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Summary of the PhD Thesis

Mechanizmy aktivace a modulace iontových kanálů specifických pro nociceptivní neurony

Mechanisms of Activation and Modulation of Ion Channels Specific for Nociceptive Neurones

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Abstract

Human body detects potentially damaging stimuli by specialized sensory nerve endings in the skin, the nociceptors. Their membranes are equipped with ion channels, molecular sensors, coding the outside stimuli into the trains of action potentials and conducting them to the higher brain centers. The most prominent group of transduction ion channels is the transient receptor potential (TRP) channel family followed by ion channels responsible for generation and conduction of action potentials from the periphery to the brain, the voltage-gated sodium channels (VGSCs). Understanding the mechanisms how particular stimulus is encoded and processed is of particular importance to find therapeutics for various types of pain conditions.

We characterized the properties of VGSC subtypes $Na_V1.9$ and $Na_V1.8$ at high temperatures. We showed that $Na_V1.9$ undergo large increase in current with increasing temperatures and significantly contribute to the action potential generation in dorsal root ganglion (DRG) neurons.

Ciguatoxins (CTXs) are sodium channels activator toxins causing ciguatera fish poisoning, a disease manifested by sensory and neurological disturbances. We elucidated the mechanism of CTX-induced cold allodynia, a pathological phenomenon where normally innocuous cool temperatures are perceived as pain. We showed that CTX actions manifest in TRPA1-expressing peptidergic C-fibers and also A-fibers in a TRPA1-independent way. The most potent ciguatoxin subtype, P-CTX-1 (Pacific-Ciguatoxin Subtype-1), did not directly activate TRPA1, but this channel was stimulated through an indirect mechanism. CTXs are also effective in releasing calcitonin-gene related peptide (CGRP) from nerve terminals. We showed that P-CTX-1 induces CGRP release from the mouse skin mainly through $Na_V1.9$, and the combined activation of $Na_V1.7$ and $Na_V1.1$.

Next, we investigated the actions of crotalphine, a 14-amino acid analgesic peptide from the venom of rattlesnake *Crotalus durissus terrificus*, on peripheral nervous system. We found that crotalphine selectively activates and subsequently desensitizes TRPA1, thus exerting the analgesic effects.

In the next part, we focused on determining the mechanism of action of well-known topical remedy for pain, the camphor, on nociceptors and elucidating the molecular action of camphor on TRPV1 specifically.

In the last part, we introduced an improved thermal gradient behavioral assay for testing the temperature preference of mice in an unbiased circular running track. It allowed discerning exploratory behavior from thermal selection behavior. This setup shed light on different temperature preference of TRPA1-/-, TRPM8-/- and TRPM8/A1/-/- mice.

Abstrakt

Lidský organizmus detekuje potencionální škodlivé podněty z okolí pomocí specializovaných volných nervových zakončení v kůži, nociceptorů. Buněčné membrány těchto neuronů jsou vybaveny iontovými kanály, molekulárními senzory, které kódují vnější podněty ve formě akčních potenciálů a vedou je z periferie do vyšších mozkových center. Jedna z významných skupin těchto iontových kanálů je specifická podskupina teplotně citlivých TRP (transient receptor potential) receptorů následovaná iontovými kanály, které generují a vedou akční potenciály: napětově řízenými sodíkovými kanály. Porozumění molekulárním mechanizmům, které se na procesech aktivace těchto iontových kanálů podílejí, je zásadním předpokladem pro nalezení nových terapeutických přístupů pro léčbu bolesti.

Charakterizovali jsme vlastnosti sodíkových kanálů podtypu $Na_V1.9$ a $Na_V1.8$ při působení vysokých (nociceptivních) teplot. Ukázali jsme, že aktivita $Na_V1.9$ kanálů zesílená se stoupající teplotou významně přispívá ke vzniku a vedení akčního potenciálu v neuronech zadních kořenů míšních (DRG).

Ciguatoxiny (CTX) jsou aktivační toxiny sodíkových kanálů, které způsobují závažné onemocnění ciguatera projevující se poruchami senzorických vjemů. Objasnili jsme mechanizmus CTX-indukované chladové alodynie, což je patologický jev, při kterém dochází ke vnímání neškodné teploty jako bolest. Ukázali jsme, že CTX působí prostřednictvím skupiny TRPA1-pozitivních, peptidergních C-vláken a specifické skupiny A-vláken, kde je působení CTX nezávislé na TRPA1. P-CTX-1 (Pacific-Ciguatoxin Subtype-1) neaktivuje TRPA1 přímo, ale jeho aktivita je potencována nepřímým mechanizmem. P-CTX-1 také účinně stimuluje uvolňování CGRP (Calcitonin Gene-Related Peptide) z nervových zakončení. Aplikace P-CTX-1 na myší kůži způsobí uvolnění CGRP převážně aktivací kanálů $Na_V1.9$ a kombinace $Na_V1.7$ a $Na_V1.1$.

V další části jsme osvětlili analgetický mechanizmus působení crotalphinu, 14-aminokyselinového peptidu z jedu chřestýše brazilského *(Crotalus durissus terrificus)*, na periferní nervový systém. Zjistili jsme, že crotalphine selektivně aktivuje a následně desenzitizuje TRPA1, a tím dochází k analgetickému působení.

Věnovali jsme se určení mechanizmu působení známé přírodní účinné látky proti bolesti, kafru, na nociceptory a na molekulární úrovni na TRPV1 receptor.

V poslední části předložené dizertační práce jsme vytvořili a testovali nové experimentální zařízení: kruhový termální gradient pro behaviorální experimenty. Účelem tohoto zařízení je kvantitativně vyhodnotit pohyb myši a sledování teploty povrchu, na kterém se pohybuje, a který jako svoji teplotně komfortní zónu vyhledá. Ukázali jsme rozdílné teplotně preferenční vlastnosti knockout myší TRPA1-/-, TRPM8-/- a TRPM8/A1/-/-.

Introduction

Peripheral nervous system is an essential component of human body responsible for maintaining fundamental homeostatic processes. It detects information about the outside world and the conditions an individual is facing and enables the organism to adapt and survive in constantly changing environment. The stimuli are transduced into trains of action potentials which are conducted to the central nervous system and are perceived by brain as touch, vibration, temperature and pain. Nociceptors, specialized peripheral nerve fibers responsible for mediating pain, guard the organism against potentially damaging stimuli: chemical, mechanical or extreme temperatures (Basbaum et al., 2009; Dubin and Patapoutian, 2010). Understanding how particular stimulus is encoded at the periphery and further processed by the brain is of particular importance. Besides protective function, dysregulation or nerve injury can lead to unwanted debilitating pain conditions like chronic and neuropathic pain. Nociceptors can detect broad spectrum of stimuli ranging from innocuous temperature to noxious cold and hot, including chemical and mechanical detection. It thus remains unclear how all these stimuli are coded and to what extent innocuous temperature perception overlaps with the perception of pain. Therefore, elucidation of molecular mechanisms and neuronal pathways involved in various types of pain are subject of tremendous scientific effort and a precondition to find effective therapies.

Temperature modulation of voltage-gated sodium channels

Voltage-gated sodium channels (VGSCs) expressed on nociceptive neurons, are responsible for the rising phase of the action potential. They conduct the information from the periphery to the central nervous system via C- and Aδ-fibers. There are 9 structurally related pore-forming α-subunits of VGSCs whereas the main subtypes responsible for signaling in nociceptive fibers are $Na_V1.7$, $Na_V1.8$, $Na_V1.9$ in C-fibers and $Na_V1.6$, $Na_V1.7$ and $Na_V1.8$ in Aδ-fibers (Ahern et al., 2016; Bennett et al., 2019).

One of the key abilities of nociceptors is to transmit the sensory temperature information. The peripheral nervous system has to deal with a profound modulation of conductive ion channels. The structure of these proteins and their lipid environment they are embedded in are profoundly sensitive to temperature changes. Even couple of degrees difference may alter the kinetics of the channel gating and even lead to their inactivity (Volgushev et al., 2000; Zimmermann et al., 2007).

One important approach how to reveal the specific role of ion channels is the use of knockout animals of a specific gene of interest. Regarding the perception of nociceptive stimuli among VGSCs this approach revealed the crucial role of $Na_V1.7$ in nociception, namely inflammation-induced pain, thermal hyperalgesia after burn injury and loss-of-function mutations leading to congenital insensitivity to pain (Nassar et al., 2004; Cox et al., 2006; Shields et al., 2012; Shields et al., 2018). On the other hand, $Na_V1.8$ is a sodium channel subtype with relatively slow kinetics and high voltage threshold (compared to $Na_V1.7$) broadly expressed on nociceptive C-fibers. This channel is reported to be the only VGSC subtype not being inactivated at low temperatures (Zimmermann et al., 2007). In contrast to $Na_V1.7$ and $Na_V1.8$, $Na_V1.9$ has been reported to set the action potential threshold and due to its ultra-slow kinetics and non-inactivating, persistent sodium current not to contribute to action potential rising phase (Cummins et al., 1999; Dib-Hajj et al., 2015).

Temperature is a crucial parameter influencing the conduction of action potentials and thus modulating many behavioral aspects. The mammalian system is balanced to maintain core body temperature between 36° to 38°C. Temperature increase above these values by only couple of °C leads to conduction block and to protein denaturation, serious medical conditions and death. Therefore, the rapid signaling of potential danger at high temperatures has to be maintained. We focused on researching sodium channel subtypes at high temperatures, especially the role of $Na_V1.8$ and $Na_V1.9$.

Ciguatoxin and cold allodynia

Ciguatoxins (CTXs) are one of the most potent toxins described causing ciguatera fish poisoning. They are well known sodium channel activator toxins causing a number of neurological disturbances including pain, pruritus, cold allodynia, paresthesias, dysaesthesias and a number of other symptoms e.g. nausea and diarrhea (Strachan et al., 1999; Lewis, 2001). Cold allodynia is a sensory disturbance which is characterized by burning and stabbing pain in response to mild cooling. Ciguatoxins belong to the group of ichtyosarcotoxins and are produced by dinnoflagellates of the genus *Gambierdiscus* in cirmuctropical regions. The most common way of poisoning is eating fish which has acquired the ciguatoxins through bioaccumulation via the marine food chain. There are several related variants of these toxins of which the Pacific-ciguatoxin-1 (P-CTX-1) is the most potent isoform (Lewis, 2001).

Characterization of the precise molecular mechanism how these toxins influence the nervous system and cause cold allodynia will contribute to the knowledge how cold and temperature sensing works in general and overlaps with nociception sharing the same neural pathways. Elucidating the molecular mechanisms behind this phenomenon would be of significant importance in the effort of finding novel and more effective therapeutic pain targets.

Analgesic effects of crotalphine and campher

Crotalphine is a 14- amino acid structural analog to an analgesic peptide that was first identified in the crude venom from the South American rattlesnake *Crotalus durissus terrificus* (Giorgi et al., 1993; Konno et al., 2008). Previous studies speculated that opioid receptors or endogenous opioids are involved in the analgesic mechanism of crotalphines`s action (Picolo et al., 2000; Konno et al., 2008). Oral, intravenous and intraplantar doses of crotalphine lead to long-lasting (5 days) analgesia in inflammatory pain models in rats, which was suppressed by the kappa-opioid receptor antagonist norbinaltorphimine (Konno et al., 2008) and by intraplantar injection of a CB2 receptor antagonist AM630 (Machado et al., 2014). In contrast to opioids, the analgesic effects of crotalphine are not accompanied by withdrawal symptoms and tolerance (Gutierrez et al., 2008). However, crotalphine does not exert its effect through direct activation of opioid receptors. Therefore, the aim was to identify direct molecular targets in nociceptive pathway.

Camphor is an organic substance from *Cinnamomum camphora* tree used as a topical remedy for its antipruritic and analgesic properties. The proposed mechanism of camphor`s analgesic properties was through partial activation and subsequent desensitization of TRPV1 receptor (Xu et al., 2005). Camphor is a lipophilic substance also activating TRPV3 (Moqrich et al., 2005) and TRPM8 (Vogt-Eisele et al., 2007; Selescu et al., 2013) and is a blocker of TRPA1 (Xu et al., 2005). TRPV1, the receptor for capsaicin and heat (> 43°C), is expressed mainly in small diameter DRG nociceptive neurons. Camphor potentiates heat-evoked responses and shifts the voltage-dependence of TRPV1 activation to more negative voltages (Xu et al., 2005). Here we elucidated the molecular mechanism of camphor action on native cutaneous nociceptors and binding on TRPV1 (Marsakova et al., 2012; Vetter et al., 2013).

Ion channels in thermal preference behavior

Researching molecules involved in temperature perception or thermoregulation such as TRP ion channels relies heavily on experiments performed in animal models. Creating knockout animal and finding the behavioral phenotype is of profound significance. One of the limiting parameters in researching the role of thermosensitive proteins is the lack of suitable behavioral assays. Therefore, we focused on designing a special behavioral assay to quantify temperature sensitivity, cold/hot hypersensitivity or hyperalgesia.

Since the identification of TRPM8 as the major cold sensing molecule also responsible for menthol detection (McKemy et al., 2002; Peier et al., 2002; Bautista et al., 2007; Dhaka et al., 2007; Knowlton et al., 2011), it has been substantial debate to which extent other ion channels are involved in cold sensing. Other candidate has been TRPA1 receptor (Story et al., 2003; Kwan et al., 2006;

Karashima et al., 2009), which cold sensitivity has been proved as purified protein in lipid bilayers (Moparthi et al., 2014). To better understand the role of these channels in cold sensation, we tested thermal sensitivity in TRPM8-, TRPA1- and TRPM8/A1-deficient mice.

Aims of the study

- To determine the contribution of voltage-gated sodium channel subtypes $Na_V1.8$ and $Na_V1.9$ to action potential generation at noxious temperatures.
- To elucidate the mechanism of action of ciguatoxins in cold allodynia.
- To establish the role of specific voltage-gated sodium channel subtypes on CGRP release caused by ciguatoxin.
- To elucidate the mechanism of analgesic action of crotalphine.
- To characterize actions of camphor on nociceptors and specifically on TRPV1 receptor at the molecular level.
- To characterize TRPM8-/-, TRPA1-/-, and TRPM8/A1-/- mice in a novel circular gradient thermal preference assay.

Materials and methods

Patch-clamp Electrophysiology

Whole-cell patch-clamp recordings were conducted with an Axopatch 200B amplifier/Clampex 10.4 software (Molecular Devices) or EPC 10USB/Patchmaster software (HEKA Elektronik). Glass pipettes were fabricated with a P-1000 Micropipette Puller (Sutter), with 1.8-3 MΩ resistance for voltage-clamp configuration and 3.5-6 MΩ resistance for current-clamp configuration. The bath solution contained (in mM) 140 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 20 glucose, and 0.1 $CdCl_2$, adjusted to pH 7.4 with NaOH. For voltage-clamp recordings, 20 mM TEA-Cl was added. Pipette solution for voltage-clamp recordings on $Na_V1.9$ channel, if not otherwise indicated, contained (in mM) 120 CsCl, 10 NaCl, 10 HEPES, 10 EGTA, 2.2 $MgCl_2$, 1.9 $CaCl_2$, 5 TEA-Cl, 4 MgATP, and 0.2 Na_2GTP, pH 7.3, adjusted with CsOH. CsF-based pipette solution contained (in mM) 140 CsF, 10 NaCl, 10 HEPES, 1 EGTA, and 5 TEA-Cl, pH 7.3 with NaOH, and was also used for voltage-clamp experiments with N1E-115 and ND7/23 cells. 500 or 1500 nM TTX (tetrodotoxin) was added as indicated. The pipette solution for current-clamp recordings contained (in mM) 135 potassium gluconate, 4 NaCl, 3 $MgCl_2$, 0.3 Na-GTP, 2 Na_2-ATP, 5 EGTA, and 5 HEPES, adjusted to pH 7.3 with KOH. Currents were sampled at the rate of 20–100 kHz, based on the protocol type. Series resistance was compensated for (65–85%).

Calcium Imaging

Dissociated DRG neurons from WT (wild-type) and all cell lines used were plated on Poly-D-Lysin-coated glass coverslips. Recordings were performed on an Olympus IX71 inverse microscope with a 20 objective. Fura-2 was excited at 340 and 380 nm with a Polychrome V monochromator (Till Photonics). Images were exposed for 20 ms and acquired at a rate of 1 Hz with a 12-bit CCD camera (Imago Sensicam QE, Till Photonics). Data were recorded and further analysed using TILLvisION 4.0.1.3 software (Till Photonics). To assess Ca^{2+} responses to cold/warm stimulation and also for the patch-clamp experiments at different temperatures, a system for fast superfusion of the cultured cells was used (Dittert et al., 2006).

Animal behavior

The protocol for *in vivo* experiments in animals was reviewed by the local animal ethics committee (University of Erlangen) and approved by the local district government. Experiments involving live animals were conducted in accordance with the International Association for the Study of Pain Guidelines for the Use of Animals in Research. Where indicated, mice were anesthetized with sevoflurane and injected with TTX (20 µl, 3 µM) intracutaneously in the plantar hindpaw skin. Mice

were placed in an acrylic glass chamber with a Kevlar grid surface. The Hargreave`s infrared probe (UgoBasile) was placed under the plantar surface, and the latency was automatically recorded as soon as the mouse withdrew. Cutoff time of the heat pulse was set to 30 s to avoid tissue damage. Mice were habituated 1 d before the experiment and also 1 h before the first measurement. Paw withdrawal latency was measured 4–10 min after the mouse had received the TTX injection and had regained consciousness. TTX effects lasted for maximum 10 min. Eight mice per genotype were measured, and at least two consecutive measurements were performed on the same hindpaw with minimal time difference of 3 min and maximal of 6 min between measurements. Values measured on the same hindpaw were averaged. The measurements were repeated with the same mouse groups after 5 d, and TTX was applied on the contralateral side. The experimenter was blinded to the genotype and injection.

Cell Culture

DRGs were isolated and cultured as previously described (Zimmermann et al., 2007). In brief, DRGs from T1-L6 were isolated mice and collected in DMEM supplemented with 50 mg/ml gentamicin (Sigma), 100U/ml penicillin, 100 mg/ml streptomycin and 0.25mg/ml amphotericin B (Invitrogen). DRGs were then incubated for 30 min at 37°C and 5% CO_2 in dissociation media containing 1mg/ml collagenase (Sigma) and 0.1mg/ml protease (Sigma). After three wash steps, cells were triturated through a flame-polished glass Pasteur pipette and cultured for 24h in TNB 100 solution supplemented by TNB 100 lipid-protein complex, 1 nM NGF, 100 mg/ml streptomycin and penicillin (all from Biochrom, Berlin, Germany) and 200 mg/ml glutamine (Invitrogen, Carlsbad, USA) or Neurobasal medium supplemented with B27, 1 nM NGF and 100 mM glutamine (all from Invitrogen, Mulgrave, VIC, Australia) for high content imaging.

Reagents and Toxins

P-CTX-1 (>90% purity) was isolated from moray eel *(Gymnothorax javanicus)* liver as previously described (Lewis et al., 1991), stored as a concentrated stock in 50% methanol/50% H_2O and routinely diluted in the presence of 0.1-0.3% bovine serum albumin (BSA) to avoid loss to plastic. All other reagents were from Sigma-Aldrich (aufkirchen, Germany) unless otherwise stated. Crotalphine was synthesized by the American Peptide Co. (Sunnyvale, CA) (Konno et al., 2008).

Circular gradient temperature preference assay

Our circular assay was assembled in 2 configurations, small and large (Touska et al., 2016). Both configurations consist of a ring-shaped 1.5 cm thick aluminum disk that provides a circular running track for the mouse to move freely. The dimensions of inner and outer ring diameter are 28 cm and 40 cm for the smaller, and 45 cm and 57 cm for the larger assembly. Inner walls of plexiglass and outer walls of aluminum, both of 12 cm height with circumferences of 88 and 126 cm or 141 and 179 cm, respectively, confine a circular surface area of 640 cm^2 or 960 cm^2, respectively, with a width of 6 cm on which a temperature gradient is equilibrated. The surface is stained in frosted orange (eloxadized aluminum with minimal light reflection) to provide sufficient contrast for mouse detection, but also to allow surface temperature control with an infrared camera. During measurements the running track is uniformly illuminated and the mouse behavior is videotaped with a regular CCD camera. The outer walls are made non-transparent to mask positional cues. The thermal gradient is constantly equilibrated across the aluminum disk using 2 feed-back controlled Peltier-based plates (TE Technology, Traverse City, MI, USA) in combination with a custom-built temperature controller (Labortechnik Franken, Röthenbach, Germany). To optimize thermal conductivity, the Peltier plates are lubricated with a thin layer of thermoconductive paste and opposite sides of the aluminum ring are screwed tightly onto the plates. The stability of the surface temperature gradient is monitored before each measurement with an infrared camera (T400 series, FLIR€ Systems GmbH, Frankfurt, Germany). Deviations of less than ± 1 °C were tolerated, but readjusted. A transparent lid is labeled to divide the small ring in 15 and the large ring in 22 even-sized and one larger zone. Each individual zone covers a surface area of 40 cm^2 while the one larger

zone covers 80 cm^2 and marks the coldest area. It is required for unambiguous offline analysis of videotaped behavior with a custom-engineered. The symmetric assembly yields 2 semi-circles of even temperature on opposite sides. Consequently, for each thermal zone measured values are provided in duplicate and values of each 2 zones of equal temperature are added; in our assemblies 8 and 12 zones resulted.

Results

To determine the contribution of sodium channel subtypes $Na_V1.8$ and $Na_V1.9$ to heat-resistant action potentials.

Most polymodal C-fibers undergo conduction block between 50°C-60°C with some nerve fibers still firing action potentials even above 60°C for couple of seconds without suffering irreversible damage (Touska et al., 2018). Whole-cell patch-clamp recordings on dissociated mice DRG neurons showed extensive differences between characteristics of sodium channel isoforms at different temperatures. Especially TTXr subtype $Na_V1.9$ underwent remarkable speeding of activation kinetics. By increasing the temperature from 20° to 30° its peak current amplitude increased 4-fold, and conductance 3.8-fold **(Fig. 1A, B)**. Compared to $Na_V1.8$-mediated current where the increase of peak current was only 1.6-fold and conductance 1.7-fold **(Fig. 1C, D)**. We further increased the temperature and compared $Na_V1.9$ current at 20°C, 37°C, and 43°C. The peak current amplitude increased 4.9-fold at 37°C, n = 15 compared with 20°C, n = 17, and the conductance increased 4.3-fold at 37°C (n=15) compared with 20°C, n=16. At 43°C, peak current and conductance were not significantly different from 37°C **(Fig. 1E, F)**. We next measured TTXs subtypes from native currents of neuronally-derived cell line N1E-115 and ND7/23 containing native TTXs currents. The current amplitudes in ND7/23 were 1.0 and 0.7 comparing 20°C with 43°C and in N1E-115 1.2 and 0.7, respectively (Touska et al., 2018).

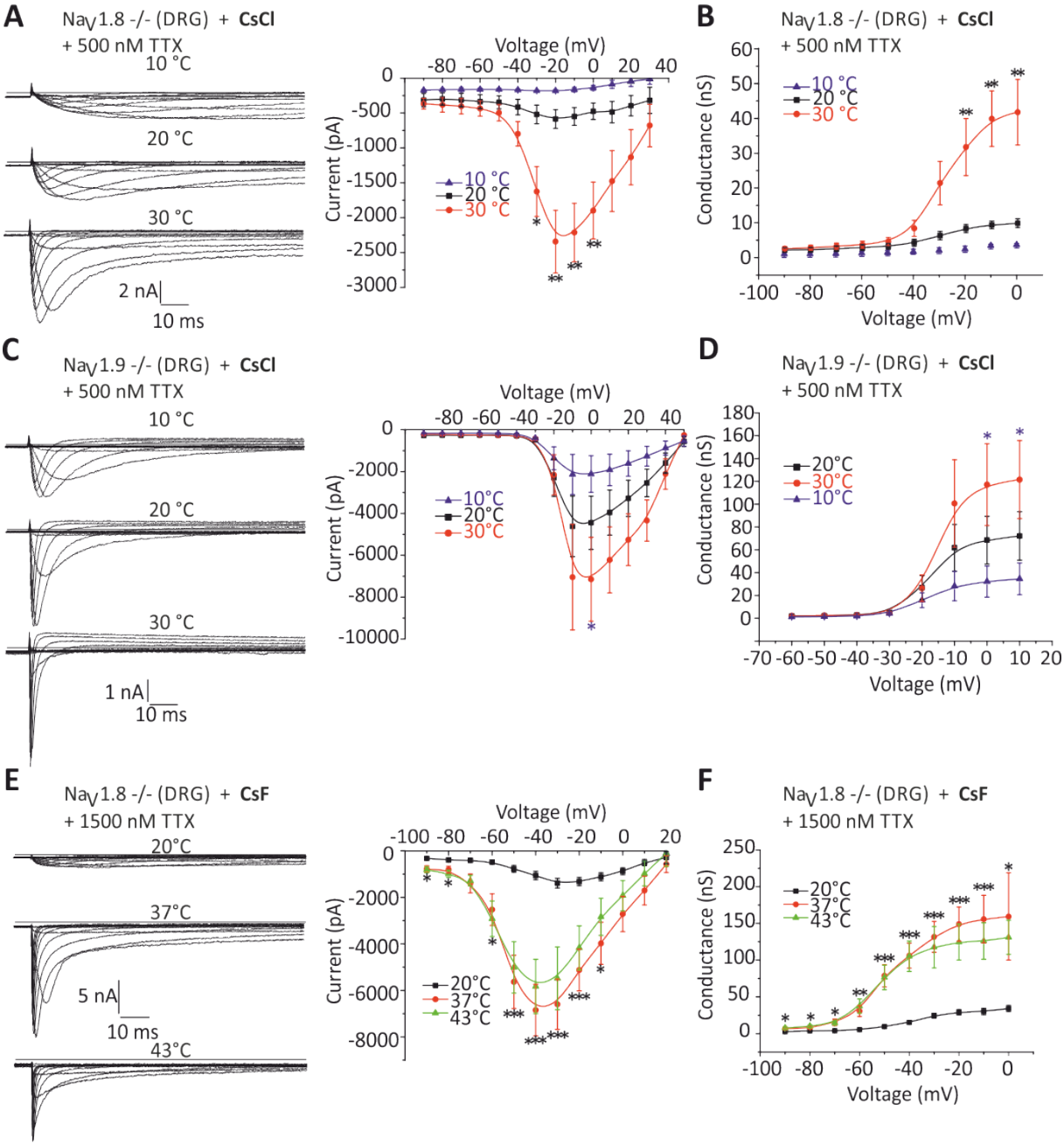

Figure 1. $Na_V1.9$ undergoes a larger warming-induced gain of function than $Na_V1.8$. Neurons were held at −90 mV and stimulated with 500-ms voltage pulses from −90 mV up to +50 mV. **(A, C, and E, left panels)** Sample traces of the specific voltage-gated sodium currents at three indicated temperatures. **(A, C, and E, right panels; and B, D, and F)** The effect of temperature on the voltage-dependence of activation and the conductance, respectively. **(A–D)** Presumptive $Na_V1.9$ currents from a cultured small-diameter $Na_V1.8$-/- DRG **(A and B)** and $Na_V1.8$ current from a $Na_V1.9$-/- DRG **(C and D)**, both recorded in the presence of 500 nM TTX with a CsCl-based pipette solution at 10°C, 20°C, and 30°C. An increase in temperature from 20°C to 30°C enlarged the $Na_V1.9$ (CsCl) peak current amplitude 4.0-fold (−585.8 ± 135.7 pA, to −2342.8 ± 448.8 pA, n = 9, P = 0.002, and the conductance 3.8-fold (8.3 ± 1.2 nS, n = 8, to 31.5 ± 8.1 n = 6, P = 0.006). In contrast, the $Na_V1.8$ peak current amplitude increased 1.6-fold (−4444.3 ± 1275.6 to −7147.6 ± 2005.5 pA, n = 8) and the conductance 1.7-fold (68.6 ± 20.8 nS to 117.1 ±35.8 nS, n = 8, P > 0.05). **(E and F)** Presumptive $Na_V1.9$ current from small-diameter $Na_V1.8$-/- DRG, acquired with a CsF-based pipette solution and in the presence of 1500 nM TTX at 20°C, 37°C, and 43°C, showed a 4.9-fold increase in the peak current amplitude at 37°C compared with 20°C (-1388.7 ± 208.2 pA, n = 15, to -6844.0 ± 1108.4 pA, n = 17, P = 0.0001) and a 4.3-fold increase in the conductance (24.5 ± 3.4 nS, n = 15, to 106.1 ± 17.1 nS, n = 16, P = 0.0003). At 43°C, peak current and conductance were not significantly different from 37°C. Data are depicted as means ± SEM. P < 0.05; **, P < 0.01; and ***, P < 0.001 (one-way ANOVA with Tukey HSD). *Figure adapted from (Touska et al., 2018).*

Contribution of $Na_V1.9$ on action potential threshold

In our study, we analyzed the temperature dependence of action potential generation in DRG neurons derived from $Na_V1.8$- and $Na_V1.9$-deficient mice and compared it with that measured from DRG neurons from WT mice (derived from C57BL/6). TTX (500 nM) was used to block TTXs Na_V channels. The action potential threshold was shifted from -32.3 ± 2.4 mV (n=20) to -14.6 ± 2.8 mV (n = 15) when $Na_V1.9$ was missing **(Fig 2A, B)**. Further temperature increase depolarized the membrane potential to +3.3 ± 4.9 mV by 18 mV. In WT neurons, the temperature increase (37°C) depolarized the membrane potential only to -26.6 ± 2.4 mV, n = 23. In $Na_V1.8$-decifient neurons, the action potential threshold was not significant to WT **(Fig. 2A-C)**. In our experiments, we were able to generate action potentials in $Na_V1.8$-deficient mice in the presence of 500 nM TTX by injecting current steps with a clear inflection **(Fig. 2C, D)**. Consistent with our voltage-clamp results, the upstroke of $Na_V1.9$-mediated action potential was strikingly speeded at 37°C compared to 20°C. The slope of the rising phase of action potential was accelerated 5.8-fold (n = 17) **(Fig 2D, F)**. In $Na_V1.8$-mediated action potentials, the acceleration was 2.3-fold (n = 5) faster, not significantly different from action potentials from the combination of both $Na_V1.8$ and $Na_V1.9$, 2.5-fold (n = 21) **(Fig. 2E, F)**.

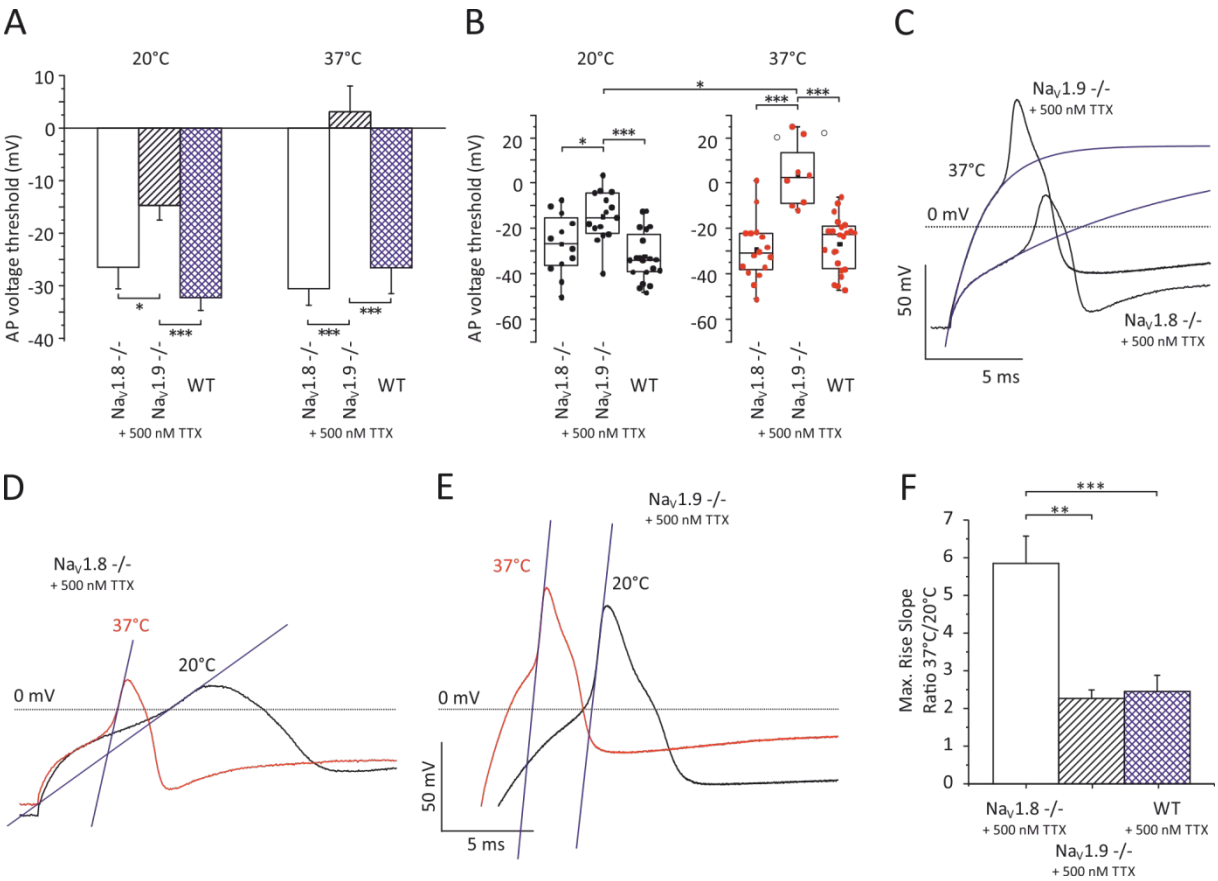

Figure 2. TTXr sodium channel subtype $Na_V1.9$ generates action potentials at 37°C and becomes essential for reaching the action potential threshold when the temperature rises. **(A)** Contribution of sodium channel subtypes $Na_V1.9$ and $Na_V1.8$ to the action potential threshold at 20°C **(left columns)** and 37°C **(right columns)**. All measurements were performed in the presence of 500 nM TTX in the bath solution in DRGs derived from C57BL/6J or $Na_V1.9$- or $Na_V1.8$-deficient mice. At 20°C and 37°C, the action potential threshold of the combined $Na_V1.8$ and $Na_V1.9$ current was −32.3 ± 2.4 mV, n = 20, and 26.6 ± 2.4 mV, n = 23, respectively. In the absence of either $Na_V1.8$ or $Na_V1.9$, the action potential thresholds at 20°C were depolarized to −26.8 ± 3.9 mV, n = 12 and -14.6 ± 2.8 mV, n = 15, respectively. At 37°C, lack of $Na_V1.9$ depolarized the threshold of the $Na_V1.8$-dependent action potentials to +3.3 ± 4.9 mV, n = 8. **(B)** Distribution and statistical analysis of the threshold values of individual measurements as displayed in A. **(C)** Typical action potentials from $Na_V1.8$-/- and $Na_V1.9$-/- DRG neurons at 37°C in the presence of 500 nM TTX with extrapolation of the threshold, as indicated by the blue curves. **(D–F)** Typical action potentials from $Na_V1.8$-/- and $Na_V1.9$-/- DRGs at 20°C and 37°C with

extrapolation of the rise slope (superimposed blue lines) to illustrate the effect of warming. The speeding of the upstroke was largest **(D)** for the putative $Na_V1.9$-mediated action potentials ($Na_V1.8$-/- with 500 nM TTX, n = 17) and reached 5.8-fold, whereas it was only 2.3-fold **(E)** for the $Na_V1.8$-mediated action potentials ($Na_V1.9$-/- with 500 nM TTX n = 5) and 2.5-fold **(F)** for action potentials generated by a combination of $Na_V1.8$ and $Na_V1.9$ (n = 21). Data are depicted as means ± SEM. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 (one-way ANOVA with Tukey HSD). *Figure adapted from (Touska et al., 2018).*

We further investigated the role of TTXr VGSC subtypes in repetitive action potential firing in response to depolarizing current stimuli at 20°C and 37°C. $Na_V1.9$-mediated action potentials (measured in $Na_V1.8$-deficient mice with 500 nM TTX) were able to fire repetitively in 10 out of 17 neurons with a mean of 8.2 ± 2.1 action potentials per 200-ms current pulse, whereas, at 20°C, only 3 of 17 neurons produced repetitive firing with 2.3 ± 0.9 action potentials in response to the same stimulus. These results were not significantly different from TTXr WT neurons ($Na_V1.8$ and $Na_V1.9$). Interestingly, $Na_V1.8$-mediated action potentials were not able to fire repetitively in all cells tested (n = 15) (Touska et al., 2018).

We assessed the contribution of $Na_V1.8$ and $Na_V1.9$ in behavioral experiments using the Hargreave`s assay **(Fig 3A, B)**. Here, an infrared beam is directed on the mouse hindpaw and the latency until the hindpaw is withdrawn is measured. Both genotypes displayed an increased reaction time which became significant after injecting 20 µl of 3 µM TTX intracutaneously in the paw under light sevoflurane anesthesia. The latencies subsequently increased from 12.1 ± 1.1 to 16.3 ± 1.5 s (n = 8, P < 0.001) compared with C57BL/6J.

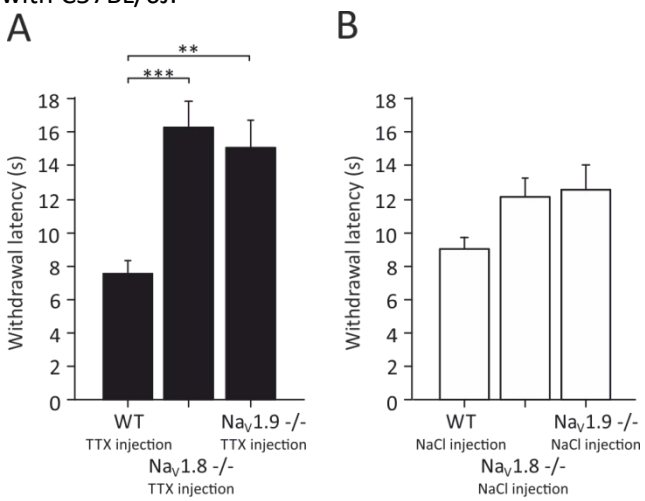

Figure 3. Fast-gated TTXs Na_V channels reduce the heat pain threshold. $Na_V1.8$-/-, $Na_V1.9$-/-, and littermate mice were subjected to heat pain threshold measurement with the plantar radiant heat test (Hargreave`s apparatus). **(A)** After intraplantar administration of 3 µM TTX in a volume of 20 µl, the withdrawal latency was almost doubled in $Na_V1.8$-/- (***, P < 0.001) and $Na_V1.9$-/- (**, P < 0.01; individual trials). **(B)** The same mice were measured before the application of TTX and showed a tendency to increased withdrawal latency (n = 8 per group). Data are depicted as means ± SEM. *Figure adapted from (Touska et al., 2018).*

Mechanism of action of ciguatoxin in cold allodynia

CTX is a well-known VGSC channel activator toxin causing debilitating conditions with sensory and neurological disturbances. One of the most prominent manifestations of ciguatera poisoning is pain, pruritus, general neurological disturbances and especially cold allodynia.

Our results confirmed that intraplantar injection of P-CTX-1 caused prominent dose-dependent cold allodynia in mice. Mice exhibited spontaneous pain behavior in form of paw lifting, flinching, shaking at room temperature exacerbated by cold temperature and relieved by warming

up to 42°C. This spontaneous pain faded after 45-60 minutes and in most cases cold allodynia occurred and persisted for several hours (Vetter et al., 2012).

Symptom of cold allodynia is reported in the majority of ciguatera poisoning cases (76 – 94% patients) suggesting that CTX affects specific nociceptive pathways. We addressed the question, which DRG subpopulation is P-CTX-1 sensitive. At low concentration (1 nM), P-CTX-1 affected the majority of peptidergic, CGRP- (82%) and TRPA1- (95%) positive neurons, 42% of NF200-positive cells (a marker of large myelinated A-fiber neurons), 66% of peripherin-positive neurons (marker of unmyelinated C-fiber and thinly myelinated Aδ-fiber-associated neurons). Among IB4-positive cells, only 12% were ciguatoxin sensitive. With increasing concentration of ciguatoxin the majority of DRG neurons were further activated manifesting the effects of ciguatoxin as a global VGSC channel activator (Vetter et al., 2012).

We found that P-CTX-1 induced a new sensitivity to those DRG neurons which were not cold sensitive prior the P-CTX-1 application (in 39% of initially cold-insensitive neurons) **(Fig. 4A-D)**. This effect was less pronounced in the absence of P-CTX-1, comparing first and subsequent second cold stimulus on the same cell (8.9% sensitized to cold, **Fig. 4E**) and was dependent on TRPA1 (effect absent in TRPA1-/- neurons) **(Fig. 4D)**.

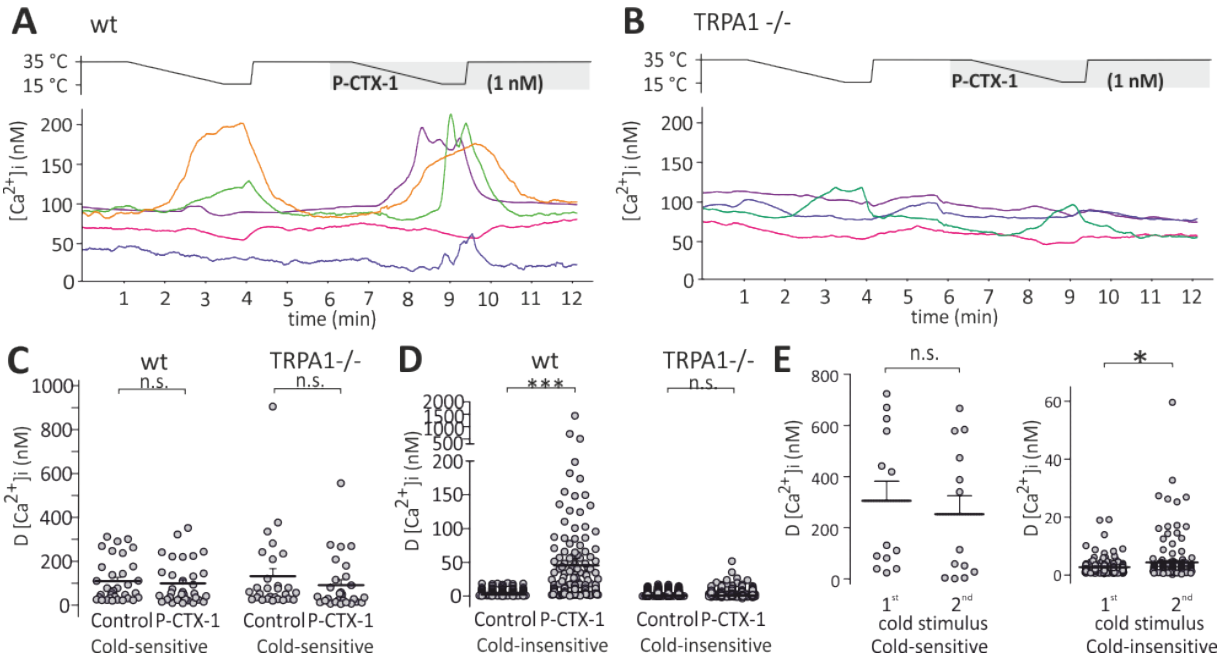

Figure 4. Ciguatoxin induces new sensitivity to cold in cultured DRG neurons via TRPA1. **(A–D)** Cold responses and cold sensitization by P-CTX-1 (1nM) in cultured DRG neurons from **(A)** WT and **(B)** TRPA1-/-mice. **(C)** Ca^{2+} responses of cold-sensitive cultured neurons were not significantly affected by P-CTX-1 but **(D)** P-CTX-1 induced novel sensitivity to cooling in previously cold-insensitive neurons, which was absent in TRPA1-/- neurons. **(E)** Cold sensitization to a second cold stimulus was less pronounced in WT neurons in the absence of P-CTX-1. Statistical significance was determined using a paired, two-tailed Student's *t*-test; *P < 0.05; ***P < 0.001; NS, P > 0.05. Data are presented as mean ± SD. *Figure adapted from (Vetter et al., 2012).*

P-CTX-1 induced ongoing activity in C-fiber nociceptors in *ex-vivo* recordings from murine skin-saphenous nerve preparations, which was markedly reduced in TRPA1-deficient animals compared to WT (Vetter et al., 2012). P-CTX-1 caused massive action potential firing activity in these fibers which ceased after cooling. This pronounced effect mediated by TRPA1-positive neurons was also confirmed by behavioral analysis of TRPA1 (Vetter et al., 2012). Ciguatoxin-induced cold allodynia was markedly reduced in TRPA1-/- mice but not TRPM8-/-, TRPV1-/-, or TRPC5-/-. However TRPA1 is not directly activated by P-CTX-1 (up to 100 nM) in heterologous expression systems

(HEK293 cells) tested in patch-clamp and Ca-imaging experiments suggesting an indirect involvement of TRPA1 in ciguatoxin action.

P-CTX-1 activated also A-fibers devoid of TRPA1. Within this subpopulation, cooling-induced action potential activity occurred. This activity was inhibited by warming above 33°C. Also in whole-cell current-clamp experiments in one subpopulation of DRG neurons, P-CTX-1 caused persistent firing and membrane potential depolarization in the range from 10-15 mV and, thus, causing hyperexcitability and action potential firing in DRG neurons **(Fig 5A-C)**. Notably, in one subpopulation of DRG neurons, this persistent firing occurred in cool/cold temperatures and ceased with warming. This action potential firing was caused by P-CTX-1-induced activation of TTXs **(Fig. 5D, E)** and TTXr **(Fig. 5F, G)** VGSCs, respectively.

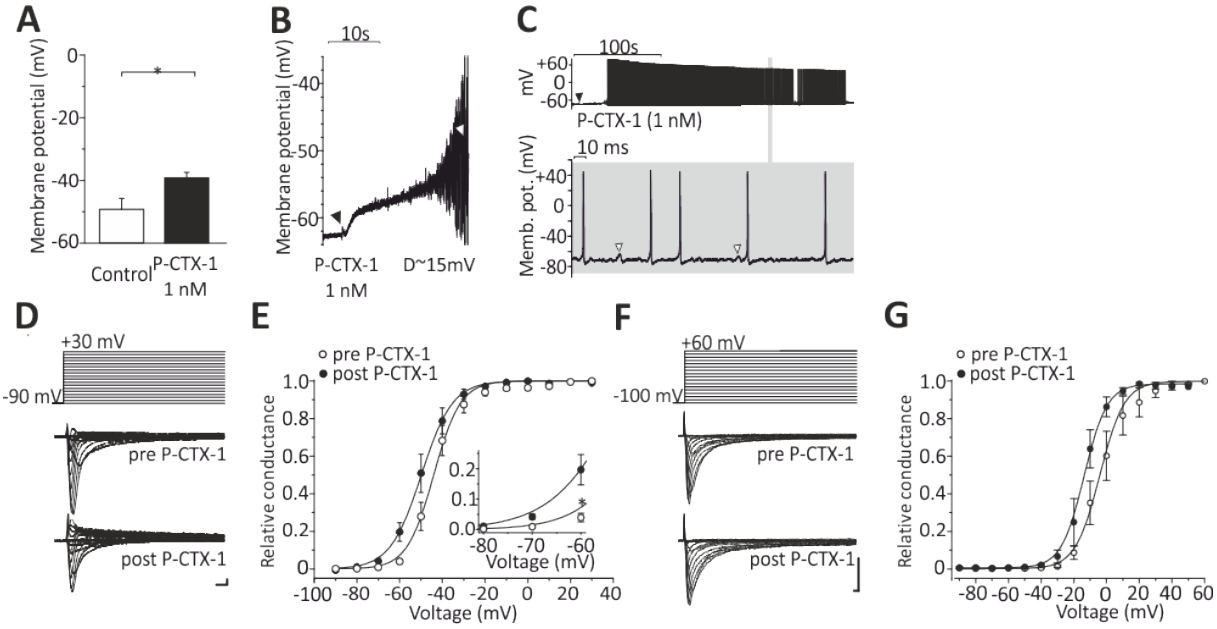

Figure 5.(A - C) Representative examples of P-CTX-1-induced depolarization. **(B)** Application of P-CTX-1 (black arrow) caused depolarization of membrane potential followed by action potential firing (white arrow: first action potential). **(C)** Upper panel: ciguatoxin-induced depolarization rapidly leads to series of action potentials. Detail expanded in lower panel: white arrows: membrane oscillations, frequently followed by action potentials. **(D)** Representative TTXs current traces recorded from large-sized DRG neurons (average diameter of 42.9 ± 1.4 µm). Upper lane: voltage protocol; middle: traces before and lower: traces after perfusion with P-CTX-1 (1 nM). **(E)** Effect of P-CTX-1 (1 nM) on the voltage-conduction relationship of TTXs channels measured in mouse DRG neurons (n = 9). **(F)** Representative recording of current traces recorded from ND7/23 cells heterologously expressing $Na_V1.8$. Upper lane: voltage protocol; middle: traces before and lower: traces after perfusion with P-CTX-1 (1 nM). **(G)** Effect of P-CTX-1 (1 nM) on the voltage-conduction relationship of $Na_V1.8$ heterologously expressed in ND7/23 cells (n = 5); scale bars in **(D, F)** represent 1 ms and 1 nA; all data are presented as mean ±SEM. *Figure adapted from (Vetter et al., 2012).*

Ciguatoxins evoke potent CGRP release by activation of Na_V-channel subtypes $Na_V1.9$, $Na_V1.7$ and $Na_V1.1$

P-CTX-1 is effective in releasing CGRP from skin. Using ELISA (enzyme-linked immunosorbent assay), we investigated which ion channels are involved in this process. Using transgenic mice and pharmacology, we showed that for the main fraction of CGRP release carried the $Na_V1.9$ and the combination of $Na_V1.7$ and $Na_V1.1$ sodium channel subtypes **(Fig. 6B)**. P-CTX-1-induced CGRP release was reduced by 42% in $Na_V1.9$-/- compared to C57BL/6J control group which further increased the block to 78% by adding 1 µM ICA-121431 ($Na_V1.1$ blocker). $Na_V1.7$-/- alone did not show any significant difference from control group, but further blocking $Na_V1.1$ in $Na_V1.7$-/- mice reduced CGRP release by 34% **(Fig 6A)**.

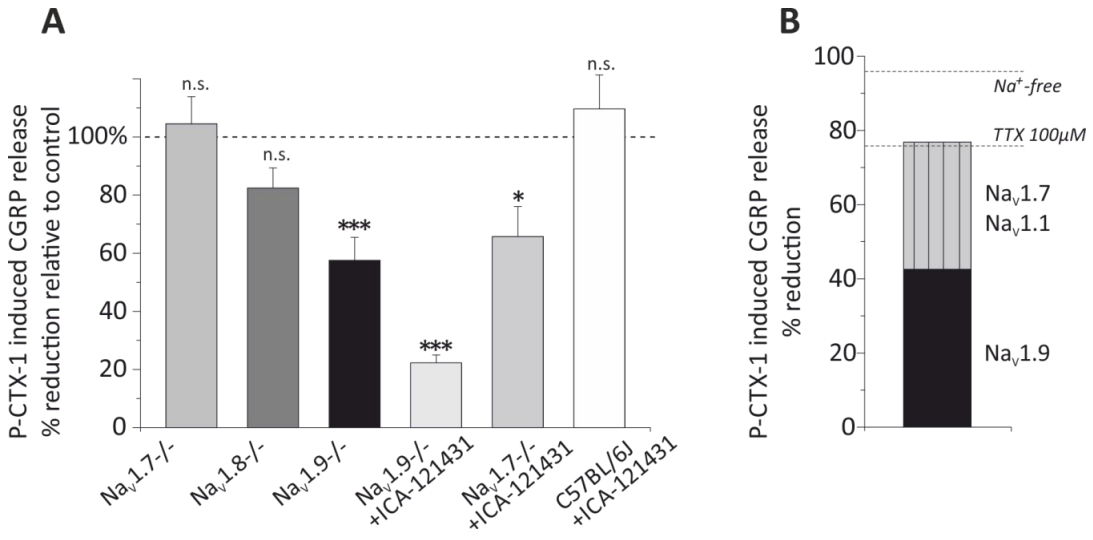

Figure 6. P-CTX-1 induced CGRP release is mediated by Na_V channel subtypes $Na_V1.9$, $Na_V1.7$ and $Na_V1.1$. The columns represent P-CTX-1 induced immunoreactive (i)CGRP release (in pg/ml), as measured in P-CTX-1 incubation step of the experiment. The values were baseline subtracted and error bars represent mean ± SEM. Statistical comparisons were performed with the *t*-test. Significance is indicated with * for P< 0.05, ** for P < 0.01 and *** for P < 0.001; n.s. relates to no significant difference. **(A)** Columns show % reduction of CGRP-release from transgenic mice compared to the respective littermates or C57BL/6J control group. The sample sizes were: $Na_V1.8$ -/-: n = 8, $Na_V1.7$-/-: n = 6, $Na_V1.9$-/-: n = 8, $Na_V1.9$-/- + ICA-121431 ($Na_V1.1$ blocker): n = 5, $Na_V1.7$-/- + ICA-121431: n = 4, C57BL/6J + ICA-121431: n = 4. Significant reduction of P-CTX-1induced CGRP release occurred in $Na_V1.9$-/-and amounted to 42% and 1 µM ICA-121431 further increased the block to 78%. In $Na_V1.7$-/- 1 µM ICA-121431 reduced CGRP release by 34% (P = 0.0002 and P = 0.03, respectively, indicated by asterisks). **(B)** Schematic calculation of the major contributors to the 1 nM P-CTX-1-induced CGRP release. Dashed lines illustrate the reducing effect of sodium deprivation and the TTX-blocked fraction. The sodium channel contribution is composed of $Na_V1.9$ (42%) and $Na_V1.7$ + $Na_V1.1$ (34%). Note that both may contain some voltage-gated calcium channels effect and also other Na_V channel subtypes might contribute with minor fraction. *Figure adapted from (Touska et al., 2017).*

Crotalphine is partial TRPA1 agonist with analgesic effects

Crotalphine is a 14-amino acid structural homologue of an native analgesic venom isolated from the South American rattlesnake *Crotalus durissus terrificus*. Although analgesic effects of this peptide are well established, its direct mechanism of action remained unresolved. The aim of our study (Bressan et al., 2016) was to investigate the effect of crotalphine on ion channels in peripheral pain pathways. Using whole-cell patch-clamp electrophysiology, we demonstrated that crotalphine (1 µM) induced small inward currents in the presence of extracellular calcium mediated by TRPA1 in DRG neurons, thus confirming the activating effects previously seen in calcium imaging experiments (Bressan et al., 2016). The currents induced by 2 subsequent applications of crotalphine were 0.4 ± 0.1 nA and 0.7 ± 0.2 nA, respectively. The average maximal response induced by carvacrol was 6.4 ± 0.2 nA (n = 4). Paradoxically, low concentrations of crotalphine (0.01 µM) produced higher peak currents than a higher subsequent concentration (10 µM), suggesting a bimodal effect on TRPA1. Crotalphine-induced currents were smaller in the presence of extracellular calcium (1.25 mM) than in calcium-free solution. Importantly, crotalphine did not produce any significant effects in DRG neurons from TRPA1-/- mice.

Camphor inhibits M-channel-mediated potassium current in native nociceptors

Camphor (2 mM) sensitized 25-30% of mechano-sensitive C-fibers to cold and also induced novel responsiveness to cold in some of the thermoinsensitive fibers (Vetter et al., 2013). In DRG neurons, camphor blocked 48-53% of potassium current at positive potentials. We further evaluated

the subtype specificity of potassium channels and found that the most blocking effect exerted camphor on $K_V7.2$ channels with half-maximal block of 500 µM. The M-channel, which is formed by $K_V7.2/K_V7.3$ heteromultimers, was less sensitive with 1.42 mM camphor of half-maximal blocking effect. Camphor also induced pronounced and reversible decrease in rheobase current by 31% in current-clamp patch clamp experiments which indicates increased excitability. Camphor also partially activated and subsequenty desensitized heterologously transfected TRPM8 channels in HEK293 cells (Vetter et al., 2013).

Pore helix domain is critical to camphor sensitivity of TRPV1 receptor

TRPV1-mediated currents through the T633A mutant were smaller than in WT which reached only 6.1% ± 3.2 % of the maximal response to 10 µM capsaicin when measured at -70 mV (n=7) (Marsakova et al., 2012). TRPV1 chimera (Δ15:Y627-C634) was completely insensitive to camphor. In this chimera, the pore helix (Y627-C634) was replaced with the counterpart form TRPV2, a camphor-insensitive homolog. Camphor also induced changes in spatial distribution of PIP_2 on the inner leaflet of the plasma membrane. Altogether, these data suggest that camphor modualtes TRPV1 by affecting its overall gating equilibrium (Marsakova et al., 2012).

Thermal gradient

We evaluated temperature preference behavior within the innocuous temperature range of TRPM8-/-, TRPA1-/- and TRPM8/A1-/- mice. First, we compared if the slope of thermal gradient and surface area have influence on thermal selection of male C57BL/6J mice (n = 14-20, a background strain of all used knockouts). Temperature gradient of 25°C in innocuous temperature range between 15°C to 40°C in both small **(Fig. 7B)** and large **(Fig 7A)** assembly was established. That results in 3.57°C and 2.27°C between virtual adjacent fields in small and large assembly, respectively. There was no difference in exploratory behavior or preferred temperature location at the end of the observation time after 60 minutes. The mice located at 32.9° ± 2.4°C in the small assembly and 33.3° ± 3.1 °C in the large assembly (Touska et al., 2016).

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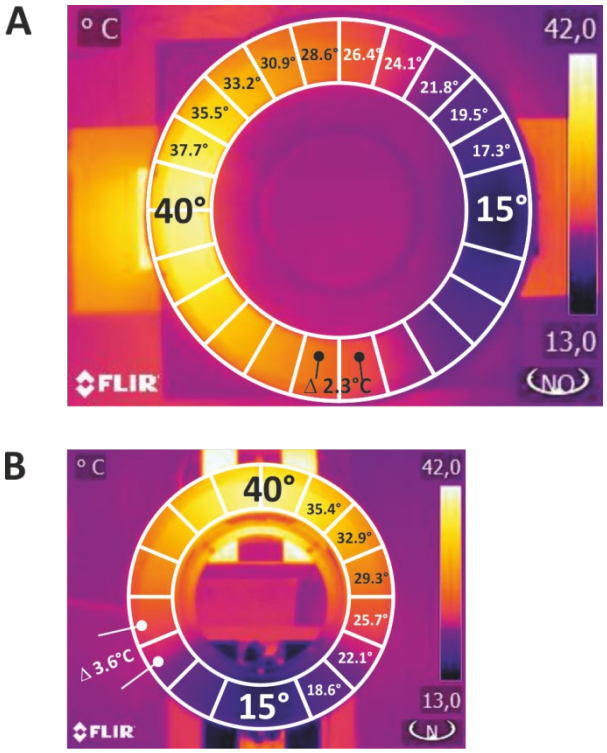

Figure 7. Circular gradient temperature preference assay depicted as infrared photograph with overlaid schematic zone illustrations. **(A)** Larger 12 – zone assembly, **(B)** Smaller 8 – zone assembly. Inside the circular disc, the mice move freely and are enclosed by plexiglass walls and lid with zones indicated. The assay is heated and cooled on opposite sides creating a symmetric temperature gradient. For data analysis each zones of even temperature on opposite sides are summarized to yield 8 or 12 zone histograms. Each zone has the same size. The temperature gradient for larger assay (A) is 2.27°C per zone (0.31°C/cm) or (B) 3.57°C (0.47°C/cm). *Figure adapted from (Touska et al., 2016).*

The major cold-sensitive transduction channel is generally accepted to be TRPM8 (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). In the next step, we compared TRPM8-/- mice with WT **(Fig. 8A-C)**. The preferred temperature where the mice settled in the last 15 minutes of 1 hour screening time was reduced from 32.9° ± 2.4°C to 29.7° ± 3.5°C. In the 12 – zone assay, there was interestingly no significant difference between TRPM8-/- and WT mice. TRPM8-/- mice also showed marked avoidance of warmer zones especially during the first 45 minutes of 1 hour test trial **(Fig. 8)**.

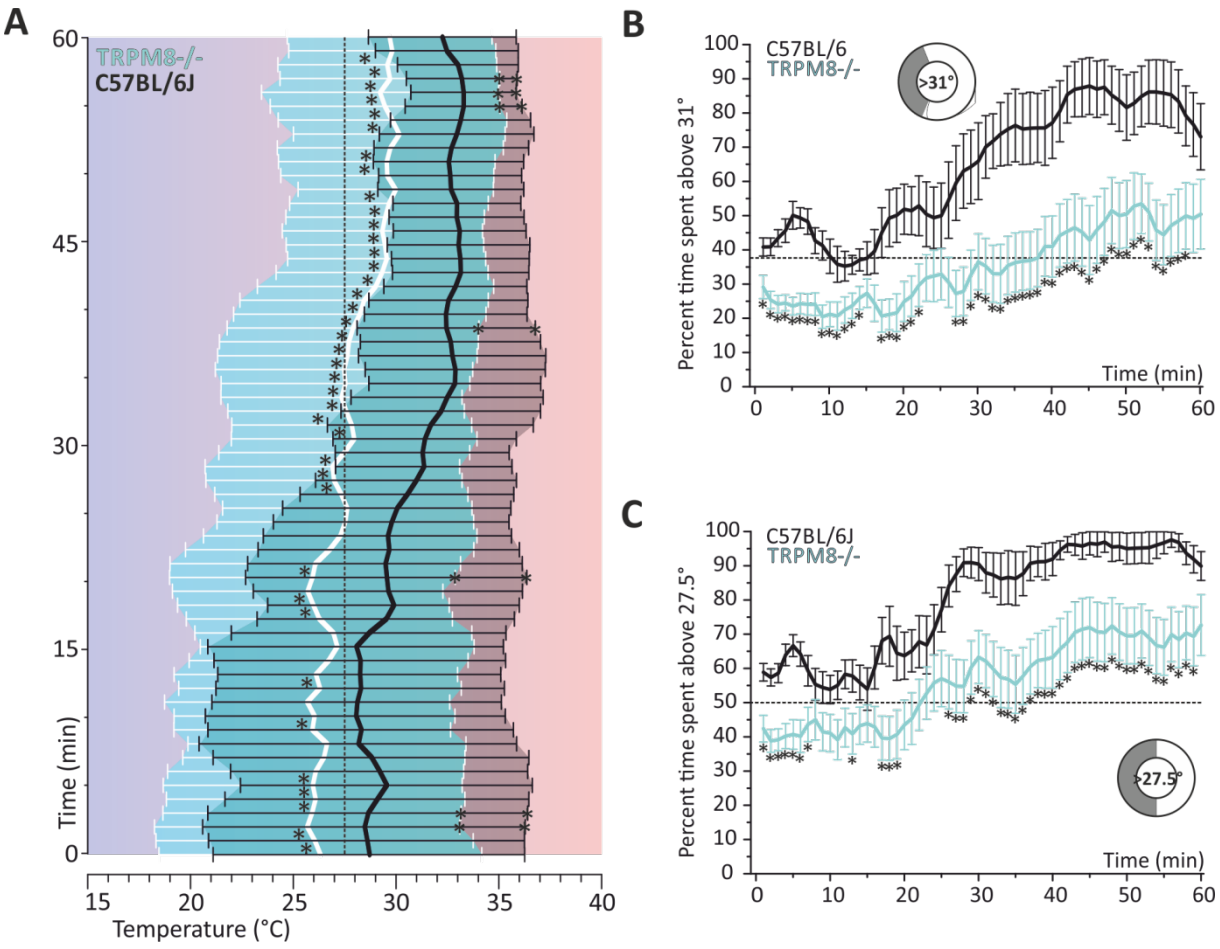

Figure 8. TRPM8-/- and WT mice in 8 – zone assay show marked warm avoidance and reduced avoidance of innocuous cool. **(A)** Preference temperature time course throughout 60 mins. Temperature preference behavior of TRPM8-/- (n = 21) is markedly shifted to cooler temperatures compared to control mice (n = 14). Data were subjected to a 3-point averaging procedure. Error bars represent SD for TRPM-/- (cyan), and control (black). Asterisks indicate significant difference ($P < 0.05$, *t*-test). **(B,C)** Time courses of temperature selection. **(B)** Percent time spent above 31°C. **(C)** Percent time spent above 27.5%. Error bars represent SEM. *Figure adapted from (Touska et al., 2016).*

We further analyzed TRPA1-/- and TRPM8/A1/-/- in the 12 – zone assay with a gradient from 15° to 40°C. The TRPA1-deficient mice did not differ from WT in the final preferred temperature reached (33.1 ± 2.5°C; P = 0.9, ANOVA). Interestingly, TRPA1-/- mice were markedly faster in recognizing warmer zones as preferable. They needed less time, 14 minutes, to spent more than 50% of all time at temperatures above 32°C **(Fig. 9B)**, compared to 28 min for WT and 30 min for TRPM8-/-. Additional lack of TRPM8 (TRPM8/A1 double knockouts) further decreased cold avoidance markedly and the mice settled with the weighted average at 29.8 ± 3.6°C, 3.5°C colder temperatures than in WT (P = 0.0003, ANOVA) and 2.7°C lower than in TRPM8-/- (P = 0.005, ANOVA) **(Fig 9A)**. The time course of thermal selection also showed that double knockouts locate to the zone above 32°C only randomly (33% likelihood throughout the experiment) and locate for at least 50% of the time above 27.5°C after 12 min, similar to the TRPM8-/- **(Fig 9C)**, but never spent more than 75% of their time above 27.5°C **(Fig. 9C)**. Similar results were obtained in the large ring assay with the temperature range from 5° to 30°C with shallow (0.3°C/cm) gradient. The difference between double knockout and WT was 3.5°C and 2.7°C, respectively (Winter et al., 2017).

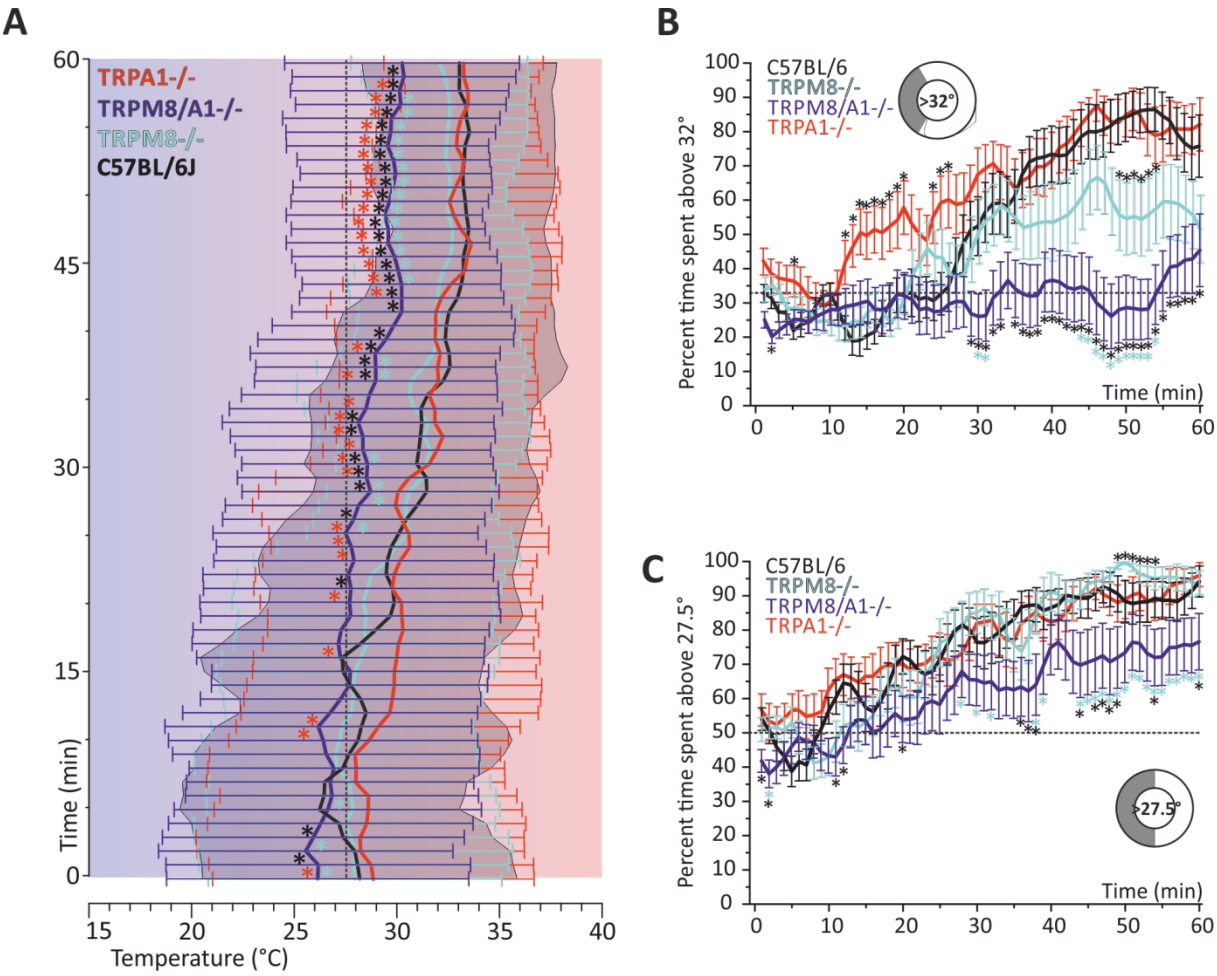

Figure 9. (A) Temperature preference time course (60 min) of TRPM8-/- (cyan), TRPA1-/- (red), TRPM8/A1-/- (blue) and control C57BL/6J (black) in 12 – zone assay. In the second half hour the double knockout separates from other 3 phenotypes, the mice prefer significantly colder temperatures (about 3°C). Error bars represent SD, data were subjected to a 3-pt averaging procedure; gray area: SD of control mice; all asterisks compare TRPM8/A1-/- to respective other strain indicated by color. **(B,C)** Percent time spent in the warmer semicircle above **(B)** 32°C and **(C)** above 27.5°C. Values are calculated in 1min resolution (averaging from 60 values per min) and subjected to a 3pt averaging procedure. Error bars represent SEM. Figure adapted from *(Touska et al., 2016).*

Discussion

Heat-resistant action potentials in primary afferent sensory neurons

In contrast to neurons of the CNS where optimal temperature around 36°C-38°C is maintained, PNS has to deal with changes over a wide temperature range. This feature underlies different set of VGSC subtypes in both systems. Whereas CNS expresses predominantly $Na_V1.1$, $Na_V1.2$ and $Na_V1.6$, PNS relies on TTXr channel subtypes $Na_V1.8$, $Na_V1.9$ and TTXs subtype $Na_V1.7$. It has been generally accepted until now, that slow activation and inactivation characteristics of $Na_V1.9$ do not contribute to the rising phase of the action potential (Cummins et al., 1999; Dib-Hajj et al., 2015). We found $Na_V1.9$ current to be substantially potentiated at rising temperatures and also supporting repetitive firing increasingly with rising temperature. 4-fold increase in peak inward current and conductance and 6-fold increase in the slope of the action potential emphasizes an active role in action potential generation in polymodal nociceptive afferents. We showed for the first time that $Na_V1.9$ can generate action potential on its own with current-clamp patch-clamp

experiments. Deficits in heat withdrawal assay measured with the Hargreave's apparatus demonstrate important roles for both TTXr subtypes $Na_V1.8$ and $Na_V1.9$ over different temperatures.

Ciguatoxins and cold allodynia

Ciguatera is a serious disease caused by ciguatoxins. It affects thousands of people worldwide annually causing serious neurological symptoms including pain and distorted temperature perception. P-CTX-1 has been characterized as one of the most potent VGSC channel activator toxins also blocking potassium channels and thus generally stimulating excitability and action potential firing in primary afferent neurons. Besides these general effects on neuronal excitability, our understanding of the physiological and pathophysiological mechanisms and particular molecular targets in most prominent symptom of ciguatera, cold allodynia, has been poorly elucidated.

We showed an involvement of TRPA1-containing nociceptive pathways in ciguatoxin-induced allodynia. Although TRPA1 is not directly activated by P-CTX-1, it promotes novel cold sensitivity in cells previously not cold sensitive, an effect not present in TRPA1-deficient mice. TRPA1 is potentiated by Ca^{2+} ions and a variety of second messengers, which could be the way of indirect action of P-CTX-1 on this ion channel. Behavioral experiments showed the involvement of TRPA1, but not the major cold sensor, TRPM8. Our study thus provides evidence of TRPA1-expressing neurons to play significant part in physiological and pathological cold sensing which has been further confirmed by BOLD (Blood Oxygenation Level Dependent) signal using fMRI (functional magnetic resonance imaging) with deficits in TRPA1-/- mice compared to WT.

The other group of polymodal fibers involved was devoid of TRPA1 and was characterized by action potential activity potentiated by cooling and inhibited by warming in single-fiber and current-clamp patch clamp recordings. This effect was exclusively VGSC channel – mediated and inhibited by TTX. P-CTX-1 elicits peripheral activation in C-fibers as well as *de-novo* sensitization and activation in A-fibers and provides a profound insight in the mechanism of cold transduction and processing.

We showed next that crotalphine activates TRPA1 channels in heterologously transfected HEK293T cells and DRG neurons in a concentration and a time-dependent manner and the activation is followed by Ca^{2+}-dependent desensitization. Nevertheless, crotalphine does not produce any pain-related symptoms, but analgesic effects, which are consistent with the fact that the current amplitude elicited by crotalphine is relatively small, compared to full agonists like carvacrol. These results suggest that crotalphine may produce only a partial activation of TRPA1, subsequently followed by desensitization of the channel. Behavioral studies showed profound antihyperalgesic effects after oral application of crotalphine administered to mice in the ciguatoxin, bradykinin and zymosan evoked pain models. These analgesic effects were blocked by TRPA1 blocker HC030031 and were not present in TRPA1-deficient mice. Our findings thus support the conclusion that TRPA1 is one of the most important transduction ion channels in nociceptive pathways detecting not only chemical irritants and inflammatory mediators but also cold.

Ciguatoxins – mediated CGRP release

Ciguatoxins are the most potent drugs in inducing CGRP release identified. After eliminating that CGRP release is mediated by TRP channels with experiments on respective knockout animals, the focus aims at the primary ciguatoxin target, VGSC channels. We have shown that P-CTX-1 largely affects TRPA1-positive DRG neurons (Vetter et al., 2012), which are largely coexpressed with CGRP and $Na_V1.8$. However, high concentrations of TTX (100 μM) caused a 74% reduction of CGRP release and a complete lack of Na^+ ions in the bath solution resulted in 96% CGRP inhibition. CGRP release relies on Ca^{2+} - dependent exocytosis and, from our results, the activation of combination of TTXr and TTXs Na_V channels $Na_V1.9$, $Na_V1.7$ and $Na_V1.1$ and subsequent activation of voltage-gated calcium channels is sufficient for the most CGRP releasing effect of P-CTX-1.

Crotalphine partially activates and desensitizes TRPA1

We showed next that crotalphine activates TRPA1 channels in heterologously transfected HEK293T cells and DRG neurons in a concentration and a time-dependent manner. The activation is followed by Ca^{2+}-dependent desensitization. Although crotalphine activates nociceptive pathways, it does not produce any pain-related symptoms, but analgesic effect, which is consistent with the fact that the current amplitude elicited by crotalphine is relatively small, compared to full agonist carvacrol. These results suggest that crotalphine may produce only a partial activation of TRPA1, subsequently followed by desensitization of the channel. Behavioral studies showed profound antihyperalgesic effects after oral application of crotalphine administered to mice in the ciguatoxin, bradykinin and zymosan evoked pain models. These analgesic effects were blocked by TRPA1 blocker HC030031 and were not present in TRPA1-deficient mice. Here we show that partial TRPA1 activation leads to initiation of signaling cascades that interacts with opioid pathways to elicit analgesic effects.

Camphor in nociceptive pathways

Our data indicate that camphor binds to the outer pore domain of the TRPV1 receptor, particularly involving the residue T633, a residue located in the middle of the pore helix that is also critical for direct activation of TRPV1 by protons (Ryu et al., 2007). Replacing this residue with alanine reduced the current amplitude leaving the capsaicin response intact (capsaicin binds to the S2-S4 linker). We replaced the N-terminal portion of the pore helix (Y627-C634) with its counterpart from TRPV2. The resulting chimera was completely insensitive to camphor.

Camphor has promiscuous effects on various in channels on nociceptors. One of the effects it exerts is the sensitization of terminal nerve endings, measured on the mice skin preparation, to cold. We identified potassium current that was blocked by camphor through K_V7 channels (M-current). The amplification of cold response by potassium current inhibition has been described and directly affects the excitability (Madrid et al., 2009; Noel et al., 2009). Although this effect itself is not enough to trigger the action potential firing, it enhances the excitability in combination with other effects, for example the partial activation of TRPM8.

Crotalphine and camphor are partial agonists of two nociceptive TRP channels with desensitizing properties. Nevertheless, the mechanisms of action are completely different. Camphor likely binds directly to the outer pore domain of TRPV1 which activates TRPV1 followed by strong desensitization and thus prevention of further channel opening, on the other hand enhances cold transduction by inhibiting potassium current through M-channel (Kv7). In contrast, crotalphine application leads to activation of TRPA1 which activates intracellular pathways leading to analgesic actions through opioid receptors. Our results highlight the importance of natural substances in the science and development of potential therapeutic strategies.

Thermal preference

Temperature-based assays for mouse models are widely used and necessary to assess the contribution of ion channels and molecules involved in temperature perception and thermoregulation and can also be used for various drug screening purposes. We speculate that the key aspect of why we saw a significant difference in the small (8 – zone) assay between TRPM8-/- and WT and not in the large 12 – zone assay is the steeper temperature gradient. That indicates that TRPM8 primarily detects the temperature difference of adjacent places. CC-fibers (cold sensitive C-fibers) detect differential cold sensitivity with the primary cold sensor being TRPM8, which is missing in the knockout animals (Toro et al., 2015). The lack of input from these fibers may also reflect a distort input from warm and cold fibers, which might be better compensated from other temperature transducer with the less steep gradient.

Our experiments revealed a substantial contribution of TRPA1 to innocuous cold perception but only in combination with knockout of TRPM8 in TRPM8/A1-/- animals with reduced cold aversive behavior compared to TRPM8-/- and WT. TRPM8 has been described as a major detector of

innocuous cold a probably functional TRPM8 alone is sufficient to compensate for TRPA1. TRPA1 might amplify TRPM8-mediated signal or alone function only in more extreme temperatures. This result is consistent with our previous finding of a reduction in BOLD signal in an fMRI screening of TRPA1-/- mice in response to stimulation of the paw with temperatures around 15°C (Vetter et al., 2012).

Conclusions

- $Na_V1.9$ undergoes gain-of-function measured by voltage-clamp and current-clamp electrophysiology at noxiously high temperatures and is capable of generating action potentials *per se* with an increased frequency upon warming.
- We showed that VGSC subtypes $Na_V1.8$ and $Na_V1.9$ are essential for conduction of action potentials in C-fiber nociceptors at high temperatures.
- Ciguatoxin induced cold allodynia partly relies on TRPA1 positive nerve fibers and DRG neurons.
- Ciguatoxin caused cold allodynia in A-fibers and in a specific subgroup of DRG neurons, it was TRPA1 independent and the effect was mediated by VGSC channels rendering neurons to fire action potentials in cool/cold temperatures which ceased after warming.
- Ciguatoxin caused prominent CGRP release via $Na_V1.9$ and the combination of $Na_V1.1$ and $Na_V1.7$.
- Crotalphine exerts analgesic effects by partial selective activation and subsequent desensitization of TRPA1.
- Camphor sensitizes a subpopulation of menthol-sensitive cutaneous nociceptors in the mouse to cold by reducing the outward potassium current mainly through K_V7 (M-current).
- We provided novel insights into the structural basis of how camphor modulates TRPV1 by affecting its overall gating equilibrium by altering the short helical segment within the permeation pore as well as the spatial distribution of lipids on the inner membrane leaflet.
- We designed and developed a novel automated, circular gradient assay for assessment of mice temperature preference behavior. TRPM8-/- mice showed avoidance of warm compared to WT but only in assay with a steeper temperature gradient (0.47°C/cm) but not with a less steeper gradient (0.31°C/cm). TRPM8/A1/-/- mice showed even greater warm avoidance compared to TRPM8-/- and control (C57BL/6J), showing contribution of previously disputed TRPA1 ion channel to cold perception.

Publications

List of publications related to the PhD Thesis:

Touska F, Turnquist B, Vlachova V, Reeh PW, Leffler A, Zimmermann K
Heat-resistant action potentials require TTX-resistant sodium channels $Na_V1.8$ and $Na_V1.9$
Journal of General Physiology, 150(8):1125-1144, 2018, **IF(2018) 4.258**
Author contribution: Designed and performed patch-clamp experiments, performed behavioral experiments, analyzed the data, participated in designing the figures and writing the manuscript.

Vetter I, **Touska F**, Hess A, Hinsbey R, Sattler S, Lampert A, Sergejeva M, Namer B, Sharov A, Eberhardt M, Engel M, Cabot PJ, Wood JN, Vlachova V, Reeh PW, Lewis RJ, Zimmermann K
Ciguatoxins activate specific cold pain pathways to cause burning pain from cooling
EMBO Journal, 31:3795-808, 2013, **IF(2018) 11.227** (Times cited: 64)
Author contribution: Performed patch-clamp experiments, performed calcium imaging experiments, analyzed the data.

Touska F*, Sattler S*, Malsch P, Lewis R, Reeh PW, Zimmermann K
Ciguatoxins evoke potent CGRP release by activation of voltage-gated sodium channel subtypes $Na_V1.9$, $Na_V1.7$ and $Na_V1.1$
Marine Drugs, 15(9) pii: E269, 2017, **IF(2018) 3.772** (Times cited: 3)
Author contribution: Participated in performing CGRP release experiments, analyzing the data and designing the figures
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Bressan E, **Touska F**, Vetter I, Kistner K, Kichko TI, Teixeira NB, Picolo G, Cury Y, Lewis RJ, Fischer MJ, Zimmermann K, Reeh PW
Crotalphine desensitizes TRPA1 ion channels to alleviate inflammatory hyperalgesia
Pain, 157(11):2504-2516, 2016, **IF(2018) 6.029** (Times cited: 11)
Author contribution: Participated in designing and performing patch-clamp experiments and analyzing the data.

Vetter I, Hein A, Sattler S, Hessler S, **Touska F**, Bressan E, Parra A, Hager U, Leffler A, Boukalova S, Nissen M, Lewis RJ, Belmonte C, Alzheimer C, Huth T, Vlachova V, Reeh PW, Zimmermann K
Amplified cold transduction in native nociceptors by M-channel inhibition
Journal of Neuroscience, 33:16627-41, 2013, **IF(2018) 6.074** (Times cited: 19)
Author contribution: Designed and performed the current-clamp experiments, analyzed the data

Marsakova L, **Touska F**, Krusek J, Vlachova V
Pore helix domain is critical to camphor sensitivity of TRPV1
Anesthesiology, 116(4):903-17, 2012, **IF(2018) 6.424** (Times cited: 11)
Author contribution: Participated in performing experiments and analyzing the data.

Touska F*, Winter Z*, Mueller A, Vlachova V, Larsen J, Zimmermann K
Comprehensive thermal preference phenotyping in mice using a novel automated circular gradient assay
Temperature (Austin), 2;3(1):77-91, 2016, **Indexed in Pubmed Central**
Author contribution: Participated in performing experiments, analyzing the data and writing the manuscript
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Winter Z, Gruschwitz P, Eger S, **Touska F**, Zimmermann K:
Cold Temperature Encoding by Cutaneous TRPA1 and TRPM8-Carrying Fibers in the Mouse
Frontiers in Molecular Neuroscience, 10:209, 2017, **IF(2018) 3.720** (Times cited: 7)
Author contribution: Participated in analyzing the data, calculated statistics, participated in designing the figures and writing the manuscript.

Boukalova S, **Touska F**, Marsakova L, Hynkova A, Sura L, Chvojka S, Dittert I, Vlachova
Gain-of-function mutations in the transient receptor potential channels TRPV1 and TRPA1: how painful?
Physiological Research, 63 Suppl 1:S205-13 (review), 2014, **IF(2018) 1.701** (Times cited: 11)
Author contribution: Participated in writing the manuscript.

List of publications unrelated to the PhD thesis:

Petzold J, Aigner TB, **Touska F**, Zimmermann K, Scheibel T, Engel FB:
Surface Features of Recombinant Spider Silk Protein eADF4(kappa 16)-Made Materials are Well-Suited for Cardiac Tissue Engineering.
Advanced Functional Materials, 36 (27), 2017, **IF(2018) 15.621** (Times cited: 9)

Touska F, Marsakova L, Teisinger J, Vlachova V
A "cute" desensitization of TRPV1,
Current Pharmaceutical Biotechnology, 12(1):122-9 (review), 2011, **IF(2018) 1.516** (Times cited: 41)

Vyklický L, Nováková-Tousová K, Benedikt J, Samad A, **Touska F**, Vlachová V
Calcium – Dependent Desensitization of Vanilloid Receptor TRPV1: A Mechanism Possibly Involved in Analgesia Induced by Topical Application of Capsaicin
Physiological Research, 57 Suppl 3:S59-68, (review), 2011, **IF(2018) 1.701** (Times cited: 59)

References

Ahern, C.A., J. Payandeh, F. Bosmans, and B. Chanda. 2016. The hitchhiker's guide to the voltage-gated sodium channel galaxy. *The Journal of general physiology*. 147:1-24.

Basbaum, A.I., D.M. Bautista, G. Scherrer, and D. Julius. 2009. Cellular and molecular mechanisms of pain. *Cell*. 139:267-284.

Bautista, D.M., J. Siemens, J.M. Glazer, P.R. Tsuruda, A.I. Basbaum, C.L. Stucky, S.E. Jordt, and D. Julius. 2007. The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature*. 448:204-208.

Bennett, D.L., A.J. Clark, J. Huang, S.G. Waxman, and S.D. Dib-Hajj. 2019. The Role of Voltage-Gated Sodium Channels in Pain Signaling. *Physiological reviews*. 99:1079-1151.

Bressan, E., F. Touska, I. Vetter, K. Kistner, T.I. Kichko, N.B. Teixeira, G. Picolo, Y. Cury, R.J. Lewis, M.J. Fischer, K. Zimmermann, and P.W. Reeh. 2016. Crotalphine desensitizes TRPA1 ion channels to alleviate inflammatory hyperalgesia. *Pain*. 157:2504-2516.

Colburn, R.W., M.L. Lubin, D.J. Stone, Jr., Y. Wang, D. Lawrence, M.R. D'Andrea, M.R. Brandt, Y. Liu, C.M. Flores, and N. Qin. 2007. Attenuated cold sensitivity in TRPM8 null mice. *Neuron*. 54:379-386.

Cox, J.J., F. Reimann, A.K. Nicholas, G. Thornton, E. Roberts, K. Springell, G. Karbani, H. Jafri, J. Mannan, Y. Raashid, L. Al-Gazali, H. Hamamy, E.M. Valente, S. Gorman, R. Williams, D.P. McHale, J.N. Wood, F.M. Gribble, and C.G. Woods. 2006. An SCN9A channelopathy causes congenital inability to experience pain. *Nature*. 444:894-898.

Cummins, T.R., S.D. Dib-Hajj, J.A. Black, A.N. Akopian, J.N. Wood, and S.G. Waxman. 1999. A novel persistent tetrodotoxin-resistant sodium current in SNS-null and wild-type small primary sensory neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 19:RC43.

Dhaka, A., A.N. Murray, J. Mathur, T.J. Earley, M.J. Petrus, and A. Patapoutian. 2007. TRPM8 is required for cold sensation in mice. *Neuron*. 54:371-378.

Dib-Hajj, S.D., J.A. Black, and S.G. Waxman. 2015. NaV1.9: a sodium channel linked to human pain. *Nature reviews. Neuroscience*. 16:511-519.

Dittert, I., J. Benedikt, L. Vyklicky, K. Zimmermann, P.W. Reeh, and V. Vlachova. 2006. Improved superfusion technique for rapid cooling or heating of cultured cells under patch-clamp conditions. *Journal of neuroscience methods*. 151:178-185.

Dubin, A.E., and A. Patapoutian. 2010. Nociceptors: the sensors of the pain pathway. *The Journal of clinical investigation*. 120:3760-3772.

Giorgi, R., M.M. Bernardi, and Y. Cury. 1993. Analgesic effect evoked by low molecular weight substances extracted from Crotalus durissus terrificus venom. *Toxicon : official journal of the International Society on Toxinology*. 31:1257-1265.

Gutierrez, V.P., K. Konno, M. Chacur, S.C. Sampaio, G. Picolo, P. Brigatte, V.O. Zambelli, and Y. Cury. 2008. Crotalphine induces potent antinociception in neuropathic pain by acting at peripheral opioid receptors. *European journal of pharmacology*. 594:84-92.

Karashima, Y., K. Talavera, W. Everaerts, A. Janssens, K.Y. Kwan, R. Vennekens, B. Nilius, and T. Voets. 2009. TRPA1 acts as a cold sensor in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 106:1273-1278.

Knowlton, W.M., R.L. Daniels, R. Palkar, D.D. McCoy, and D.D. McKemy. 2011. Pharmacological Blockade of TRPM8 Ion Channels Alters Cold and Cold Pain Responses in Mice. *PLoS One*. 6:e25894.

Konno, K., G. Picolo, V.P. Gutierrez, P. Brigatte, V.O. Zambelli, A.C. Camargo, and Y. Cury. 2008. Crotalphine, a novel potent analgesic peptide from the venom of the South American rattlesnake Crotalus durissus terrificus. *Peptides*. 29:1293-1304.

Kwan, K.Y., A.J. Allchorne, M.A. Vollrath, A.P. Christensen, D.S. Zhang, C.J. Woolf, and D.P. Corey. 2006. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron*. 50:277-289.

Lewis, R.J. 2001. The changing face of ciguatera. *Toxicon : official journal of the International Society on Toxinology*. 39:97-106.

Lewis, R.J., M. Sellin, M.A. Poli, R.S. Norton, J.K. MacLeod, and M.M. Sheil. 1991. Purification and characterization of ciguatoxins from moray eel (Lycodontis javanicus, Muraenidae). *Toxicon : official journal of the International Society on Toxinology*. 29:1115-1127.

Madrid, R., E. de la Pena, T. Donovan-Rodriguez, C. Belmonte, and F. Viana. 2009. Variable threshold of trigeminal cold-thermosensitive neurons is determined by a balance between TRPM8 and Kv1 potassium channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 29:3120-3131.

Machado, F.C., V.O. Zambelli, A.C. Fernandes, A.S. Heimann, Y. Cury, and G. Picolo. 2014. Peripheral interactions between cannabinoid and opioid systems contribute to the antinociceptive effect of crotalphine. *British journal of pharmacology*. 171:961-972.

Marsakova, L., F. Touska, J. Krusek, and V. Vlachova. 2012. Pore Helix Domain Is Critical to Camphor Sensitivity of Transient Receptor Potential Vanilloid 1 Channel. *Anesthesiology*. 116:903-917.

McKemy, D.D., W.M. Neuhausser, and D. Julius. 2002. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature*. 416:52-58.

Moparthi, L., S. Survery, M. Kreir, C. Simonsen, P. Kjellbom, E.D. Hogestatt, U. Johanson, and P.M. Zygmunt. 2014. Human TRPA1 is intrinsically cold- and chemosensitive with and without its N-terminal ankyrin repeat domain. *Proceedings of the National Academy of Sciences of the United States of America*. 111:16901-16906.

Moqrich, A., S.W. Hwang, T.J. Earley, M.J. Petrus, A.N. Murray, K.S. Spencer, M. Andahazy, G.M. Story, and A. Patapoutian. 2005. Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. *Science*. 307:1468-1472.

Nassar, M.A., L.C. Stirling, G. Forlani, M.D. Baker, E.A. Matthews, A.H. Dickenson, and J.N. Wood. 2004. Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. *Proceedings of the National Academy of Sciences of the United States of America*. 101:12706-12711.

Noel, J., K. Zimmermann, J. Busserolles, E. Deval, A. Alloui, S. Diochot, N. Guy, M. Borsotto, P. Reeh, A. Eschalier, and M. Lazdunski. 2009. The mechano-activated K+ channels TRAAK and TREK-1 control both warm and cold perception. *The EMBO journal*. 28:1308-1318.

Peier, A.M., A. Moqrich, A.C. Hergarden, A.J. Reeve, D.A. Andersson, G.M. Story, T.J. Earley, I. Dragoni, P. McIntyre, S. Bevan, and A. Patapoutian. 2002. A TRP channel that senses cold stimuli and menthol. *Cell*. 108:705-715.

Picolo, G., R. Giorgi, and Y. Cury. 2000. delta-opioid receptors and nitric oxide mediate the analgesic effect of Crotalus durissus terrificus snake venom. *European journal of pharmacology*. 391:55-62.

Ryu, S., B. Liu, J. Yao, Q. Fu, and F. Qin. 2007. Uncoupling proton activation of vanilloid receptor TRPV1. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 27:12797-12807.

Selescu, T., A.C. Ciobanu, C. Dobre, G. Reid, and A. Babes. 2013. Camphor activates and sensitizes transient receptor potential melastatin 8 (TRPM8) to cooling and icilin. *Chemical senses*. 38:563-575.

Shields, S.D., L. Deng, R.M. Reese, M. Dourado, J. Tao, O. Foreman, J.H. Chang, and D.H. Hackos. 2018. Insensitivity to Pain upon Adult-Onset Deletion of Nav1.7 or Its Blockade with Selective Inhibitors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 38:10180-10201.

Shields, S.D., X. Cheng, N. Uceyler, C. Sommer, S.D. Dib-Hajj, and S.G. Waxman. 2012. Sodium channel Na(v)1.7 is essential for lowering heat pain threshold after burn injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 32:10819-10832.

Story, G.M., A.M. Peier, A.J. Reeve, S.R. Eid, J. Mosbacher, T.R. Hricik, T.J. Earley, A.C. Hergarden, D.A. Andersson, S.W. Hwang, P. McIntyre, T. Jegla, S. Bevan, and A. Patapoutian. 2003. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*. 112:819-829.

Strachan, L.C., R.J. Lewis, and G.M. Nicholson. 1999. Differential actions of pacific ciguatoxin-1 on sodium channel subtypes in mammalian sensory neurons. *J Pharmacol Exp Ther*. 288:379-388.

Toro, C.A., S. Eger, L. Veliz, P. Sotelo-Hitschfeld, D. Cabezas, M.A. Castro, K. Zimmermann, and S. Brauchi. 2015. Agonist-dependent modulation of cell surface expression of the cold receptor TRPM8. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 35:571-582.

Touska, F., S. Sattler, P. Malsch, R.J. Lewis, P.W. Reeh, and K. Zimmermann. 2017. Ciguatoxins Evoke Potent CGRP Release by Activation of Voltage-Gated Sodium Channel Subtypes NaV1.9, NaV1.7 and NaV1.1. *Marine drugs*. 15.

Touska, F., B. Turnquist, V. Vlachova, P.W. Reeh, A. Leffler, and K. Zimmermann. 2018. Heat-resistant action potentials require TTX-resistant sodium channels NaV1.8 and NaV1.9. *The Journal of general physiology*. 150:1125-1144.

Touska, F., Z. Winter, A. Mueller, V. Vlachova, J. Larsen, and K. Zimmermann. 2016. Comprehensive thermal preference phenotyping in mice using a novel automated circular gradient assay. *Temperature*. 3:77-91.

Vetter, I., A. Hein, S. Sattler, S. Hessler, F. Touska, E. Bressan, A. Parra, U. Hager, A. Leffler, S. Boukalova, M. Nissen, R.J. Lewis, C. Belmonte, C. Alzheimer, T. Huth, V. Vlachova, P.W. Reeh, and K. Zimmermann. 2013. Amplified cold transduction in native nociceptors by M-channel inhibition. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 33:16627-16641.

Vetter, I., F. Touska, A. Hess, R. Hinsbey, S. Sattler, A. Lampert, M. Sergejeva, A. Sharov, L.S. Collins, M. Eberhardt, M. Engel, P.J. Cabot, J.N. Wood, V. Vlachova, P.W. Reeh, R.J. Lewis, and K. Zimmermann. 2012. Ciguatoxins activate specific cold pain pathways to elicit burning pain from cooling. *The EMBO journal*. 31:3795-3808.

Vogt-Eisele, A.K., K. Weber, M.A. Sherkheli, G. Vielhaber, J. Panten, G. Gisselmann, and H. Hatt. 2007. Monoterpenoid agonists of TRPV3. *British journal of pharmacology*. 151:530-540.

Volgushev, M., T.R. Vidyasagar, M. Chistiakova, T. Yousef, and U.T. Eysel. 2000. Membrane properties and spike generation in rat visual cortical cells during reversible cooling. *The Journal of physiology*. 522 Pt 1:59-76.

Winter, Z., P. Gruschwitz, S. Eger, F. Touska, and K. Zimmermann. 2017. Cold Temperature Encoding by Cutaneous TRPA1 and TRPM8-Carrying Fibers in the Mouse. *Frontiers in molecular neuroscience*. 10:209.

Xu, H., N.T. Blair, and D.E. Clapham. 2005. Camphor activates and strongly desensitizes the transient receptor potential vanilloid subtype 1 channel in a vanilloid-independent mechanism. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 25:8924-8937.

Zimmermann, K., A. Leffler, A. Babes, C.M. Cendan, R.W. Carr, J. Kobayashi, C. Nau, J.N. Wood, and P.W. Reeh. 2007. Sensory neuron sodium channel Nav1.8 is essential for pain at low temperatures. *Nature*. 447:855-858.

Curriculum Vitae

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