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

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Statement of authorship:

I hereby declare, that I have written this thesis independently. I have cited all the used information sources. This thesis serves only and exclusively for my PhD graduation at the Faculty of Science of the Charles University in Prague. Any part of this thesis was not used in support of an application for any other academical degree.

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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/-/-.

Keywords

nociception, pain, Na_V channel, TRP channel, $Na_V1.9$, $Na_V1.8$, TRPM8, TRPA1, TRPV1, ciguatoxin, crotalphine, camphor, temperature preference behavior, temperature gradient

Klíčová slova

nocicepce, bolest, Na_V kanál, TRP kanál, $Na_V1.9$, $Na_V1.8$, TRPM8, TRPA1,TRPV1, ciguatoxin, crotalphine, kafr, teplotní preference, teplotní gradient

Abbreviations

Aδ-fibers	Group A delta nerve fibers
BOLD	Blood Oxygenation Level Dependent
C-fibers	Group C nerve fibers
CGRP	Calcitonine Gene-Related Peptide
CTX	Ciguatoxin
DRG	Dorsal Root Ganglion
DTR	Diphtheria Toxin Receptor
fMRI	functional Magnetic Resonance Imaging
Na_V	Voltage-Gated Sodium Channel
P-CTX-1	Pacific-Ciguatoxin Subtype-1
Scn11a	Gene encoding $Na_V1.9$ protein
TRP	Transient Receptor Potential ion channel family
TRPA1	Transient Receptor Potential Ankyrin subfamily member 1
TRPC5	Transient Receptor Potential Canonical subfamily member 5
TRPM8	Transient Receptor Potential Melastatin subfamily member 8
TRPV1	Transient Receptor Potential Vanilloid subfamily member 1
TRPV3	Transient Receptor Potential Vanilloid subfamily member 3
TTX	Tetrodotoxin
TTXr	Tetrodotoxin resistent
TTXs	Tetrodotoxin sensitive
VGSC	Voltage-Gated Sodium Channel
WT	Wild Type

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 (Woolf and Ma, 2007; Basbaum et al., 2009; Dubin and Patapoutian, 2010). Nociception, and the perception of pain, is normally elicited only by noxious stimuli i.e. by stimuli extreme enough to reach the threshold of nociceptors which further conduct this information to higher brain centers where it is perceived as pain. 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. How crucial the nociceptors are for the integrity of an organism show the rising number of identified channelopathies with either loss of function or gain of function mutations. They lead either to loss-of-pain sensation or exacerbated pain perception. Both conditions have an immense impact on the quality of life (Cox et al., 2006; Kremeyer et al., 2010; Zhang et al., 2013; Boukalova et al., 2014; Leipold et al., 2015; Bennett et al., 2019).

Nerve fibers leading from the periphery to the central nervous system have unique pseudounipolar architecture (Woolf and Ma, 2007). The periphery is connected with the dorsal horn spinal cord with one single axon process which occludes the cell body and directly synapses with second order neurons of dorsal horn spinal cord. This has the advantage that the signal coming from the periphery is not biased or modulated by the spatially and morphologically different cell body (Woolf and Ma, 2007).

Classification of fibers

C-fibers and Aδ- fibers are responsible for detection of nociceptive input. Fibers are classified based on their specificity for stimulus type (mechanical, hot, cold) and conduction velocity

(Zimmermann et al., 2009; Dubin and Patapoutian, 2010; Winter et al., 2017). C-fibers are the slowest conducting neurons with the conduction velocity around 0.5 - 1 .5m/s and encode long lasting, undefined pain. In contrast Aδ- fibers are lightly myelinated with conduction velocities around 1.6-12 m/s. Aδ- fibers are responsible for relaying fast pain like pinprick or acute heat stimulus. C-fibers are small-diameter and conduct poorly localized, slow pain. Both classes of nociceptors are represented by multiple groups based on their further properties (Zimmermann et al., 2009; Dubin and Patapoutian, 2010; Winter et al., 2017). One important group consist of fibers which undergo sensitization from silent mode to active mode. During normal physiological conditions, the fiber is non-responsive and becomes active first after sensitization with certain mediators (Schmidt et al., 1995). The exact role of Aδ- and C-fibers in temperature perception is still a matter of debate. Both, Aδ-fibers and C-fibers are involved in detecting noxious and innocuous temperature (Milenkovic et al., 2014; Bokiniec et al., 2018).

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 and play essential part in electrical excitability and conduct the information from the periphery to the central nervous system. The α-subunits of these ion channels gate rapidly (within ms) and create a selective, permeable transmembrane pathway for sodium ions.

There are 9 structurally related pore-forming α-subunits in mammalian nervous system ($Na_V1.1$-$Na_V1.9$) (Ahern et al., 2016; Bennett et al., 2019). These are functionally divided into tetrodotoxin sensitive (TTXs) $Na_V1.1$, $Na_V1.2$, $Na_V1.3$, $Na_V1.4$, $Na_V1.6$, $Na_V1.7$) and TTX-resistant ($Na_V1.8$, $Na_V1.9$) subtypes. TTXs are inhibited by nanomolar concentrations of TTX and TTXr by millimolar concentrations of TTX, with one exception, $Na_V1.5$, the cardiac sodium channel subunit, which is inhibited by intermediate TTX concentrations. The nervous system is characterized by different expression of Na_V channel subtypes. 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.

The peripheral nervous system is subjected to a modulation of ion channels in various environments. The structure of these proteins, and their lipid environment they are embedded in, are profoundly sensitive to temperature changes (Griffin and Boulant, 1995; Volgushev et al., 2000; Zimmermann et al., 2007). Even couple of degrees difference may alter the kinetics of the channel gating and even lead to their inactivity.

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 where it is responsible for carrying substantial amount of current (Renganathan et al., 2001). This channel is reported to be the only sodium channel subtype not being inactivated at low temperatures in vitro (Zimmermann et al., 2007) and necessary for sensing prolonged extreme, damaging cold temperatures (Luiz et al., 2019). 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 slow kinetics and non-inactivating, persistent sodium current not to contribute to the 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 a couple of °C leads to a conduction block and to protein denaturation which may end up in serious medical conditions and death. Therefore, the rapid signaling of a 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. It is a group of lipophilic, polycyclic polyether toxins (Lewis and Holmes, 1993). 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 (Hogg et al., 1998; Dechraoui et al., 1999; Strachan et al., 1999; Yamaoka et al., 2009). 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 circumtropical 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 based on their origin in the Caribbean, the Indian and Pacific Ocean where 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 camphor

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 crotalphine`s action (Picolo et al., 2000; Konno et al., 2008). Oral, intravenous and intraplantar doses of crotalphine lead to a 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 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, thermoregulation and thermoregulatory behavior such as TRP ion channels relies heavily on experiments performed in animal models. Therefore, creating knockout animal models and finding the behavioral phenotypes is of profound significance. One of the limiting parameters in researching the role of thermosensitive

proteins is the lack of suitable behavioral assays. In our study, we focused on designing a special behavioral assay to precisely quantify temperature sensitivity, cold/hot hypersensitivity or hyperalgesia in knockout mice model.

Since the identification of TRPM8 as the major cold sensing molecule also responsible for menthol detection and establishing its role as the principal cold transduction ion channel (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. TRPC5 has been shown to be cold sensitive, but without any identified behavioral phenotype (Zimmermann et al., 2011). Other cold sensing candidate has been TRPA1 receptor (Story et al., 2003; Kwan et al., 2006; Karashima et al., 2009), sensor of environmental irritants and pungent compounds, potentiated by inflammatory mediators (Bandell et al., 2004; Bautista et al., 2006), which cold sensitivity has been proved as a purified protein in lipid bilayers (Moparthi et al., 2014). Nevertheless, in mice deficient for TRPA1 behavioral phenotype has not been seen in 2-temperature choice assay (Knowlton et al., 2010) and TRPV1-DTR mice (diphtheria toxin receptor), which lack TRPV1 and also TRPA1 and TRPV1-DTR/TRPM8-DTR mice, which lack TRPV1, TRPA1 and TRPM8, weren`t different from TRPM8-DTR in response to piece of dry ice applied under the hind paw (Pogorzala et al., 2013).

The rising number of potential proteins involved in thermosensitivity, and conflicting results from different assays, require accurate characterization of thermal selection behavior under precisely defined experimental conditions. Therefore, we improved a thermal gradient assay design that enables more precise, automated and bias-free assessment of murine thermal preference (Touska et al., 2016).

Aims of the study

The general idea of the thesis was to characterize the role of specific ion channels in specific physiological and pathophysiological conditions and to elucidate the molecular mechanisms of action on nociceptors involved in temperature and chemical sensing namely:

- 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.

Discussion

Heat-resistant action potentials in primary afferent sensory neurons

In contrast to neurons of the CNS where the optimal temperature around 36°-38°C is maintained, PNS has to deal with changes over a wide temperature range. This feature is reflected by a different set of VGSC subtypes in both systems. Whereas CNS expresses predominantly TTXs subtypes $Na_V1.1$, $Na_V1.2$ and $Na_V1.6$, PNS relies on TTXr channel subtypes $Na_V1.8$, $Na_V1.9$ and TTXs subtype $Na_V1.7$ (Bennett et al., 2019). The main functional difference between VGSCs isoforms lies in the kinetics of the activation and inactivation. TTXs subtypes ($Na_V1.1$, $Na_V1.6$ and $Na_V1.7$) represent fast activation kinetics, i.e. they open and close very fast. TTXr subtype $Na_V1.8$ has slow kinetics but carries the majority of the current and is the major contributor to the action potential in a large population of nociceptive neurons (Renganathan et al., 2001). The biophysical properties of $Na_V1.9$ are unique compared to other VGSC isoforms. Its kinetics is "ultra-slow" creating a persistent, non-inactivating current around -70 mV to -40 mV, which is around the resting membrane potential (Cummins et al., 1999; Renganathan et al., 2001; Rush et al., 2007; Dib-Hajj et al., 2015). This persistent so called "window current" and its ultra-slow kinetics suggests that $Na_V1.9$ does not contribute to the rising phase of the action potential as is the case for all other sodium channel subtypes mentioned previously. The evidence rather suggests that $Na_V1.9$ is a threshold channel that regulates resting membrane potential and prolongs the depolarizing response to subthreshold stimuli (Cummins et al., 1999; Herzog et al., 2001; Dib-Hajj et al., 2015). It reduces action potential threshold and induces action potential firing (Baker et al., 2003). The sensitization of $Na_V1.9$, the potentiation of the current and hyperpolarizing shift of activation by inflammatory mediators has been well established (Baker et al., 2003; Rush and Waxman, 2004; Maingret et al., 2008; Vanoye et al., 2013; Hockley et al., 2014). We found that $Na_V1.9$ current is substantially potentiated by rising temperatures and also supports repetitive firing increasingly with rising temperature (Touska et al., 2018). 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 neurons. We showed with current-clamp patch-clamp experiments that $Na_V1.9$ can generate action potentials *per se* and besides its role as a threshold channel and amplifier of subthreshold stimuli and its role in inflammatory pain, it plays a physiological role also at high temperatures. 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 (Minett et al., 2014; Touska et al., 2016; Hoffmann et al., 2017). It has been recently reported that $Na_V1.9$ is important for the perception of pain in response to noxious cold and has been shown to attenuate cold allodynia

induced by the anticancer drug oxaliplatin (Lolignier et al., 2015) and also cold neuropathic pain (Leo et al., 2010) . Further pathological cold pain states linked to gain of function mutations of *Scn11a* gene coding $Na_V1.9$ protein are emerging (Leipold et al., 2015). Also, the membrane resistance of neurons increases with cooling and potassium conductance decreases, which requires less transduction current to trigger action potentials (Volgushev et al., 2000; Zimmermann et al., 2007). Thus, activation of $Na_V1.9$ may serve as an additional mechanism how to maintain nerve conductance with increasing temperature. Overall our findings postulate the important function of both ion channels, $Na_V1.8$ and $Na_V1.9$, through the entire temperature spectrum, with important functional contribution of $Na_V1.9$ to electrogenesis at rising 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 (Strachan et al., 1999; Birinyi-Strachan et al., 2005; Inserra et al., 2017). 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 variety of second messengers, which could be the way of indirect action of P-CTX-1 on this ion channel (Zurborg et al., 2007). 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 a significant part in physiological and pathological cold sensing which has been further confirmed by fMRI and the detection of BOLD (Blood Oxygenation Level Dependent) signal in TRPA1-/- mice compared to WT. We illustrated a reduced BOLD signal in brain areas associated with emotion (amygdala), pain (cingulate cortex) and cold processing (S1 and S2 somatosensory cortices) (Vetter et al., 2012).

The other group of polymodal A-fibers and large-diameter DRG neurons (soma of A-fiber neurons) involved in ciguatoxin-induced cold allodynia was devoid of TRPA1 and was characterized by action potential activity potentiated by cooling and inhibited by warming in single-fiber and current-clamp electrophysiological recordings. This effect was exclusively sodium 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 into the mechanism of cold transduction and processing.

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 aimed 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 effects, which are consistent with the fact that the current amplitude elicited by crotalphine is relatively small, compared to the 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 interact 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 channel, 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 S4-S5 region). 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 ion channels present 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 that directly affects the excitability of nociceptors (Madrid et al., 2009; Noel et al., 2009). Although this effect itself is not sufficient 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 it enhances cold transduction by inhibiting potassium current through M-channel (K_V7). In contrast, crotalphine application leads to activation of TRPA1 which activates intracellular pathways leading to analgesic actions through opioid receptors. Together, 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. This 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 knock out animals (Toro et al., 2015). The lack of input from these fibers may also reflect a distorted input from warm and cold fibers, which might be better compensated from other temperature transducer with the less steep gradient.

Our experiments revealed substantial contribution of TRPA1 to innocuous cold perception but only in combination with knock out 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 on 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 at 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

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**
Author contribution: Performed patch-clamp experiments, performed calcium imaging experiments, analyzed the data.

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Author contribution: Participated in performing CGRP release experiments, analyzing the data and designing the figures
*these authors contributed equally

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Author contribution: Participated in designing and performing patch-clamp experiments and analyzing the data.

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Author contribution: Designed and performed the current-clamp experiments, analyzed the data

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Author contribution: Participated in performing experiments and analyzing the data.

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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
*these authors contributed equally

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**
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**
Author contribution: Participated in writing the manuscript.

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