicating that these subtypes do not contribute to the sanshool sensitivity of trigeminal neurons. In contrast, KCNK18 transcript levels were significantly more abundant (13.1 ± 1.2-fold) in trigeminal ganglia than in CGNs at day 2 or 7, suggesting that this subtype has an important role in mediating sanshool sensitivity of primary

sensory neurons. Indeed, among sanshool-sensitive KCNK subtypes, KCNK18 contributes significantly to the background K^+ current in cultured DRG neurons[6]. Moreover, *in situ* hybridization histochemistry suggests that KCNK18 transcripts are expressed by a significant fraction of primary sensory neurons[7]. However, this analysis is likely to overestimate the prevalence of KCNK18 expression because of the high sequence conservation among KCNK subtypes, with consequent hybridization with *in situ* probes to numerous KCNK transcripts. We therefore used a more stringent PCR-based analysis to correlate KCNK18 gene expression with sanshool sensitivity among trigeminal neurons. We collected cytoplasmic contents from small groups (2–3) of cells that were either sanshool sensitive or sanshool insensitive as determined by calcium imaging (**Fig. 3b**) and subjected them to RT-PCR using KCNK18-specific primers. These results clearly showed that the expected KCNK18 fragment was amplified from all sanshool-responsive neurons, but was not observed in any sanshool-insensitive samples (**Fig. 3c**).

Discussion

Our data clearly show that KCNK3 and KCNK18 are the principal sanshool-sensitive subtypes in sensory neurons, whereas in CGNs, KCNK3 and KCNK9 predominate, thereby accounting for the bulk of cellular sensitivity to sanshool. Our pharmacological results with hydroxy-β-sanshool indicate that KCNK3 homomeric channels are not primary contributors to the excitatory effects of sanshool, where KCNK18 or KCNK3/KCNK9 heteromeric complexes must account for these actions in sensory

neurons or CGNs, respectively. Further analysis of the relative contributions of these channels to sanshool sensitivity must await the development of additional subtype-selective antagonists or the generation of triple KCNK3-, KCNK9- and KCNK18-deficient mice, because animals lacking any single KCNK subtype show compensatory upregulation of related channels[2, 8]. Similarly, the analysis of such animals will show whether additional ion channels or receptors contribute to sanshool sensitivity at the behavioral level.

Methods

Neuronal cell culture, calcium imaging and electrophysiology. Primary cultures of mouse CGN were prepared from postnatal day 7 (P7) mouse cerebellum as described[9]. Whole-cell patch-clamp recordings were made at 7–9 d.i.v. in an extracellular solution containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose, 25 mM sucrose, 5 mM HEPES (all from Sigma) and 1 μM tetrodotoxin citrate (Tocris), pH 7.3. Internal pipette solution containing 150 mM potassium methyl sulfate, 10 mM KCl, 4 mM NaCl, 10 mM HEPES, 0.4 mM tetrasodium GTP and 4 mM dimagnesium ATP, pH 7.25, was used for all recordings. Agonist was applied via a local perfusion barrel system (Automate Scientific). For calcium imaging, cells were loaded with 10 μM Fura-2-AM (Molecular Probes) at 22–25 °C for 60 min in extracellular Ringer's solution. Cells were illuminated using a xenon light source and filter wheel (Lambda LS and Lambda-10, Sutter Instruments) for 300 ms, alternately at 350 nm and 380 nm (band-pass filters from Chroma Technology). Fluorescence emission at >480 nm (long-pass filter from Chroma Technology) was captured with an intensified CCD camera (Hamamatsu) and

was digitized, background corrected and analyzed with the MetaFluor imaging system (Molecular Devices) Background-corrected 340/380 ratio images were collected every 3 s. $[Ca^{2+}]_i$ was determined from the relationship $[Ca^{2+}]_i = K^*(R - R_{min})/(R_{max} - R)$, where R is the F340/F380 ratio, R_{min} and R_{max} are the ratios at 0 Ca^{2+} and saturating Ca^{2+} (10 mM), respectively, and K^* is the apparent dissociation constant[10].

PCR. For quantitative PCR (qPCR) experiments, total RNA samples were isolated from cultured trigeminal ganglion neurons or CGNs and reverse transcribed as described above. qPCR experiments were performed using Sybr Green reagents and Applied Biosystems 7300 Real-Time PCR system. All experiments were performed in triplicate. Amplification of ribosomal protein L19 (RPL19) transcripts was used as a standard and for normalization of all qPCR reactions. 100-250bp fragments were amplified using specific primers for each gene (**Table 1**).

For RT-PCR analysis of individual sensory neurons, cells were examined for sanshool sensitivity by calcium imaging and 2–3 cells in each category were aspirated into a large-diameter glass electrode filled with lysis buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 U μl^{-1} RNasin (Promega)) and flash frozen. Reverse transcription was performed using both murine Moloney leukemia virus and avian reverse transcriptases at 37 °C for 1 h. The product was diluted 1:5 and used as the template for PCR experiments.

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Figure Legends

Figure 1 Sanshool excites CNS neurons that express KCNK3, KCNK9 or KCNK18. (**a**) Representative calcium response of cultured cerebellar granule neurons (CGNs) in response to sanshool (100 μM; left). Representative electrophysiological response of a CGN to sanshool (100 μM; orange) or protons (pH 6.5; blue) during whole-cell voltage-clamp recording (holding potential = –60 mV) (right). (**b**) Comparison of sanshool-evoked calcium responses by CGNs cultured for 2 d (top) versus 7 d (bottom) (days *in vitro*, d.i.v.). (**c**) Quantitative PCR analysis of sanshool-sensitive KCNK transcripts from CGN cultured for 7 d.i.v. versus 2 (n = 3–4).

Figure 2 Sanshool excites sensory neurons that express KCNK3, KCNK9 or KCNK18. (**a**) Quantitative PCR analysis of sanshool-sensitive KCNK expression in cultured trigeminal (TG) sensory neurons versus CGN cultured for 2 d.i.v. (n = 3–4). (**b**) Representative calcium imaging experiment used to identify sanshool-sensitive cells. Cells 1 and 2 are sanshool-sensitive, whereas cells 3 and 4 are insensitive. (**c**) Representative PCR analysis of KCNK18 expression in sanshool-positive and sanshool-negative sensory neurons. Lane 1 contains a sample amplified from cDNA prepared from cells 1 and 2; lane 2 contains a sample amplified from cDNA prepared from cells 3 and 4 (see above). n = 6 samples for sanshool sensitive neurons and 3 for sanshool insensitive neurons; each sample contained 2–3 cells. Note the presence of control RPL19 product in all samples (right).

Figure 3 Quantitative PCR analysis of KCNK transcripts from cerebellar granule and trigeminal sensory neurons **(a)** Comparison of KCNK channel subtype expression in cerebellar granule neurons (CGN) on day 2 (left) versus day 7 (right) in culture (n=3 per condition). **(b)** KCNK channel subtype expression in trigeminal (TG) neurons (n=3).

Table 1

Primer sequences used for qPCR experiments		
KCNK1	5′	GTTCCTGTATACATTTCTACCCTCTTG
	3′	CATAATCTGTTCAGGGGAGAGG
KCNK2	5′	CACTGTGAGTTTTGCACATGG
	3′	GGGACTGGACTTTTTCTGAATC
KCNK3	5′	TGCTCGTGCCTCTGGTACA
	3′	CGTGGACACCGAGCTGAT
KCNK4	5′	CATCCAAAAAGCCTTCCAGA
	3′	ATTTGGCAACCACTGGACTC
KCNK5	5′	TTGCTGATTCAGGCACGTAG
	3′	GAGAACCACATGCCAAACCT
KCNK6	5′	TTGATGCTCTGCATGGCTAC
	3′	TCCCCCGTGTGACTTTCTAC
KCNK9	5′	CTACTGGAGGGAGAAGTTGCGGAGA
	3′	ACATCATCATCATCATCATCGTCATC
KCNK10	5′	GCAGCTTTCCCTTAGACCAG
	3′	CCAGGGACATTCATTTTGGA
KCNK13	5′	CTGTTTTGTGGCTTTCAGCA
	3′	CCGCTATCCAGTTTCCTCAG
KCNK16	5′	GTCTTCTGTGTCTTCTATGCTCTGAT
	3′	AGAGATGGGGATTTTCTGTGATCTA
KCNK18	5′	CTCTCTTCTCCGCTGTCGAG
	3′	AAGAGAGCGCTCAGGAAGG
RPL19	5′	AGCCTGTGACTGTCCATTCC
	3′	GGCAGTACCCTTCCTCTTCC

Table 1 Specific primers used in qPCR experiments. Primers were designed to amplify a specific 100-250 base pair fragment of each known functional KCNK gene for use in qPCR experiments. qPCR experiments were performed as described in **Methods.**

Figure 1

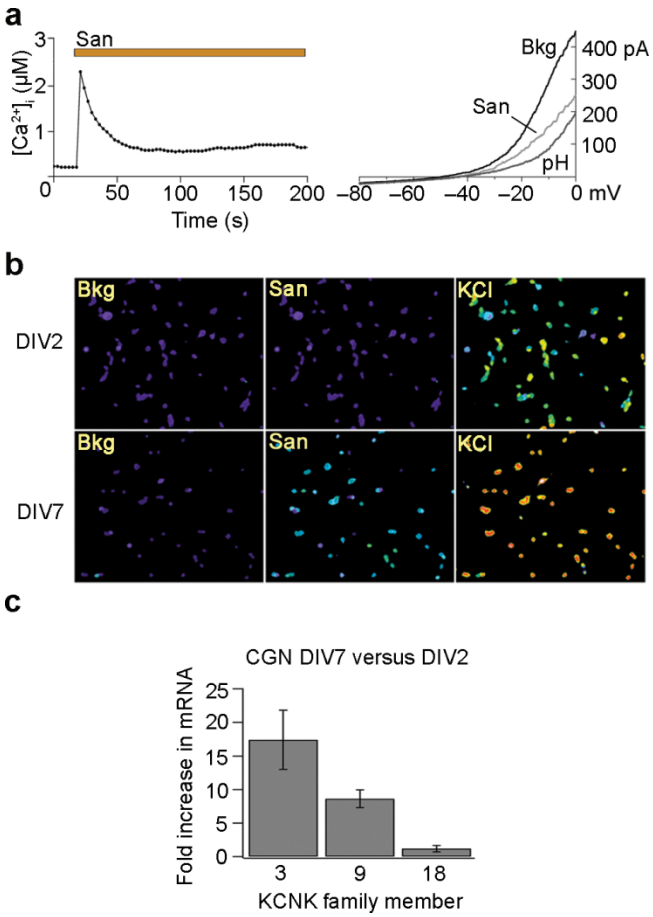

a
San
[Ca2+]i (μM)
3
2
1
0
50
100
150
200
Time (s)
Bkg
400 pA
300
San
200
100
pH
–80
–60
–40
–20
0 mV
b
Bkg
San
KCl
DIV2
DIV7
c
CGN DIV7 versus DIV2
Fold increase in mRNA
25
20
15
10
5
0
3
9
18
KCNK family member

Figure 2

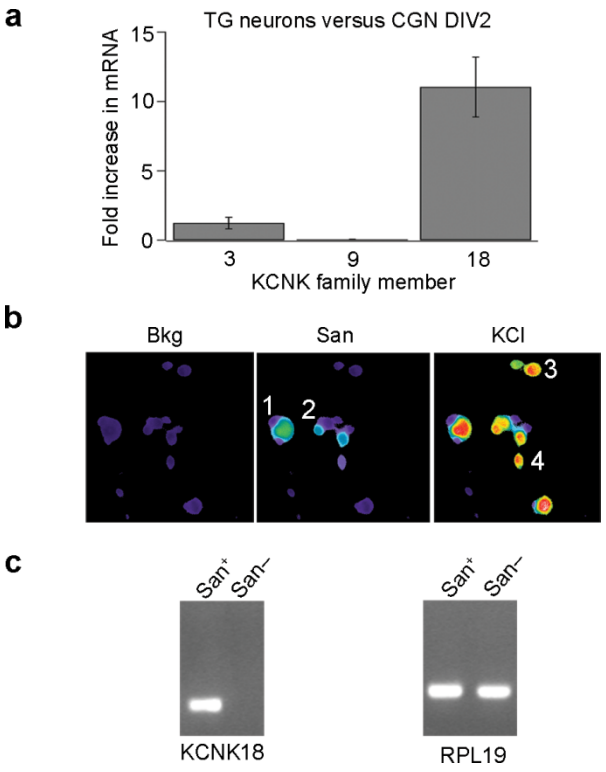
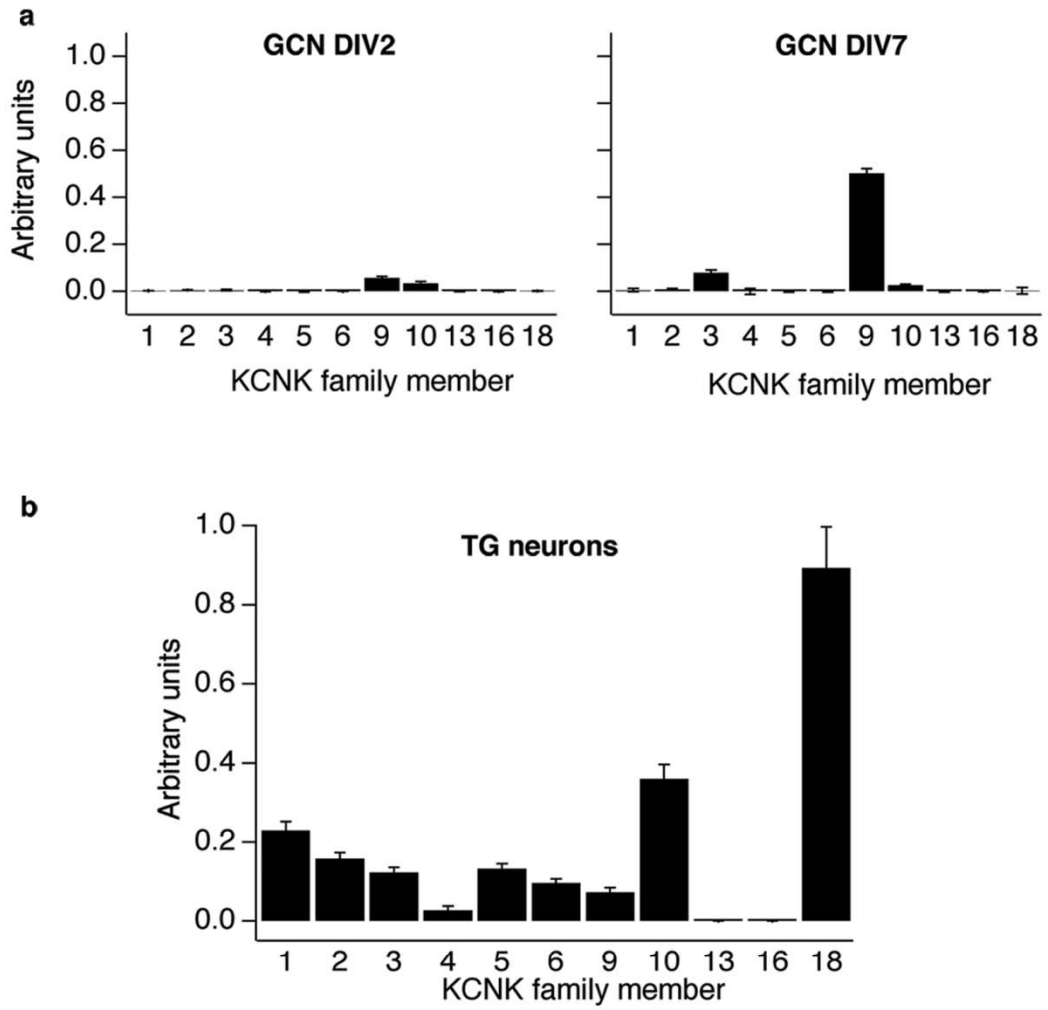

a
TG neurons versus CGN DIV2
Fold increase in mRNA
15
10
5
0
3
9
18
KCNK family member
b
Bkg
San
KCl
1
2
3
4
c
San+
San–
KCNK18
San+
San–
RPL19

Figure 3


a
GCN DIV2
GCN DIV7
Arbitrary units
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 9 10 13 16 18
KCNK family member
b
TG neurons
Arbitrary units
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 9 10 13 16 18
KCNK family member

Chapter 5

Future Directions

Conclusions and Future Directions

Further determination of the cellular and molecular sites of action for sanshool and related alkylamides

It is interesting that anandamide, a structurally similar molecule to hydroxy-α-sanshool, does not activate either sensory neurons or cerebellar granule cells (not shown). However, it has been previously shown that this molecule is able to inhibit KCNK3 and KCNK9 more potently than sanshool. How then, does the application of sanshool, acting through the same mechanism activate neuronal populations which express the relevant KCNK family members? To excite neurons, the inhibition of KCNK channels leads to the rise in the resting membrane potential of the cell. This rise activates voltage gated ion channels including voltage gated sodium and calcium channels. The T-type voltage gated calcium channels ($Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$) are considered the low-threshold voltage gated channels and can be activated by potentials only slightly more positive than the resting membrane potential. Therefore only a small rise in membrane potential is necessary to trigger an action potential. However, it has been shown that anandamide can directly inhibit T-type calcium channels at the same concentration at which it can inhibit KCNK potassium channels. Therefore, presumably with no low-threshold voltage gated channels that can be activated, anandamide does not directly lead to the influx of calcium in the types of neurons examined.

If hydroxy-α-sanshool is to act through this mechanism, we can hypothesize that as opposed to anandamide, this sanshool molecule should not inhibit T-type calcium

channels. While we have not directly testing this yet, previous studies on the chemical determinants of anandamide support this idea. In this work, changes in the length of the alkyl chain and position of the saturated carbon-carbon bonds did not strongly affect the inhibition of T-type calcium channels, changing the four double bonds from *cis* to *trans* almost completely eliminated the inhibition of these channels by anandamide. Of the alkylamides tested in our work that lead to neuronal activation, all contain at least two *trans* double bonds and hydroxy-α-sanshool contains three. If viewed as anandamide analogues, these molecules would not be expected to inhibit T-type calcium channels. Based on this hypothesis, investigating the effects of these alkylamides on T-type calcium channels may begin to provide for a more detailed molecular mechanism of neuronal activation by these molecules.

Generation and analysis of KCNK3, 9, and 18 triple knockout mice

Undoubtedly, further confirmation of the role of KCNK3, 9, and 18 in sensory and general neuronal biology will await the generation and analysis of mice lacking these genes. To date, knockouts of KCNK3 and KCNK9 have been reported as well as the KCNK3/KCNK9 double deficient line. In addition, a putative pore-dead mutant of KCNK18 has been reported. However, despite reports of KCNK18 playing a major role in sensory neurons, there was no discernible reported sensory or molecular phenotype in these animals. Due to the reported upregulation of KCNK channels in other studies of knockouts, it is not possible to determine if this lack of a phenotype is due to a lesser role for KCNK18 or, more likely, due to the necessary compensatory effect in order to maintain normal signaling. Therefore, to properly examine the role of sanshool

sensitivity, a triple KCNK3, 9, and 18 knockout would be ideal. In such a model it would be possible to confirm or rule out the contribution of additional components of sanshool sensitivity. Cellular and behavioral tests could examine a direct role for these channels in low-threshold mechanosensation as well as the detection of noxious stimuli. However, the interpretation of these results will need to be performed carefully as the deletion of several background potassium channels could have a range of varying effects. If there is a deficit in the expression of background potassium channels, the resting membrane potential in these cells could be dramatically higher. This alteration could lead to either an increase in overall sensitivity in the neurons or could prevent the recovery phase of an action potential and prohibit the firing of repetitive action potentials. The deletion of these KCNK genes could lead to further upregulation of other background channels resulted in altered regulation of the current. In the most severe phenotype, if these genes are essential the deletion of multiple KCNK family members could lead to a decrease in the viability of the neurons where they are usually expressed. Regardless of these concerns, when all possible outcomes are taken into account, the generation of these triple knockout mice will most likely lead to new and useful finding regarding the role of KCNK channels in sensory biology.

Another interested possibility would involve the creation of a KCNK18 knock-in mouse. Through mutational analysis, specific amino acid residues necessary for the inhibitory actions of sanshool can be identified and altered. A mouse containing a KCNK18 with these mutations would be predicted to retain an otherwise normal background potassium conductance, but lack sanshool sensitivity. Using this animal would eliminate the concerns about upregulation of other sanshool sensitive KCNK

family members and it would still express a functional KCNK18. Challenging cultured sensory neurons isolated from these animals would likely provide the most definitive answer to the role of KCNK18 in sanshool sensitivity in sensory neurons.

Non-neuronal roles for sanshool-sensitive KCNK channels

Members of the KCNK potassium channel family are not limited to the nervous systems, but are widely expressed throughout the body. There is strong evidence for the role of several members, including KCNK1 and KCNK5, in kidney function. Recently, multiple studies have suggested a role for KCNK3, KCNK9, and KCNK18 in T cell function. The precise role of these channels in an immune cell is less obvious as they are not classically excitable cells. However, these channels could still regulate the influx of calcium in these cells, possibly in an opposite way. During cellular signaling, when calcium selective or non-selective ion channels are opened, the influx of calcium is driven by both the concentration gradient as well as the potential difference across the membrane. Therefore, by inhibiting a background potassium channel, sanshool could raise the membrane potential, thereby decreasing the driving force, leading to less influx of calcium. In this scenario, the application of sanshool would have an inhibitory effect on T cells in contrast to the excitatory role on sensory neurons. This potential mechanism would be particularly interesting in the case of the alkylamides E1 and E2 isolated from *Echinacea* as this plant has been traditionally used for its immunomodulatory effects.

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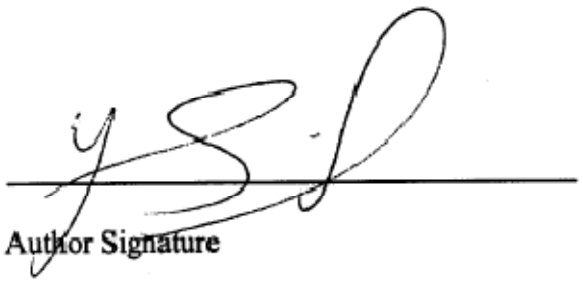

Author Signature

1/6/09

Date