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TRANSIENT RECEPTOR POTENTIAL CHANNELS IN HUMAN DISEASE: FROM SENSORS TO SUPPRESSORS

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Cheers with love,

Laura, Lauriiii, Lulu, Laurita

Table of Contents

List of abbreviations

4αPDD	4α-phorbol-12,13-didecanoate
AA	Arachidonic acid
AITC	Allyl isothiocianate
AMC	Arthrogryposis Multiplex Congenita
ANK	Ankyrin
ARD	Ankyrin repeat domain
BDNF	Brain-derived neurotrophic factors
BG	Background
BMP	Bone morphogenetic protein
BO	Brachyolmia
CA	Cinnamaldehyde
Ca^{2+}	Calcium
CaM	Calcium bound calmodulin
CMT	Charcot-Marie-Tooth
DAG	Diacylglycerol
dHMN	Distal hereditary motor neuropathy oa. CMT, SMA
DRG	Dorsal Root Ganglia
dSMA	(congenital) Distal Spinal Muscular Atrophy
EET	Epoxyeicosatrienoic acids
FDAB	Familial digital arthropathy-brachydactyly
FGF	Fibroblast growth factor
GDNF	Glial-derived neurotrophic factor
GOF	Gain of function
GPCR	G-Protein-Coupled receptor
hDRG	Human dorsal root ganglion
hESC	Human embryonic stem cells
HMSN2C	Hereditary motor and sensory neuropathy type 2C
hSCDS	Human stem cell-derived sensory
IP3	Inositol trisphosphate
iPSC	Induced pluripotent stem cells
KO	Knockout
MAPPIT	Mammalian Protein Protein Interaction Trap
MD	Metatropic Dysplasia
MO	Mustard oil

NGF	Nerve growth factor
NGF	Neuronal growth factor
NO	Nitric Oxide
NSAID	Nonsteroidal Anti-Inflammatory Drugs
PBD	PIP_2-protein binding domain
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
POA	Pre-optic area
PPI	Protein Protein Interaction
PRR	Proline rich region
PS	Pregnenolone sulphate
ROS	Reactive oxygen species
RR	Ruthenium red
SC	Stem cell
SD	Skeletal dysplasia
SEDM-PM2	Spondylo-Epiphyseal Dysplasia, Maroteaux type (pseudo-Morquio syndrome type2)
SMA	spinal muscular atrophy
SMDK	SpondyloMetaphyseal Dysplasia, Kozlowski type
SNARE	Soluble NSF Attachment REceptor
SPSMA	ScapuloPeroneal Spinal Muscular Atrophy
TG	Trigeminal Ganglia
TGF-β	Transforming Growth factor
TIR – FRAP	Total Internal Reflection – Fluorescence recovery after photobleaching
TIRF	Total Internal Reflection Fluorescence
TM	Transmembrane
TRP	Transient Receptor Potential channel (A: ankyrin, C: canonical, M: melastin, ML: mucolipin, P: Polycystin, V: vanilloid)
VAMP	Vesicle Associated Membrane Proteins
VEGF	Vascular epithelial growth factor
WT	Wild-type
ZARD	ZC4H2-associated rare disorder

Summary

Transient receptor potential (TRP) channels are a family of cation channels containing 27 members which are organized in six subfamilies (A: ankyrin, C: canonical, M: melastin, ML: mucolipin, P: Polycystin, V: vanilloid) based on sequence homology. Functional TRP channels are built as tetramers of subunits characterized by long intracellular N- and C-termini and by six transmembrane (TM) domains, of which TM4 is considered the voltage-sensing domain whereas the loop between TM5 and TM6 from four subunits complement into a functional pore. Notwithstanding this common architecture, TRP channels have variable expression patterns and functions and can be activated by a myriad of stimuli. Most TRP channels are Ca^{2+} permeable, whereby activation results in intracellular Ca^{2+} signals to induce signal transduction. The effect of this intracellular Ca^{2+} increase however, can differ vastly, triggering intracellular processes, preserving homeostasis or leading to depolarization in excitable cells. Furthermore, a small group of TRP channels are directly activated by Ca^{2+}, thereby shaping the cellular Ca^{2+} response. To add further complexity, TRP channels themselves can be downstream targets of Ca^{2+}. Most TRPs are modulated by intra- and extracellular calcium in a channel-specific manner, which allows fast feedback regulation.

Considering the broad roles of TRP channels, it is of no surprise that malfunction or dysregulation of channel function contributes to several hereditary or acquired diseases. In this thesis, we delved into two different channelopathies, in order to unravel the underlying mechanisms or explore a potential role for treatment.

First, we investigated the remarkable spectrum of TRPV4-dependent pathologies. In human, TRPV4 mutations are the direct cause of a plethora of hereditary pathologies, usually divided into three categories; skeletal dysplasia's, characterized by a malformation of the skeleton, arthropathies affecting the joints of hand and feet, and lastly several neuropathies mostly involving motor neurons. It is fascinating that mutations in one single gene can lead to such a complex pattern of phenotypes, with different cell types affected. Unfortunately, there is very little understanding about the pathophysiological mechanisms underlying these diseases so far. Interestingly, a substantial amount of mutations locates in the N-terminal ankyrin repeat domain (ARD) of the channel, a domain known for protein-protein interaction. Considering the cell-type specific effect together with the location, it has been hypothesized that disease-causing mutations affect interaction of TRPV4 with regulatory proteins. In order to identify novel interaction partners of TRPV4, we performed a Mammalian Protein-Protein Interaction Trap (MAPPIT) screen. We confirmed the zinc-finger domain containing protein ZC4H2 as a novel interacting protein and further investigated its effect on TRPV4 function. Using ratiometric Ca^{2+}-imaging experiments, we found that ZC4H2 increases both the basal activity of human TRPV4 as well as the Ca^{2+} responses evoked by different activation pathways. Using total internal reflection fluorescence (TIRF) microscopy, we further show that ZC4H2 accelerates TRPV4 turnover at the plasma membrane. Overall, these data demonstrate that ZC4H2 is a positive modulator of TRPV4, and suggest a link between TRPV4 and ZC4H2-associated rare disorders (ZARD), which show an interesting resemblance with TRPV4-pathies.

In the second part, we focused on the role of TRP channels in the somatosensory pathway. A subset of TRP channels act as molecular antennae in the nerve endings of sensory neurons, conveying physical and chemical stimuli to the central nervous system. In case of noxious stimuli (damaging temperatures or irritants), they are referred to as nociceptive TRP channels. These channels form an important target for the development of novel analgesics to battle chronic pain. One example is TRPM3, a noxious heat sensor playing a key role in acute pain sensation and inflammatory hyperalgesia in rodents. Promising studies show that knockout out or pharmacological blocking of TRPM3 attenuates symptoms of

hyperalgesia. Furthermore, TRPM3 in rodent is negatively modulated by GPCRs like opioid receptors, suggesting their involvement in the peripheral pain killing effect of opioids. However, little is known about the expression, function and modulation of TRPM3 in the human somatosensory system. Here, we studied TRPM3 in endogenous human dorsal root ganglion (hDRG) neurons from donors as well as in the more accessible model of human stem cell-derived sensory (hSCDS) neurons. Functional expression of the channel was found in 52% of hDRG and 59% of hSCDS neurons. Further characterization of the channel showed similar pharmacodynamic properties as shown in mouse sensory neurons. Finally, the μ-opioid receptor agonist DAMGO and the $GABA_B$ receptor agonist baclofen inhibited PS-evoked TRPM3 responses in a subset of hSCDS neurons. These results provide the first evidence of functional TRPM3 expression in humans, further confirming the channel as important target to develop novel analgesics. Furthermore, the model of hSCDS can be further used as an accessible *in vitro* model to characterize human nociceptive neurons, their development and the pathological consequences of inherited or acquired alterations in TRP channel functionality.

Chapter 1:
General Introduction

Part of the introduction (section 1.1.2 and 1.1.2) has been published in the following review:

Vangeel L, Voets T.
Transient receptor potential channels and calcium signaling, (2019) Cold spring harbor perspectives in biology.

1.1 Transient Receptor Potential Channels

1.1.1 An introduction to TRP channels, a versatile family

The superfamily of transient receptor potential (TRP) ion channels comprises 28 mammalian members, classified in six subfamilies based on amino acid homology (Figure 1). The ankyrin subfamily contains solely one mammalian member, TRPA1. The other subfamilies, canonical (TRPC), melastatin (TRPM), vanilloid (TRPV), polycystin (TRPP) and mucolipin (TRPML) channels, comprise three to eight mammalian members [1]. Although homology between TRP channels can be as low as 20%, they all share a similar tetrameric structure, which is also similar to that of classical voltage-gated cation channels. Each of the four subunits consists of long intracellular N- and C-termini and six transmembrane (TM) domains [2]. TM1-4 are believed to form a sensor domain, while TM5, TM6 and the interconnecting loop form a cation-conducting pore upon assembly of the four subunits [3]. In most TRP channels, the pore is rather non-selective, permeating mono- as well as divalent cations. More detailed information regarding TRP channel structures can be found in section (1.1.2).

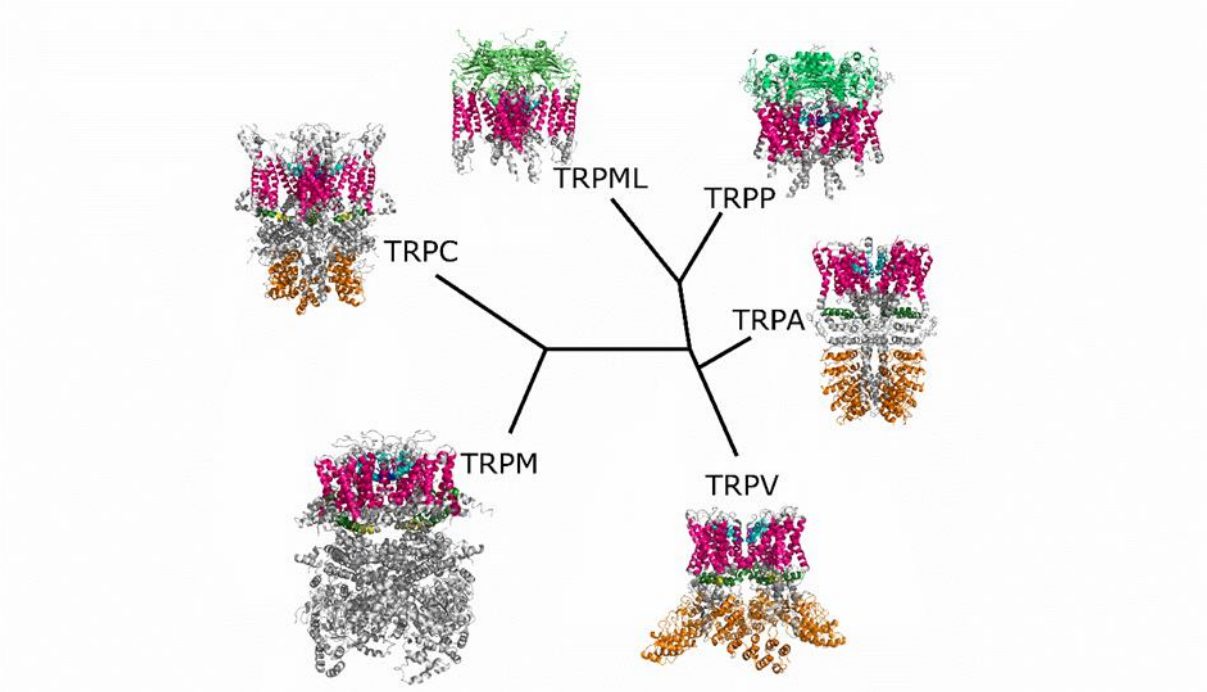


Figure 1: Cladogram of mammalian transient receptor potential (TRP) channel subfamilies and their structures. A representative structure of a member of each subfamily is illustrated: hTRML1 (PDB ID: 5WJ9), hTRPP2 (5K47), hTRPA1 (3J9P), rTRPV1 (3J5P), hTRPM4 (6BQR), and hTRPC3 (6CUD). Specific domains are indicated with the following color code: pink = transmembrane domains (S1–S6), light blue = pore region, dark blue = selectivity filter, orange = ankyrin repeat domain, dark green = TRP domain, yellow= TRP box, and lime = S1–S2 extracellular domain (luminal domain [TRPML] and polycystin domain [TRPP]).

Ever since the first *transient receptor potential* gene was cloned in 1989 [4], a continuous flood of information has been published [5]. They are widely expressed throughout the whole body in different organs, tissues and cell types (both excitable and non-excitable). They constitute molecular gateways, providing an exchange route for cations over the membrane, mostly calcium (Ca^{2+}) and sodium (Na^{+}). Intracellular Ca^{2+} is usually low, so opening of TRP channels causes a vast increase of this second messenger, triggering diverse processes including synaptic transmission, fertility, secretion and

contraction. TRP channels are therefore indispensable in many physiological processes, from sensation to homeostasis [6].

By reason, TRP proteins reside in membranes. For most TRP channels, this is the plasma membrane, where they allow movement of cations between the intra- and extra- cellular environment. They are embedded in the membrane bilayer, and often regulated by changes in this complex lipid environment [7]. Apart from the plasma membrane, some TRP channels localize in intracellular membranes [8]. For example, all three members of the TRPML subfamily are expressed in intracellular vesicles, in particular lysosomes [9]. Interestingly, some plasma membrane TRP channels are thought to be stored in intracellular vesicles, and are only transported to the plasma membrane upon stimulation with growth hormone [10]. Nevertheless, the physiological mechanism and relevance behind this is still uncertain and heavily debated. For some constitutively open TRP channels, vesicular insertion and recycling might be a way of regulating activity [11].

One remarkable characteristic of TRP channels is the promiscuousness of their gating (table 1, [12]). Most TRP channels display multiple modes of activation, which often includes chemical ligands, as well as physical stimuli such as changes in temperature, transmembrane voltage or mechanical stress [13, 14]. Since the identification of TRPV1 as the capsaicin receptor [15], the number of exo- and endogenous compounds that were identified to activate TRP channels increased exponentially [16, 17], including ligands that activate multiple TRP channels. Besides various ligands, channels can also be modulated by many other stimuli. In addition, intra- and extra- cellular ion concentrations often play a role in activation. Best examples are TRPM4 and TRPM5, the only TRP channels that do not permeate Ca^{2+}, but depend on it for activation. Additionally, channels can be influenced by Zinc (Zn^{2+}) or Magnesium (Mg^{2+}), and Ca^{2+} bound to Calmodulin (CaM). A small group of TRP channels is affected by pH and lately, multiple reports show some TRP channels to be susceptive to reactive oxygen species (ROS) by cysteine oxidation. Furthermore, phosphorylation or glycosylation of TRP channels can also influence their activity. Finally, channel function is often regulated by components of the phospholipase C (PLC) pathway, which transforms membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG).

Table 1: Activation and regulation mechanisms of TRP channels [1, 18].

	Representative ligand	[Calcium]$_i$	T°	Voltage dependence
TRPA1	AITC	+: Direct (low Ca^{2+}) −: Direct/CaM (high Ca^{2+})	❄, 🔥	↯
TRPC1				
TRPC2	DAG	−:		
TRPC3	DAG	−: CaM		
TRPC4	Gd^{3+}	−: CaM		
TRPC5	Gd^{3+}	+: Ca^{2+}	❄	↯
TRPC6	DAG			
TRPC7	DAG	−: CaM		
TRPM1	PS			
TRPM2	2′-deoxy-ADPR	+: CaM, direct		
TRPM3	PS, CIM0216	−: PIP_2 depletion	🔥	↯
TRPM4	Decavanadate	+: Direct −: PIP_2 depletion	🔥	↯
TRPM5	Ca^{2+}	+: Direct −: PIP_2 depletion	🔥	↯
TRPM6		−: PIP_2 depletion		
TRPM7	Naltriben	−: PIP_2 depletion		
TRPM8	Menthol	−: PIP_2 depletion	❄	↯
TRPML1	$PI(3,5)P_2$	−: Direct Ca^{2+} block		
TRPML2	SF-11, SN-1			
TRPML3	SF-21/41/81			
TRPP1–PKD2		+: Direct		↯
TRPP2–PKD2 L1				
TRPP3–PKD2 L2				
TRPV1	Capsaicin	−: CaM, PIP_2 depletion	🔥	↯
TRPV2	THC	−: PIP_2 depletion	🔥	
TRPV3	2-APB	+: CaM + direct	🔥	↯
TRPV4	GSK-1016790A	+: CaM −: Desensitization (?)	🔥	
TRPV5		−: CaM, Calbindin-D28K, PIP_2 depletion		
TRPV6		−: CaM, PIP_2 depletion		

❄, cold temperatures; 🔥, warm temperatures; ↯, voltage-gated.
2-APB, 2-aminoethoxydiphenyl borate; AICT, allyl isothiocyanate; CaM, calmodulin; DAG, diacylglycerol; PIP_2, phosphatidylinositol bisphosphate; PS, pregnenolone sulfate; SF, SN, sulfonamides; THC, tetrahydrocannabinol.

1.1.2. TRP channel structure

This chapter is part of the published review 'Transient receptor potential channels and calcium signaling' by Laura Vangeel and Thomas Voets in cold spring harbor perspectives in biology, 2019 [19].

Recent advantages in cryo-EM, driven by developments in detectors and single-particle analysis [20], have led to a revolution in TRP channel structural biology. As a result, detailed structures of at least one member of each TRP subfamily has been unraveled, providing insight into ion permeation and gating with resolution in the 3–4 Å range.

In general, different TRP channels structures have several features in common, including a homotetrameric organization with fourfold symmetry, and a structurally conserved TM organization that resembles that of other members of the superfamily of voltage-gated cation channels. Despite common global architectures, there are several subfamily-specific features (Figure 1). In the exoplasmic region, TRPML and TRPP members exhibit a large structured domain formed by the S1–S2 linkers, known as the luminal domain (TRPML) and the polycystin domain (TRPP), which forms a fenestrated canopy-

like structure atop the channel [21-25]. In contrast, members of the other subfamilies have only short and largely unstructured exoplasmic loops. In TRPA, TRPC, and TRPV channels, the amino terminus contains multiple ARDs, which are implicated in protein–protein interaction, trafficking, multimerization and gating. The number of ARDs ranges from three ARDs in TRPCs to 24 repeats in TRPA1. TRPM channels have relatively long amino termini but lack ARDs, as do the short amino-terminal tails of TRPP and TRPML channels. In their proximal carboxyl terminus following the S6 helix, TRPC, TRPV, and TRPM channels contain a highly conserved TRP domain, implicated in channel gating, which is not found in TRPA, TRPP, and TRPML channels. At their distal carboxy terminal, three TRPM channels exhibit an integral enzyme domain: an atypical α kinase in TRPM6 and TRPM7, and a NUDIX domain in TRPM2.

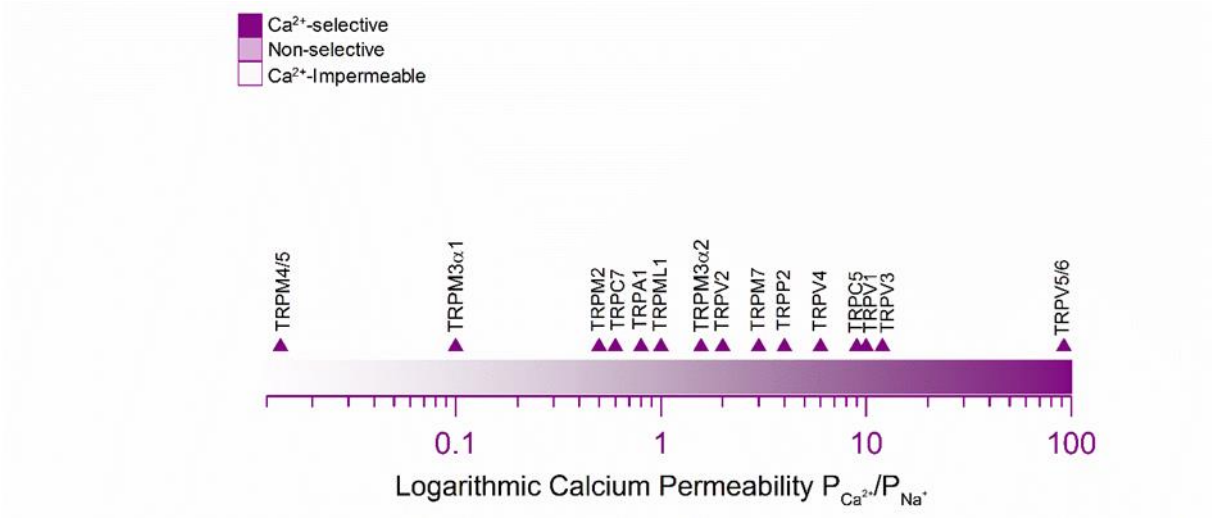


Figure 2: **Ca^{2+} permeability of transient receptor potential (TRP) channels.** On a logarithmic scale, these are examples of the indicated mammalian TRP channels illustrating the range of relative Ca^{2+} permeability (based on reversal potential measurements). Note that values are indicative, as the obtained values can vary significantly depending on cellular environment and experimental conditions.

Based upon their relative permeability to Ca^{2+} and Na^+ (P_{Ca}/P_{Na}), TRP channels can roughly be divided into three groups (Figure 2, [26]). The majority of TRP channels are Ca^{2+}-permeable nonselective cation channels with P_{Ca}/P_{Na} values in the range between 0.1 and 20. In most cell types, opening of these TRP channels will induce an inward current that is carried by a mixture of Na^+ and Ca^{2+} ions [27]. The two closely homologous epithelial TRP channels TRPV5 and TRPV6 are exquisitely Ca^{2+}-selective, with estimated P_{Ca}/P_{Na} values exceeding 100 [28, 29]. Under physiological ionic conditions, these channels will generate inward currents that are almost exclusively carried by Ca^{2+} ions, similar to the high Ca^{2+} selectivity of voltage-gated Ca^{2+} channels and store-operated Orai1 channels. TRPM4 and TRPM5 are situated on the other end of the selectivity spectrum, showing negligible permeability to Ca^{2+} ions ($P_{Ca}/P_{Na} < 0.01$). Inward currents mediated by these channels are mainly carried by Na^+ ions [30-32]. Amino acids lining the central pore are responsible to tune the Ca^{2+} selectivity of TRP channel pores, and variability in this region can explain to a large extent the differences in Ca^{2+} permeability between TRP channels.

Besides TRP channel gating shaping cellular Ca^{2+} signals, cytosolic Ca^{2+} itself also modulates the activity of several TRP channels. The action of Ca^{2+} on TRP channel gating can be both stimulatory and inhibitory, and can either be mediated via direct channel-Ca^{2+} interaction or alternatively involve cytosolic Ca^{2+}-binding proteins and/or Ca^{2+}-dependent signal transduction pathways. Recent TRP channel structures also provide interesting novel structural clues toward understanding Ca^{2+}-dependent processes that regulate channel gating (Figure 3).

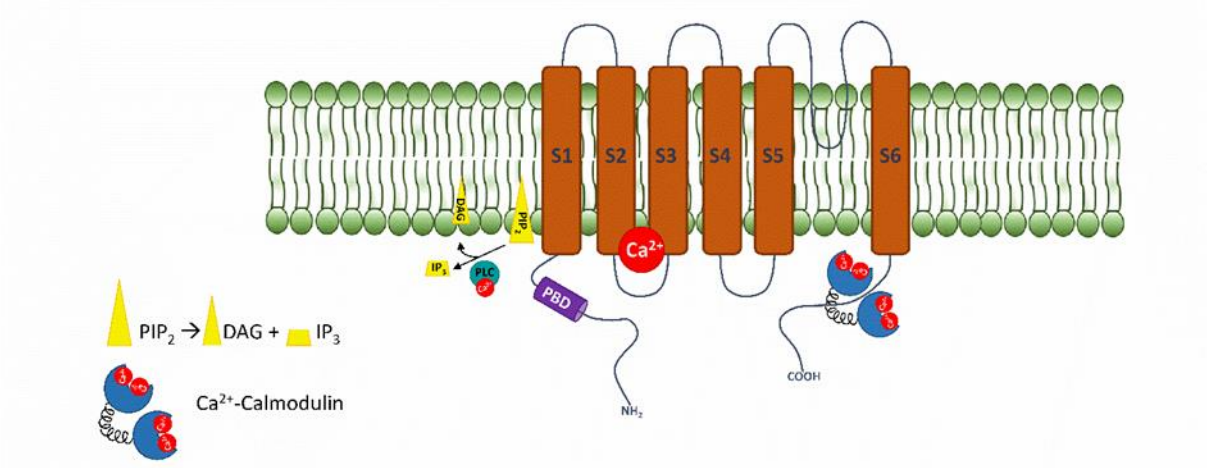


Figure 3: **Graphic presentation of a TRP channel with the possible modes of intracellular Ca^{2+} interaction for modulation channel function.** Few TRP channels exhibit a direct Ca^{2+} binding site in the cytosolic domains of the channel, these include EF-hand structures. A more common modulation happens trough cytosolic Ca^{2+} binding proteins, like calmodulin (CaM). CaM-binding domains (CaMBDs) have been identified in the cytosolic regions of different TRP channels. The effect of Ca^{2+}-CaM on TRP channel gating is channel-dependent. Ca^{2+} can also affect channel function by activation of phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphophate (PIP_2) in the plasma membrane into the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). PIP_2 hydrolysis at the plasma membrane can have a desensitizing effect on channel function, though inhibitory and bimodal effects on gating has also been observed for some TRP channels [33].

1.1.3. Trafficking of TRP channels

In addition to channel gating, a dynamic regulation of the number of functional channels in the membrane represents an important mechanism of TRP channel regulation. Like all proteins, the expression of TRP channels starts in the nucleus with transcription, followed by translation on ER-associated ribosomes, and folding and assembly in the ER. After further modification in the Golgi apparatus, TRP channels are transported in vesicles to the allocated targets, in this case plasma or intracellular membrane. After fusion, these vesicles deliver functional channels to the target membrane. TRP channels in the plasma membrane can also be re-internalized into vesicles via endocytosis (Figure 4). These recycling vesicles may remain localized near the membrane, and comprise a readily available pool of TRP channels for recruitment into or retrieval out of the membrane [34]. Vesicular trafficking mechanisms that control the efficacy and rate of insertion or internalization are therefore crucial regulators of TRP channels. In chronic pain, increased trafficking is even proposed to be connected to the pathological state [35]. For example, an increase of TRPV1 [36], TRPA1 [37] and TRPM8 [38] has been reported, which might contribute to the observed potentiation of these pain-channels in hypersensitive conditions. Although an acute increase in channel trafficking cannot always be repeated, mechanisms for channel trafficking and incorporation into the membrane are believed to be important in (patho-)physiological conditions [39].

However, not much is known about the mechanisms of this TRP channel transport. In general, vesicles are transported using molecular motors that use either actin or microtubules as intracellular pathways. SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins play a role to eventually fuse two different lipid bilayers [40]. Shortly, SNARE proteins on the plasma membrane interact with counterpart SNARE proteins in vesicular membrane and fuse the two compartments. The SNARE protein family contains more than 30 different members, among which the Vesicle Associated Membrane Proteins (VAMPs), which are resident in mammalian vesicular membrane. Endocytosis of proteins from the plasma membrane into vesicles starts by invagination of part of the plasma

membrane, leading to internalization of the therein-incorporated proteins. Coat proteins like clathrin polymerize to form an envelope around the budding vesicles. After, dynamin forms a ring around the neck of clathrin-coated vesicle and dissociates it from the membrane. These early endosomes can be recycled into the membrane or fuse with lysosomes for degradation [41]. Different Rab GTPases are also involved in several of these steps for a flawless progression of endocytosis. However, these are general mechanisms and TRP channel specific studies to unravel transport mechanisms & pathways are sparse.

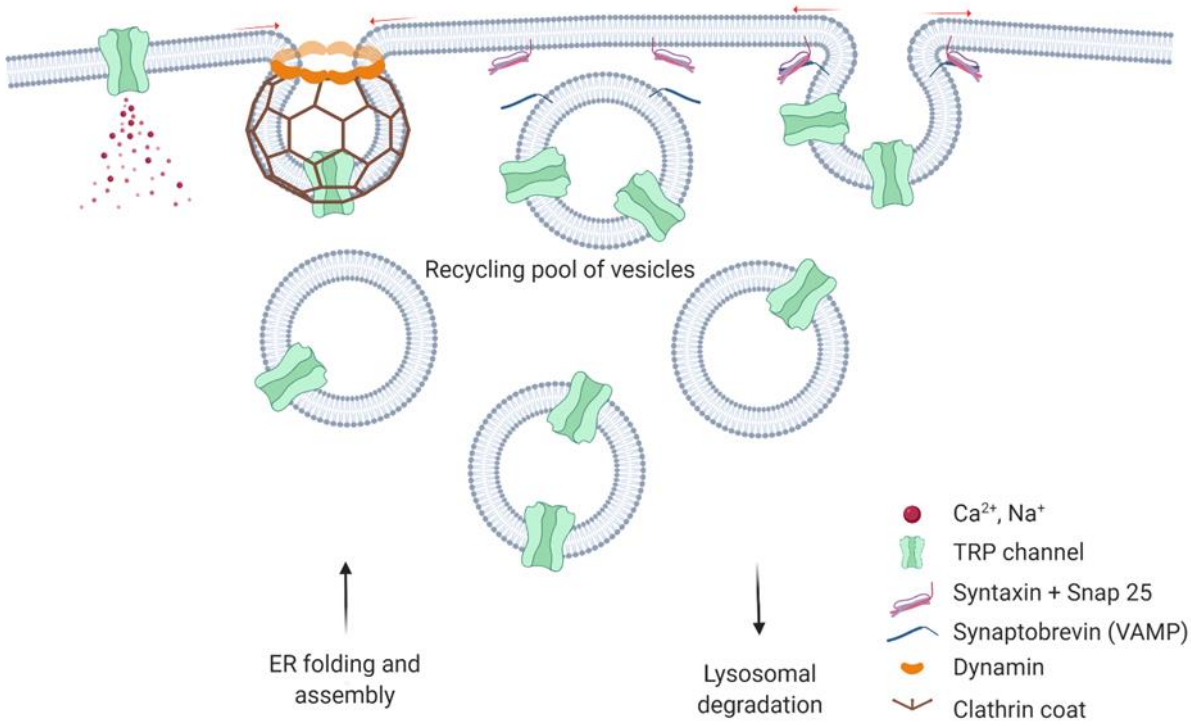


Figure 4: **Schematic overview of vesicular trafficking.** After folding and assembly of channel tetramers in the endoplasmatic reticulum (ER) and Golgi apparatus (GA), TRP channels travel in vesicles to their designated membrane. Synaptobrevin proteins in the vesicle interact with Syntaxin and Snap 25 in the membrane to form a SNARE complex. Fusion of the two membrane allows release of the TRP channels. For endocytosis, invagination of the membrane is followed by formation of a Clathrin coat around the vesicle. Ultimately, dynamin forms a ring around the neck of the vesicle and splits off the vesicle. A recycling pool of vesicles resides in the near-membrane area to enable a fast increase in channel density if necessary. Note that this is a general overview; pathways that triggers this process as well as specific proteins involved are not completely elucidated for TRP channels.

Recent studies use Total Internal Reflection Fluorescence (TIRF) microscopy to study molecular events from fluorophore-linked proteins in or near the plasma membrane [42]. The incident wave in TIRF microscopy reflects on the glass-oil interface, hereby creating an evanescent wave of which the intensity decays exponentially. The wave only penetrates 100-200nm into the specimen, exciting molecules in the restricted area of the membrane and near-membrane zone, and not deeper into the cell. Interestingly, these fluorophores can also be selectively bleached using TIRF microscopy, enabling visualization of new fluorophores incorporating into the membrane area. This technique, 'TIR-Fluorescent recovery after photo bleaching' (TIR-FRAP), can be used to study membrane trafficking events like TRP channel dynamics. For example, Gosh *et al.* used TIR-FRAP to study perimembrane TRP channel turn over and concluded that distinct modes of TRP channel integration exist, and that regulation might be channel dependent [43].

To conclude, after a period of neglect, it is acknowledged that vesicular fusion and internalization are important to control surface expression of TRP channels and thus cellular response. However, the exact mechanisms of TRP channel transport and perimembrane dynamics is yet far from elucidated [44].

1.1.4. TRP channels in human disease

Considering their widespread expression and involvement in a variety of biological processes, it is not surprising that dysregulation of TRP channels can lead to various inherited and acquired diseases [45], or 'TRP *channelopathies*'. In human, mutations in TRP channel genes are causative of a wide variety of hereditary diseases, affecting many different organs. Below, some eminent examples will be discussed.

Mutations in TRPP genes are causative of autosomal dominant polycystic kidney disease (ADPKD), which contributed to the name of the polycystin TRP family [46]. ADPKD is the most prevalent, potentially lethal, monogenic disorder in humans, which is primarily characterized by prominent fluid filled renal cysts. Due to the progressiveness of the disease, this can eventually result in fatal renal failure. Considering the high amount of intrafamilial variation, a 'two-hit mechanism' was proposed, where a second, somatic mutation in the PKD alleles is required to develop the disease. However, the exact pathophysiological mechanism behind the formation of these cysts is not completely elucidated [47]. TRPM6 and TRPM7 are one of the only TRP channels that show selectivity for Magnesium (Mg^{2+}). Both proteins where shown to be channel kinases that play a critical role in cellular Mg^{2+} uptake in the epithelium of kidney and intestine, thereby playing a role in Mg^{2+} homeostasis. Accordingly, hereditary loss-of-function mutations in TRPM6 are known to cause autosomal recessive hypomagnesemia associated with secondary hypocalcemia (HSH). Patients carrying a mutation in TRPM4 develop cardiac conduction defects (CDC), although the mechanism for this conduction block remains puzzling [48]. Recently, two *de novo* variants in TRPM3, a sensory channel also highly expressed in brain, were linked to intellectual disability and epilepsy [49]. In contrast, a GOF mutation in the other sensory channel TRPA1 was reported to cause familial episodic pain syndrome [50]. Deviant lysosomal Ca^{2+} signaling due to mutations in TRPML cause lysosomal storage disease which can result in neurodegeneration [51]. TRPV5 and TRPV6, important for Ca^{2+} reabsorption in the kidney and intestine, control Ca^{2+} homeostasis of the body. Accordingly, patients harboring mutations in the genes coding for these proteins can suffer from Ca^{2+} imbalance, which can lead to recurrent kidney stones [52] or skeletal under mineralization [53]. Gain-of-function (GOF) mutations in TRPC6 can be the underlying genetic cause of chronic kidney disease [54]. Finally yet importantly, TRPV4 harbors many known mutations that are causative of a wide spectrum of diseases, classified under the umbrella 'TRPV4-pathies'. This is more extensively described in chapter 1.2.5.

TRP channels are as well involved in multiple acquired diseases. Below, some eminent examples will be discussed.

Several TRP channels mediate mechano-, temperature and pain sensation in bladder [55]. In patients with idiopathic detrusor over activity, an increased expression of TRPM8 was reported on bladder afferents. TRPV4, important for stretch/pressure sensation in bladder urothelial, was reported to be increased in rats with experimental bladder outlet obstruction [56]. More information about TRPV4 in bladder can be found in section 1.2.4. Additionally, TRPM4 has been linked to cardiovascular physiology and disease in different manners. For example, an overexpression of the channel was detected after CNS injury in endothelial tissues of capillary vessels surrounding the injury. The unrestricted Na^{+} influx leads to cell swelling and eventually cell dead which causes secondary hemorrhage [57]. Furthermore, some TRP channels (e.g TRPV1, TRPV4, TRPA1 and TRPC6) are expressed in various tissues of the airway system. Their impeccable role in ciliary movement, barrier function and immunity demand a tight

regulation of channel function, and malfunction is associated with airway disorders like asthma [58]. Lastly, a large portion of TRP channel research in acquired diseases concerns pain. A set of TRP channels can be found in peripheral sensory neurons, where they play a role in somatosensation. Malfunction of these channels can evolve to hypersensitivity and pain. This will be more extensively discussed in section 1.3 and chapter 4.

As a silver lining, due to their important role in health and disease, TRP channels represent potential targets for pharmacological treatment [59, 60]. Note that, even before TRPV1 was cloned, the channels agonist, capsaicin, has been used in urinary tract dysfunctions to alleviate an overactive bladder. It was established later that TRPV1 is expressed in the nerves innervating the bladder, and that capsaicin treatment creates prolonged desensitization of these nerves [55]. This desensitization also lies at the basis of the widespread use of capsaicin-containing patches and creams for topical pain relief. Likewise, menthol, a plant-derived cooling compound from the mint plant, has been used for many centuries to relieve pain and itch. The development of specific and safe small molecules targeting TRP channels to treat a variety of conditions form the new aspiration.

1.2 TRPV4 and the TRPV4-pathies

1.2.1. Identification of TRPV4 as an osmosensor

TRPV4, the fourth member of the vanilloid subfamily, was identified in 2000 as a homologue of the capsaicin receptor (TRPV1) and of the C. elegans TRP channel Osm-9 that responds to extracellular hypotonicity in the physiological range [61, 62]. It was initially named 'vanilloid receptor-related osmotically activated channel (VR-OAC)' or 'OSM9-like transient receptor potential channel 4 (OTRPC4)', before the consensus nomenclature gave it its final name 'TRPV4'. TRPV4 revealed to be a polymodal channel, expressed in a myriad of tissues and involved in a multitude of functions. The *trpv4* gene is located on the long arm of chromosome 12 (12q24.11) and encodes a protein of 871 amino acids. Below is a concise overview.

1.2.2. TRPV4 activation and inhibition – the basics

TRPV4 was initially found to be activated by hypotonic **cell swelling**, suggesting a role in the regulation of cell volume and/or in the detection of mechanical stimuli. Further research revealed that swelling-induced activation is not a direct effect, but occurs via a pathway in which cell swelling activates phospholipase A_2 (PLA_2), which in turn releases arachidonic acid (AA) from membrane phospholipids. Subsequently, AA is metabolized by cytochrome-P450 (CYP) epoxygenases into epoxyeicosatrienoic acids (5,6-EET and 8,9-EET), which activate the channel in a membrane delimited manner [63-65] (Figure 5). Thus, pharmacological agents that block EET formation, including inhibitors of PLA2 and epoxygenases, inhibit activation of TRPV4 by cell swelling [66]. Note, however, that other reports suggest that hypotonic activation also involves restructuring of the F-actin cytoskelet [67] and of aquaporin 5 [68]. TRPV4 is also linked to regulatory volume decrease (RVD), the active reduction of volume after cell swelling. The channel thus mediates a tight regulation of cell volume, which is important for the maintenance of cellular homeostasis [69]. **Endogenous activators** of the TRPV4 include the endocannabinoids arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG), which both can be converted to TRPV4-activating EETs via AA (Figure 5) [65]. Using molecular dynamics simulations, a precise binding pocket for 5,6-EET was proposed with K535 in the linker between TM2 and TM3 as a crucial amino acid [70].

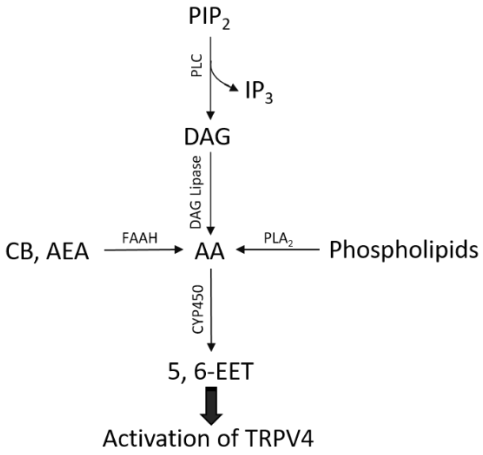


Figure 5: Pathways of TRPV4 activation. Hydrolysis of phospholipids upon membrane stretch, as well endocannabinoids (CB) like anandamide (AEA), are converted into arachidonic acid (AA). This is followed by cytochrome P450 (CYP450) mediated formation of epoxyeicosatrienoic acids (EET) of which 5,6-EET and 8,9-EET can activate the channel upon binding to TM domains. PIP_2 is essential for TRPV4 channel function and IP3 is found to sensitize channel function. Some reports suggest that DAG catalysis via lipase may lead to AA, albeit limited. Furthermore, increased DAG might activate PKC and enable TRPV4 phosphorylation, leading to sensitization of the channel. PLC: ATP-dependent phospholipase C, PLA_2: phospholipase A_2, FAAH: Fatty acid amide hydrolase.

4α-phorbol-12,13-didecanoate (4αPDD) was the first identified synthetic agonist of human and mouse TRPV4. It binds between TM3 and TM4 of the channel [71] and does not activate any other member of the TRPV family. The more potent molecule GSK-1016790A (EC_{50} = 2,1 nM in HEK cells expressing human TRPV4 [72]) binds to a similar region in TRPV4 and is the most frequently used activating ligand in TRPV4 research. Chemical inhibitors of TRPV4 include the metal-based dye ruthenium red (RR), which inhibits the TRPV4 (and several other TRP channels) in a strongly voltage-dependent manner, and the more selective antagonist HC-067047 [73].

Finally, TRPV4 is also a temperature-sensitive channel steeply activated upon warming in the range between 27°C to 35°C. This implies that the channel is constitutively active at body temperature and many physiological roles rely on this property, especially in the brain [74]. Activation by temperature is dependent on diffusible cellular compounds, as heat activation is lost in cell-free inside-out patch-clamp experiments [75]. Temperature-dependent activation occurs, however, distinct from the pathway involved in osmosensing, as it is insensitive to inhibition of PLA2 or epoxygenases.

1.2.3. Cellular modulation of TRPV4 function

Like many other TRP channels [76], TRPV4 activity is modulated by the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 was found to be an essential element for the response to heat, via an interaction with the N-terminal tail of TRPV4 [77] (Figure 6). An interaction with the N terminus, more specifically the ANK repeats was confirmed in a later study, but here the binding of PIP_2 was found to inhibit the response to 4α-PDD [78]. Therefore, the exact consequences of PIP_2 binding on TRPV4 channel function are not yet completely unraveled.

Recent work connected PIP_2 modulation of TRPV4 to Pacsin3. Pacsins are a protein family involved in vesicular trafficking and regulation of dynamin-mediated endocytosis. In 2006, Pacsin3 was shown to bind to TRPV4 and to increase the ratio of membrane versus intracellular TRPV4, indicative of a more efficient plasma membrane incorporation. The interaction required the TRPV4-specific proline-rich domain (PRR, upstream of ANK repeats), and the carboxyl-terminal Src-homology3 domain of Pacsin3.

Modulation of subcellular TRPV4 localization was proposed to occur due to changes in channel endocytosis [79]. Later, it was found that, in addition to the effects of membrane localization, Pacsin3 binding inhibits TRPV4 activation by cell swelling and heat, as well as basal channel activity, whereas gating by 4α-PDD was unaffected [80]. A recent high-resolution structure of the Pacsin3 and TRPV4 exposed more details of the binding, leading to a novel mechanistic model. In this model, TRPV4 is normally bound to PIP_2 through the N-terminal PIP_2-protein binding domain (PBD), and in this conformation a crucial proline residue (P128) in TRPV4 is mainly in the *trans* conformation. However, binding of Pacsin3 via its SH3 domain stabilizes P128 in the *cis* conformation. This leads to a reorientation of the N-terminus and release of PIP_2 interaction, resulting in desensitization of the channel. Furthermore, binding of the Pacsin3-SH3 domain to the TRPV4 N-terminus and its subsequent reorientation enables the Pacsin3 F-BAR domain to attach to the plasma membrane. The F-BAR domain plays an important role in dynamin-mediated endocytosis, which might explain the observed changes in subcellular distribution of TRPV4 in the presence of Pacsin3. Note that activation by chemical 4α-PDD is unaffected by PIP_2 and Pacsin3 [81].

OS-9, a protein ubiquitously expressed on the endoplasmatic reticulum (ER), was found the interact with the N-terminus of TRPV4 [82]. It impedes the release of the channel from the endoplasmic reticulum, hereby controlling a correct maturation of TRPV4 and prevent them from premature polyubiquitination.

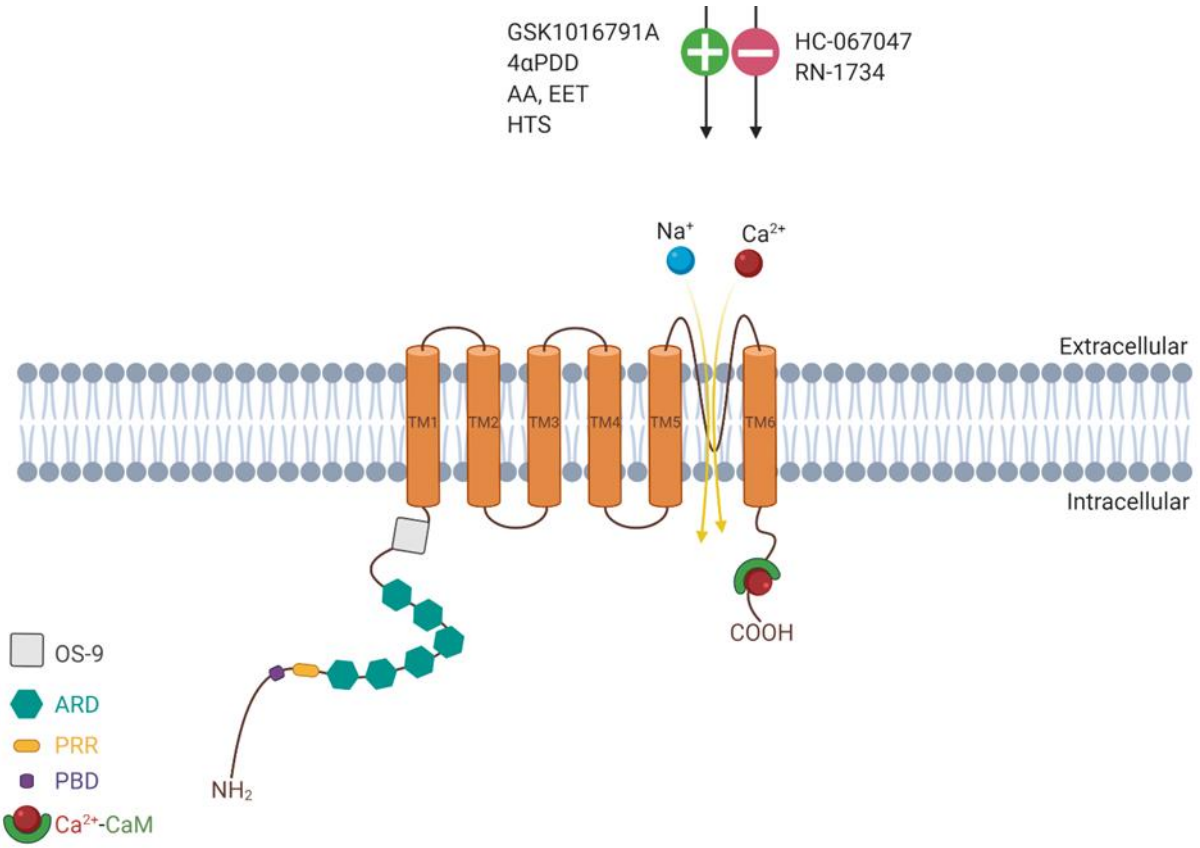


Figure 6: **Cartoon of TRPV4.** TRPV4 is characterized by six ankyrin repeats (ankyrin repeat domain, ARD) in the long intracellular N-terminus of the channel. The PIP_2-binding domain (PDB), the Proline rich region (PRR) and the OS-9 binding domain are also located in the N-terminus. As all TRP channel, TRPV4 contains six transmembrane domains (TM1-6) and a pore-forming loop between TM5 and TM6. The channel permeates mainly Ca^{2+} and Na^+ after activation by hypotonic solution (HTS), arachidonic acid (AA), epoxyeicosatrienoic acids (EET) and synthetic compounds GSK1016791A and 4αPDD. Inhibition by synthetic compound HC-067047 and RN-1734. The Ca^{2+}-Calmodulin binding spot is located in the C-terminus.

In addition, TRPV4 channels are strongly regulated by Ca^{2+}. Intracellular Ca^{2+} potentiates TRPV4 responses to hypotonicity and 4αPDD. This potentiation relies on the Ca^{2+}-CaM binding domain in the C-terminus of the channel [83]. Current activation is followed by Ca^{2+}-dependent inactivation, and increasing the extracellular Ca^{2+} concentration accelerates this decay. Mutation of phenylalanine F707, reduced the Ca^{2+}-dependent current decay, pointing to a structural rearrangement of the TM6 [84].

TRPV4 expression in the plasma membrane is also subject to changes in membrane environment. In human vascular endothelial cells, omega-3 PUFAs increase TRPV4 expression at the membrane, regulating cell activity [85]. Similarly, other studies also suggest an interaction of the channel with cholesterol through cholesterol-recognizing motifs on TM4-TM5. Binding to cholesterol in the lipid rafts and cholesterol levels are suggested to mediate channel mobility and channel function [86].

TRPV4 is also modulated by phosphorylation, and several kinases including by protein kinase C (PKC), protein kinase A (PKA), SGK1 and Src kinases were shown to sensitize the channel. Several residues, including serine162 (S162), threonine175 (T175), S189, T253 and S824 have been put forwards as sites of phosphorylation [87-89]. Furthermore, TRPV4 activation is sensitized by physical interaction with inositol trisphosphate (IP_3), which sensitizes TRPV4 to EET-mediated activation. This process provides a link between phospholipase C-coupled receptors and TRPV4 activity [90], which may also underlie the involvement of TRPV4 in serotonin-induced itch [91], as well as histamine- and serotonin-induced visceral hypersensitivity [92].

1.2.4. Physiological functions of TRPV4

As outlined above, TRPV4 acts as a molecular integrator of physical and chemical stimuli. Molecular expression of TRPV4 is found in many different cell types and organs, and the channel has been linked to a variety of physiological processes.

In the skin, TRPV4 expression can be found in keratinocytes where it contributes to the detection of warm temperatures. Furthermore, the response of TRPV4 to histaminergic pruritogens can induce itch in the skin and mediate visceral hypersensitivity [93]. In different types of epithelial and endothelial cells, TRPV4 is reported to be an essential player in the formation of junctions and maintaining barrier function. For example in skin keratinocytes, TRPV4 interacts with β-catenin, the main adaptor protein linking adherens junctions and the actin cytoskeleton. If active, TRPV4 Ca^{2+}-influx induces an actin cytoskeletal rearrangement, playing a role in the formation and maturation cell-cell junctions to obtain a tight barrier. Reasonably, knockout (KO) animals therefore suffer from increased intercellular permeability [94]. Additionally, TRPV4 proofs to be essential in gingival defense, as knock out leads to reduced amount of adherens junctions causing increased permeability [95]. Similarly, TRPV4 at the apical membrane of corneal epithelium is necessary for the formation of tight junctions to maintain a barrier function [96]. As last example, TRPV4 plays a role in endothelial Ca^{2+} signaling in the lung, mediating barrier integrity and endothelial permeability in the alveolar septal network [97]. Furthermore, TRPV4 expressed in the epithelial cells lining the airways act as key player in innate immune response. Lipopolysaccharides (LPS), an abundant component in the wall of gram-negative bacteria, rapidly activates TRPV4 in these cells, independently of Toll-like receptor 4 (TLR4). This activation induces a raise in intracellular Ca^{2+}, followed by increased ciliary beat frequency to augment mucociliary clearance (mechanical clearance of the air epithelium). Furthermore, TRPV4 dependent production and release of antimicrobial compound NO leads to bronchodilation and neutrophil infiltration in the airways [98].

TRPV4 expression is detected in urothelial cells of the bladder where its mechanosensitive properties are of crucial importance in sensing filling state of the bladder. Stretch-induced calcium release results

in ATP release, which induces paracrine signaling to neighboring cells, such as afferent nerve endings (P2X receptor) and surrounding muscle cells [99]. Ultimately, this will convey a signal that initiates voiding behavior. This inevitable role in the micturition reflex pathway denotes TRPV4 as a target to treat bladder diseases. Antagonizing TRPV4 benefits in cases of bladder over activity [73], while activation of the channel improves bladder function in detrusor nerve underactivity [100]. TRPV4 KO did exhibit spotting problems (increased number of small urine spots) and reduced ATP release. The absent stretch-response leads to enlarged bladder capacity, and consequent reduced voiding frequency [101]. Furthermore, TRPV4 is reported to be expressed in renal epithelial cells, particularly in the distal nephron and collecting ducts. This location, together with properties of TRPV4, suggests a possible role as flow sensor in these cells [102], but TRPV4 KO animals do not exhibit deficiencies that support this idea [103].

Calcium influx through TRPV4 is of crucial importance in bone tissue. Initially, TRPV4 was identified as a regulator of chondrogenic differentiation, via regulation of the SOX9 pathway. During chondrogenesis, mesenchymal cells aggregate into pre-chondrogenic condensations. This is followed by expression of cartilage specific genes, and their eventual differentiation into mature chondrocytes. The SOX9 transcription factor is essential for inducing this chondrocyte-specific gene expression, and to initiate differentiation. Activation of TRPV4 induces SOX9-dependent reporter activity in a Ca^{2+}-dependent manner, and thereby contributes to the chondrogenesis process [104]. A later study indicated that the presence of hyaluronan, an extracellular matrix component, is essential for TRPV4-induced chondrogenesis [105]. Secondly, TRPV4 is also crucial in the final differentiation of osteoclasts. In young osteoclasts, Ca^{2+} oscillations induce nuclear factor-activated T cells (NFAT) c1-responsice gene expression. Upon maturation, oscillations gradually shift into sustained Ca^{2+} signals, which are mediated by TRPV4 and sustain NFATc1-dependent gene expression. Eventually, this leads to terminal differentiation into osteoclast, the cells that break down bone. Hereby, TRPV4 contributes to normal bone remodeling and maintenance [106] and TRPV4 KO leads to defective osteoclast differentiation and subsequent increased bone mass in mice [160]. Finally, TRPV4 has been identified as the mechanosensor in osteoblasts, responsible for the integration of mechanical loading and bone remodeling. Expression of TRPV4 increases after differentiation of osteoblasts in culture under influence of bone morphogenetic protein BMP-2. When mature, the channel induces Ca^{2+} influx when activated by fluid flow, eliciting Ca^{2+} oscillations that are relevant for gene expression, differentiation and proliferation in osteoblast. Together with the fact that TRPV4 KO animals have a reduced bone loss upon unloading, this study shows an important regulatory role of TRPV4 for integrating mechanical stimuli in osteoblasts [107].

In addition, numerous other tissues express functional TRPV4, and new features appear almost weekly if not daily, underwriting the complexity of the channel. For example TRPV4 is reported to engage in shear stress adaption in vascular endothelial cells, maintaining steady vascular tone [108, 109], for example via Ca^{2+}-dependent Endothelial NO synthase 3 (eNOS) [110]. As briefly touched before, TRPV4 Ca^{2+} signaling also plays a role in innate immune-related processes [111]. Furthermore, TRPV4 is reported to be expressed in the brain (e.g. in hippocampal neurons) and exert a wide-range of functions [131]. The temperature sensor is believed to be constitutively active at brain temperature (>34°C) to enable neuronal firing and maintain normal excitability, and KO mice demonstrate reduced theta-frequency electroencephalogram (EEG) activities [95]. However, exact expression pattern and function is not completely elucidated. Similarly, there is much debate about the expression of TRPV4 in sensory neurons. Although multiple functions have been proposed [112, 113], evidence is not convincing and counteracted by the fact that little expression has been found in RNA sequencing data from human DRG and TG [114].

1.2.5. TRPV4-Pathies

Despite the many and important functions of TRPV4 throughout the body, mice with complete KO of the channel are viable and fertile and show a relatively mild phenotype. They behave normal in size, appearance, growth and body temperature [115]. TRPV4 functions are often impaired but not completely abolished, suggesting compensation mechanisms of other TRP channels triggered by the absence of TRPV4 during the development.

Mutations in the *trpv4* gene on the other hand, create a far more drastic effect in human. They are the direct cause of a number of monogenic human diseases, which are collectively classified under the umbrella of TRPV4-pathies [116]. More than 50 different mutations leading to human disease have been identified (Figure 7), and more variants are regularly reported. An up-to-date list with all the mutations, their location and associated disease, can be found in table 2. Mutations are hereditary and heterozygous, and exhibit a highly variable penetration.

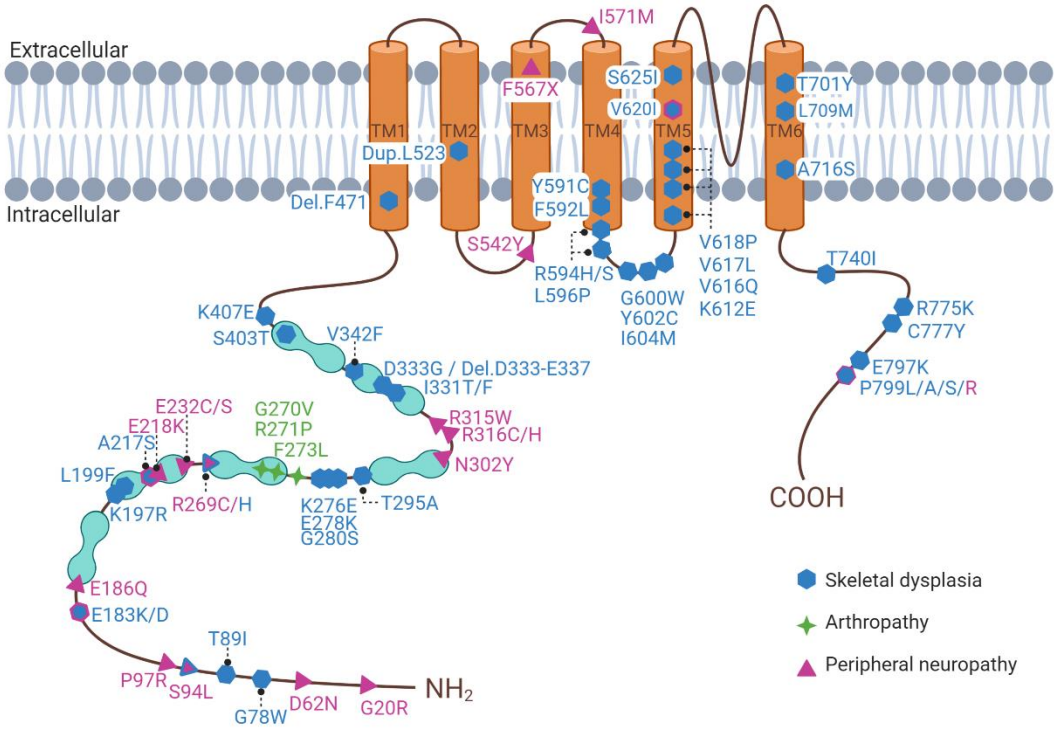


Figure 7: **Disease-causing mutations in the TRPV4 protein.** A cartoon of one TRPV4 subunit with localized mutations, color-coded for the disease they are associated with. There is a hotspot for mutations in the ankyrin repeats as well as the pore domain, however, it is not possible to deduct a clear correlation between location and disease. A complete list of mutations with references can be found below.

The spectrum of diseases caused by these mutations is equally variable, since different mutants differentially affect multiple organ systems. Disease symptoms are copious, and vary substantially between patients with the same mutations (even within families). In 2013, Nilius and Voets classified TRPV4-pathies into three groups: peripheral neuropathies, arthropathies and skeletal dysplasias [117].

Skeletal dysplasias [malformation of the skeleton]

Skeletal dysplasias are a very broad group of bone and cartilage abnormalities characterized by skeletal malformations or disproportions, such as a short trunk, and in several cases, these are caused by dominant mutations in TRPV4. Spondylo-epiphyseal dysplasia (SED), also known as pseudo Morquio disease type 2 (PM2) or Maroteaux type, is one of the typical skeletal dysplasias caused by mutations in TRPV4. A typical feature is the development of flattened vertebrae or '*platyspondyly*', which cause shortening of the trunk. Furthermore, patients exhibit short hands and feet, epiphyseal dysplasia in the hip and knees and may develop scoliosis. The long bones appear normal. There is an overlap of symptoms in patients with spondylometaphyseal dysplasia Kozlowski type (SMDK). Patients with SMDK develop a short trunk comparable to patients with PM2, but also severe scoliosis, brachydactyly (short fingers and toes), pelvic abnormalities and degenerative joint diseases. Brachyolmia type 3 (BO3) is also characterized by a short trunk and kyphoscoliosis (scoliosis in coronal and sagittal plane). Further skeletal dysplasias caused by mutated TRPV4 include parastremmatic dysplasia (PD), causing dwarfism accompanied with twisting of the limbs. Most of those malformations are present at birth or develop perinatally. At later age, patients may develop kyphoscoliosis and contractures of the large joints due to impaired bone mineralization. A similar, but often more severe disease is metatropic dysplasia (MD). Although some cases can be mild, MD can be associated with severe arthrogryposis (curved joints and club foot), and the perinatal lethal form is characterized by fetal akinesia, joint contractures and respiratory complications.

Arthropathies [diseases of the joint]

Mutations in TRPV4 also cause a particular arthropathy, known as familial digital arthropathy brachydactyly (FDAB). Patients are normal at birth, but develop deformities in the joints of hands and feet, often combined with painful osteoarthritis.

A more extensive description of all skeletal-related TRPV4 diseases and their symptoms can be found in [118].

Peripheral neuropathies [diseases affecting the peripheral nerves]

TRPV4 is also linked to several hereditary diseases of the peripheral nervous system, which can be restricted to motor neurons or involve both motor and sensory neurons.

TRPV4 is the causative gene for Charcot-Marie-Tooth type 2C (CMT2C). CMT2C is characterized by progressive degeneration of peripheral sensory and motor neurons, leading to loss of muscle and sensory function. Since myelination of the nerves appears to be unaffected, CMT2C belongs to the axonal form of CMT (CMT type 2) instead of the demyelinating form (CMT type 1). Patients may additionally suffer from vocal cord paralysis, hearing loss, diaphragmatic weakness and bladder urgency.

Sensory deficits are typically absent in congenital distal SMA (CDSMA). In SMA, an autosomal recessive disease caused by mutations in the gene encoding Survival of Motor Neuron 1 (SMN1), degeneration of motor neurons in the ventral horn leads to muscle wasting. Subsequently, patients often suffer overall muscle weakness, which initially affects the proximal muscles and respiratory distress due to loss of respiratory muscles. Autosominal dominant mutations in TRPV4 lead to CDSMA, where mainly distal limbs are affected, limiting mobility of the patients. Scapuloperoneal SMA (SPSMA) is another TRPV4-dependent neuropathy with a similar trail of motor neuron loss, this time affecting scapuloperoneal muscles (shoulder blade [scapula] and the small leg muscle groups below the knee [peroneal]). Noteworthy, some patients with SPSMA may also exhibit sensory defects not detected in the other SMA's.

This subdivision of TRPV4-pathies in these three classes is however not always bulletproof. Indeed patients with TRPV4 mutations sometimes exhibit both skeletal and neurological symptoms [119]. Furthermore, family members with the same mutation can be affected by different levels of severity, from early onset to late or from mild to severe. Even though the majority of the diseases-causing mutations are autosomal dominant, there can be incomplete disease penetrance (presentation of the phenotype), which causes a lot of variability. This information suggests that in some cases additional genetic or environmental factors or secondary (somatic) mutations play a role. Nevertheless, it is remarkable how a single nucleotide mutation in one sole gene can cause such a plethora of different diseases. How can modification of TRPV4 function cause such a complex pattern of phenotypes, with distinct diseases affecting different cell types?

At this point, very little is known about the pathophysiological processes underlying these diseases. Multiple mechanisms have been put forward to explain the development of TRPV4 diseases [117], yet none can fully explain the pathophysiology. For example, the bulk of mutations are found to be gain of function *in vitro [120, 121]*. An over activity of a TRPV4 can evidently lead to increased Ca^{2+}-influx, which might lead to altered cellular signaling. However, it remains mysterious why some gain of functions affect skeletal systems, while others damage the neuronal system. One possible explanation for the cell-type/cell-system specific effect of these disease-causing mutations is a dysregulation of interactions of mutated TRPV4 with other proteins or cellular components [122]. Altered binding with cell-specific regulatory proteins, which can either modulate TRPV4 and/or rely on TRPV4 for their correct function, may indeed explain why TRPV4 mutations can result in such diverse disease manifestations. The fact that many disease-causing mutations cluster in the N-terminal ankyrin repeats, a domain believed to be of key importance for protein-protein interactions, seems to support this hypothesis.

Table 2: **Mutations in human TRPV4.** All the reported mutations at April 2020 with their amino acid alteration/substitute, the location in the channel protein and the reported disease(s).

MUTATION	LOCATION	DISEASE	Reference
P19S	NH_2-Cytopl	Hyponatermia (COPD)	[123]
G20R	NH_2-Cytopl	dHMN	[124]
D62N	NH_2-Cytopl	CMT-2C	[125]
G78W	NH_2-Cytopl	Fetal akinesia + MD	[126]
T89I	NH_2-Cytopl	MD (lethal)	[127]
S94L	NH_2-Cytopl	dSMA + Arthrogryposis	[128]
P97R	NH_2-Cytopl	dSMA and vocal cord paralysis	[129]
E183K/D	ARD1	Spondylo-epiphyseal dysplasia, CMT2C	[130, 131]
R186Q	ARD1	CMT2C, SMA arthrogryposis with SD	[132, 133]
K197R	ARD2	MD (lethal)	[127]
L199F	ARD2	Metatropic Dysplasia (MD)	[134]
A217S	ARD2	skeletal dysplasia and peripheral neuropathy, SMDK	[119]
E218K	ARD2	CMT-2C (complex)	[124]
R232C	ARD2	Charcot-Marie-Tooth type 2C (CMT-2C), dSMA with bone abnormalities, SPSMA	[129, 133, 135, 136]
R232S	ARD2	CMT-2C	[133]

R237G/L	ARD3	CMT-2C	[137]
Q239H	ARD3	MD	[138]
R269H/C	ARD3	Charcot-Marie-Tooth type 2C (CMT-2C), dSMA, SPMA, involvement cranial nerves, arthrogryposis, skeletal abnorm.	[122, 125, 133, 135, 136, 139-144]
G270V	ARD3	FDAB	[145]
R271P	ARD3	FDAB	[145]
F273L	ARD3/4	FDAB	[145]
K276E	ARD3/4	Fetal akinesia + MD	[126]
E278K	ANK3/4	SMDK	[119, 134]
G280S	ARD3/4	MD	[146]
T295A	ANK4/4	MD	[134]
N302Y	ARD4	CMT-2C + progressive weakness	[124]
R315W	ANK4/5	CMT-2C, SPSMA, dSMA	[133, 136, 139, 141, 147]
R316C/H	ANK4/5	SPSMA, CMT-2C	[133, 135, 136, 139-141, 144]
I331T/F	ANK5	MD, SMDK	[127, 134, 136, 148]
D333G	ANK5	SMDK, MD	[134, 136, 148]
D333-E337 delinsE	ANK5	MD	[127]
V342F	ANK5	MD	[134]
S403T	ANK6	MD	[149]
K407E	ANK6	MD	[138]
F471del	TM1	MD	[127, 134]
L523dup	TM2	SMDK	[138]
S542Y	TM2-3	CMT-2C with short stature	[147]
F567X	TM3	CMT-2C	[124]
I571M	TM4	CMT-2C, also contr: benign/low penetr	[144]
Y591C	TM4-5	SMDK, Brachyolmia	[138]
F592L	TM4-5	SMDK	[134]
R594H *	TM4-5	Parastremmatic dysplasia/dwarfism, SMDK, MD	[130, 134, 136, 138, 148]
R594S	TM4-5	MD	[138]
L596P	TM4-5	SMDK	[134]
G600W	TM4-5	SMDK	[134]
Y602C	TM4-5	SEDM-PM2	[130]
I604M	TM 4-5	MD	[127, 150]

K612E	TM 4-5	MD	[151]
R616Q	TM5	Brachyolmia (BO)	[120, 134, 136, 152, 153]
F617L	TM5	MD	[127, 138]
L618P	TM5	MD	[127, 154]
L619F	TM5	MD	[155, 156]
V620I	TM5	Brachyolmia (BO), CMT-2C + SD	[120, 134, 136]
M625I	TM5	SMDK	[134]
T701Y	TM5-6	CMT-2C	[124]
L709M	TM6	SMDK	[134]
A716S	COOH-cyto	SMDK	[134, 136, 148]
T740I	COOH-cyto	Fetal akinesia + MD	[126]
R775K	COOH-cyto	MD	[134]
C777Y	COOH-cyto	SMDK	[134]
E797K	COOH-Cyto	MD, SEDM-PM2, SMDK	[127, 130, 134, 157]
P799L*/R	COOH-cyto	MD, SEDM-PM2	[119, 127, 134, 136, 138, 148, 157-159]
P799A	COOH-cyto	MD	[134]
P799S	COOH-cyto	MD	[134, 138]
P799R	COOH-cyto	MD	[134]
P799Lfs 63X	COOH-cyto	SEDM-PM2	[130]
G800D	COOH-cyto	MD	[158]
K801E	COOH-cyto	Skeletal Dysplasia	[160]
V829WfsX3	COOH-cyto	Osteonecrosis of the femoral head	[161]
N833S + E840K	COOH-cyto	Multisystem phenotyp: Neuronal + Skeleton + intellectual disability (ID)	[162]

* Common recurrent mutations (Dai *et al*, 2010), Locus localization in old studies: [163-166]

COPD = Chronic Obstructive Pulmonary Disease, dHMN = Distal hereditary motor neuropathy, CMT-2C = Charcot-Marie-Tooth type 2C, MD = Metatropic Dysplasia, d/SP SMA = distal/ ScapuloPeroneal Spinal Muscular Atrophy, SD = skeletal dysplasia, SMDK = SpondyloMetaphyseal Dysplasia - Kozlowski type, FDAB = Familial digital arthropathy-brachydactyly, SEDM-PM2 = Spondylo-Epiphyseal Dysplasia - Maroteaux type,

1.3 TRP channels in somatosensation and pain

1.3.1. Somatosensory pathway

The physiological process to detect and perceive environmental stimuli is called somatosensation. In general, it concerns the detection of a stimulus and the transduction thereof into an electrical signal, followed by further transmission of this signal until the ultimate perception in the CNS.

Somatosensory neurons are pseudo-unipolar, where one branch of the axon travels to the periphery, while the other branch travels to a second-order neuron. The peripheral innervate targets organs including the skin, internal organs, mucosa, muscles, joints, eye, nose, and mouth. Distinct categories of afferent nerve fibers exist, each with their specialized electrophysiological properties and receptive resources. They are classified into four different categories based on anatomical and conduction properties [167]. Aα and Aβ fibers have the fastest conduction (>16m/s) due to their large diameters and thickly myelinated axons. Aβ fibers often convey information of touch, as they end in specialized structures in the skin like Meissner corpuscles or Merkel's discs. Aδ fibers, which have smaller diameters and a thinner myelination layer, exhibit slower conduction velocities. Gradually, these neurons lose their myelin to terminate in the skin as free nerve endings. C-fibers are the slowest conducting nerve fibers (< 1.2 m/s); they are unmyelinated and contain the smallest cell bodies and thinnest axons. C-fibers also terminate in the skin as free nerve endings. Both C and Aδ fibers are involved in the detection of temperature.

Cell bodies of sensory nerves are clustered in specialized structures. For neurons innervating the head, that are the trigeminal ganglia (TG), which report information to second-order neurons in the sensory nucleus in the brain. Cell bodies of sensory nerves innervating the rest of the body are located in dorsal root ganglia (DRG). These are positioned in the vertebral column, lateral to the spinal cord, and transmit their information to second-order neurons in the dorsal horn. Additionally, the signal is further transmitted to, and processed by the CNS [168] (Figure 8). The brain will perceive and interpret these inputs and translate into an appropriate response, which makes the somatosensory system vital for adaptation and survival.

Interestingly, nerve endings of somatosensory neurons form specialized afferents. They are characterized by the set of molecular signal detectors on their plasma membrane, which determine which stimuli the neurons can sense and transduce. In most cases, these molecular detectors are depolarizing ion channels, like TRP channels. Being at the frontline of the sensory system, these molecular antennae use their multimodal activation properties to detect physical or chemical stimuli from the environment. After activation, a subsequent cation influx transduces the signal into neuronal depolarization. This eventually leads to the activation of voltage gated sodium channels and the generation of an action potential (AP). The AP travels further to the other end of the pseudounipolar axon, where it leads to neurotransmitter release onto second order sensory neurons in the dorsal horn (Figure 7).

Whenever somatosensation concerns a potentially damaging stimulus, it evokes pain and potentially an acute behavioral response (avoidance). This is referred to as nociception, and the responsible sensory nerves are known as 'nociceptors'. Nociceptors can be activated by noxious stimuli such as extreme temperatures, strong pressure forces and pungent compounds [169]. Nociceptive signals are primarily conducted through Aδ (acute pain) and C (slow persisting pain) fibers [170]. The nociceptive pathway represents an essential warning system that protect the body for potentially damaging situations. Section 3.4 consists of a more elaborate description of nociception and (persistant) pain.

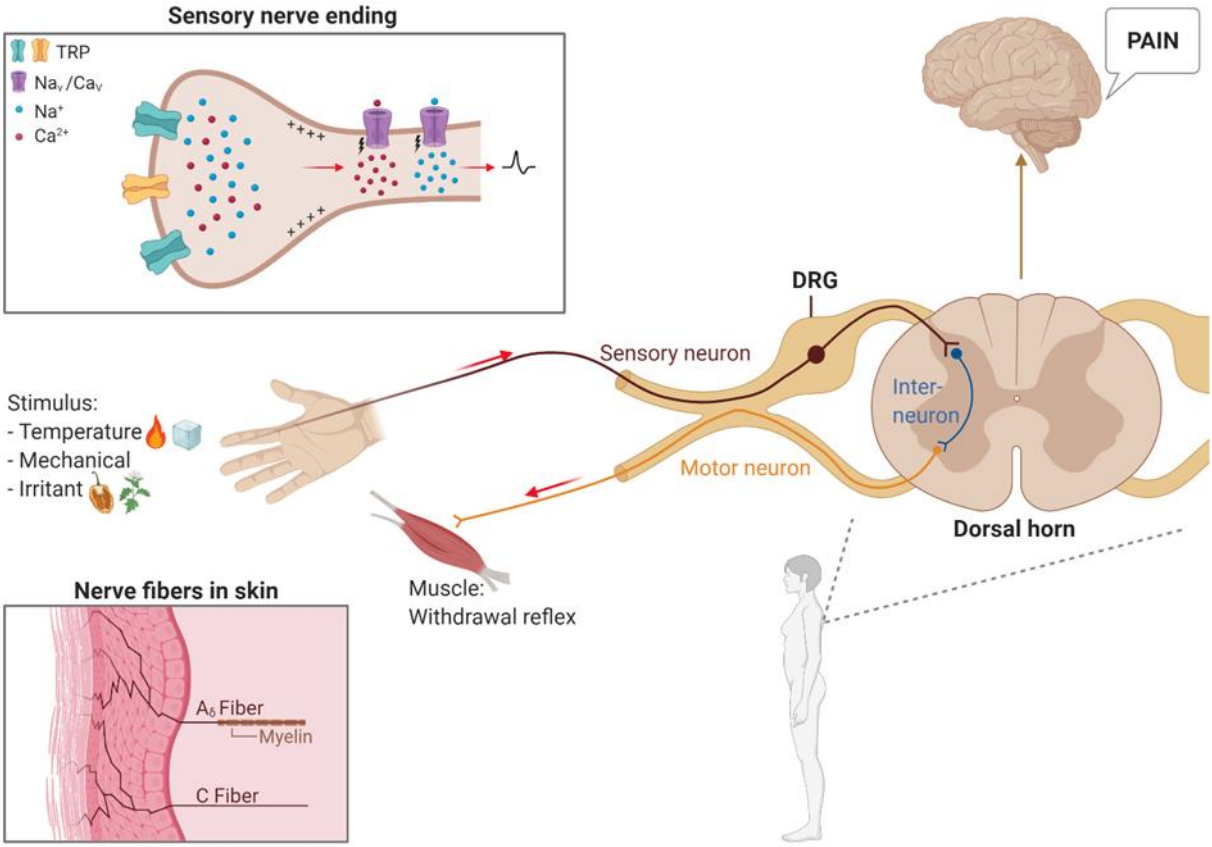


Figure 8: Somatosensory pathway of a (painful) stimulus from sensory nerves in the skin to the central nervous system (CNS). Pseudo-unipolar nerves detects stimuli in the environment of the skin via the nerve endings in the skin. Different type of nerve fibers exit, temperature information is conveyed in Aδ and C-fibers which in in the skin as free nerve endings. TRP channels represent molecular detectors in these nerve endings to transduce environmental stimuli into an electrical signal. Initial influx of Na^+ and Ca^{2+} leads to depolarization of the cells (+++), which in turn activates voltage gated channels (Na_v and Ca_v). The resulting action potential is further transmitted to cell bodies in dorsal or trigeminal root ganglia (DRG/TG). Next, the second branch of the axon conducts this signal further to a secondary neuron in the dorsal horn. Via an interneuron, fast activation of motor neurons takes place to initiate a withdrawal effect. Additionally, a signal is transmitted to the brain for perception.

1.3.2. TRP channels as primary sensors

Several TRP channels act as primary thermosensors, being activated within distinct ranges of temperatures. However, these ranges are merely an approximation, as (patho)physiological modulation of TRP channels can shift the thermal sensitivity to lower or higher temperatures. In addition, there can be a synergistic effect between temperature and chemical agonists, where the presence of an agonist modulates thermal sensitivity and *vice versa*. Temperature dependence of a TRP channel is often quantified as temperature coefficient 'Q10'. This value is defined as the rate of change of a biological or chemical process (for example current amplitude) upon a temperature increase of 10 degrees. Channels without pronounced temperature sensitivity typically have a Q10 of 1-3, whereas cold sensitive TRP channels have Q10 values <<1 and heat sensitive channels Q10 values >5. However, Q10 values should be compared cautiously, as values depend strongly on experimental conditions. Several hypotheses have been formulated to explain the biophysical and molecular mechanisms of temperature sensitivity in TRP channels [171]. An elegant explanation is provided by the two-state gating model, where the influence of temperature on channel opening and closing arises from differences in enthalpy and entropy between the open and closed channel conformation [172]. It is a matter of debate whether the

difference in enthalpy and entropy is due to conformational changes in specific 'temperature sensing domains' of the channels or rather distributed over the entire protein complexes.

Whereas temperature-sensitive TRP channels are found in various cell types, a set of four TRP channels (TRPA1, TRPM3, TRPM8 and TRPV1) are functionally expressed in somatosensory neurons, where they are crucially involved in the detection of innocuous and noxious thermal stimuli. As typical polymodal channels, these four temperature-sensitive TRP channels are also gated by specific endogenous and exogenous chemical ligands, and thus act as dual thermo- and chemoreceptors

TRPV1: TRPV1 is highly expressed in nociceptors, and is activated by heat, by pungent compouds including vanilloids such as capsaicin (the pungent compound in chili peppers) and resiniferatoxin, and by acidic pH [173]. TRPV1 KO mice lack pain responses to capsaicin, show prolonged withdrawal latencies to hot stimuli, and fail to develop inflammatory heat hypersensitivity. Although TRPV1 was the first channel discovered to be important for heat sensation, it later became clear that additional mechanisms are involved. In 2018, Vandewauw *et al.* pinpointed TRPM3 and TRPA1 as indispensable for acute heat sensation [174]. However, the role of TRPV1 becomes more prominent in an inflammatory environment, as KO mice did not suffer from inflammation-induced heat hyperalgesia [175].

TRPA1: TRPA1, which is expressed in a subset of mostly TRPV1-positive nociceptors, is activated by a long list of irritant compounds, of which mustard oil (AITC), cinnamaldehyde (CA) and allicin from garlic are the best known. Besides chemical irritants, TRPA1 shows a bidirectional temperature sensitivity, being activated by both cold and hot temperatures [176]. Independent studies reported the channel's role in the detection of both noxious cold [177] and noxious heat [174]. TRPA1 KO mice have reduced pain and inflammatory responses to AITC, and show deficits in noxious cold sensing. A family with FEPS (familial episodic pain syndrome) was identified with a gain of function mutation in TRPA1, which links the nociceptive channel TRPA1 to pain episodes [50].

TRPM8: TRPM8, which is mainly expressed in TRPV1-negative C fibers, is activated by cooling, as well as by chemical compounds such as menthol, eucalyptol and icilin, explaining the cool sensation they evoke [178]. TRPM8 KO mice lose avoidance for cool temperatures, however still responding to noxious cold [179]. They do not suffer cooling-induced hyperalgesia nor neuropathic cold allodynia.

TRPM3: TRPM3, expressed in a large subset of sensory neurons that often also express TRPV1 and TRPA1, is activated by heat and by the neurosteroid pregnenolone sulphate (PS) (Figure 9). TRPM3 KO mice lack pain responses to PS, and, like TRPV1 KO mice, show prolonged withdrawal latencies to hot stimuli and fail to develop inflammatory heat hypersensitivity [180]. The properties of TRPM3, considered a potential novel drug target to treat pain and subject of part of the research in this thesis, are discussed in more detail below.

In addition to the endogenous neurosteroid pregnenolone sulphate (PS), TRPM3 is also activated by synthetic compounds including nifedipine (Nif) and CIM0216. TRPM3 antagonists include flavonoids such as hesperetin, isosakuranetin as well as the anticonvulsant primidone. A peculiar property of TRPM3 is that it exhibits an alternative ion pathway, distinct from the central pore. This pathway can be unlocked upon simultaneous stimulation of the channel with clotrimazole (Clt) and PS, or by the strong synthetic agonist CIM0216. This additional alternative ion pathway occurs via the voltage sensor domain, and allows a large inward monovalent cation current. In contrast to the central pore, the alternative ion permeation pathway is insensitive to Ca^{2+}-dependent desensitization and resistant the nonspecific pore blocker La^{3+} [181].

Expression of TRPM3 can be found in various tissues, and initially detected in pancreatic beta cells where it potentiates insulin release. Recently, two de novo mutations in the channel were linked to developmental and epileptic encephalopathies (DEE), connected to the functional expression of TRPM3 in the brain [182]. Furthermore, TRPM3 is highly important in the peripheral nervous system. In mice, there is a high functional TRPM3 expression in almost 60% of neurons from DRG and TG, where the channel mediates responses to heat and PS [180]. Compared to TRPV1, the current-temperature relationship of TRPM3 is shifted to higher temperatures, suggesting that TRPV1 is the first heat sensor that becomes activated under normal conditions. Increases in PS in the physiological range potentiate TRPM3-mediated heat responses [183]. Recently, it was shown that the trio TRPM3, along with TRPV1 and TRPA1 form a redundant set of heat sensors essential and sufficient for detection of noxious heat. Triple knockout mice lacking all three TRP channels did not demonstrate any avoidance behavior to temperatures of 50°C, jeopardizing their body by risking burn injuries. Responses to moderate temperatures, mechanical stimuli and noxious cold remained normal [174].

Like many other TRP channels, TRPM3 is modulated by the level of phosphoinositides in the membrane, and enzymatic depletion decreases TRPM3 activity. Interestingly, the most effective PIP in sustaining TRPM3 activity is $PI(3,4,5)P_3$ instead of the common $P(4,5)IP_2$ [184]. Moreover, TRPM3 is modulated by G-protein-coupled receptors (GPCR). In short, upon activation of specific GPCRs (including the μ-opioid, GABA-B and neuropeptide Y receptors), the activated $G_{\beta\gamma}$-complex directly binds to and inhibits TRPM3. While this consists of a full block in *in vitro* overexpression systems, the inhibition was of a lesser extend in DRG neurons. Furthermore, the observed block reveals a substantial variability between different neurons, assigned to the variable expression of GPCRs in each neuron. *In vivo*, peripheral administration of morphine/DAMGO (μ-opioid agonist), PYY (neuropeptide Y agonist) or baclofen (GABA-B agonist) attenuated TRPM3-induced pain. The effect is TRPM3 specific, as DAMGO was ineffective in reducing the capsaicin-induced nocifensive behavior. These data suggest that TRPM3 is at least partially responsible for the peripheral analgesic effect of opioids [185-187].

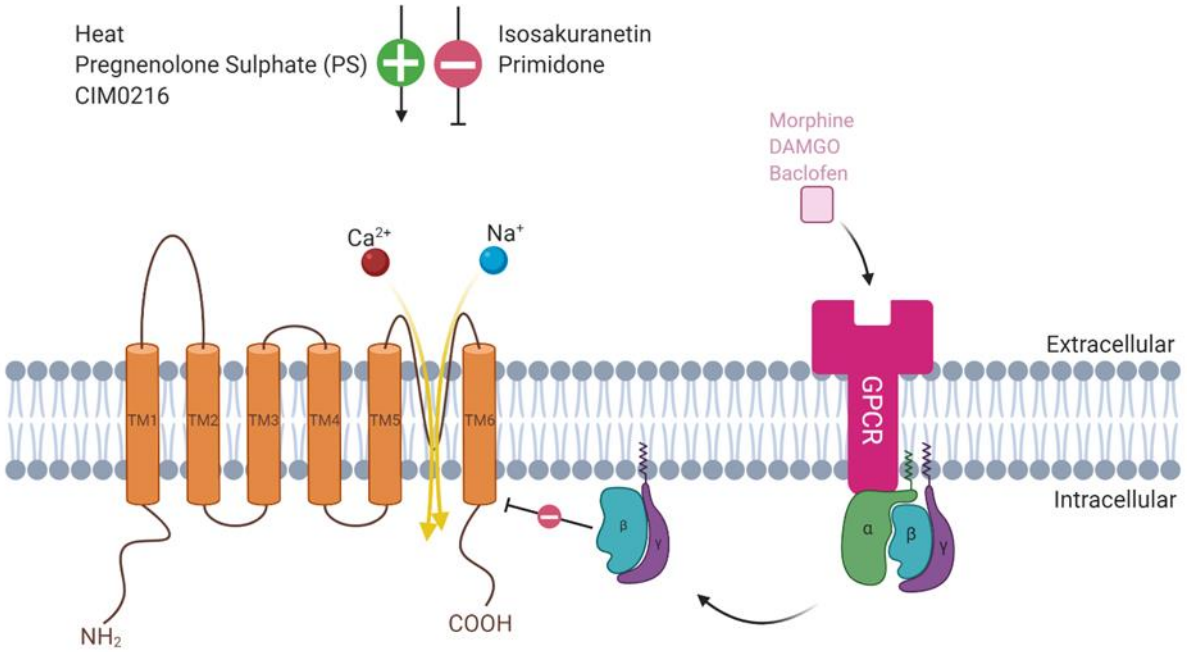


Figure 9: **TRPM3 channel function**. TRPM3 is sensitive to Pregnenolone sulphate (PS), CIM0216, depolarization and noxious heat. Currents can be antagonized by isosakuranetin or primidone. TRPM3 is modulated by different GPCRs, for example μ-opioid receptors. Upon activation by morphine or DAMGO, the activated $G_{\beta\gamma}$-complex directly binds to the channel and inhibits TRPM3-induced currents.

Recently, specific mutations in TRPM3 were found in human patients with intellectual disability and epilepsy, suggesting an important role for TRPM3 in the brain [49]. Notably, two of these identified patients reported abnormal pain perception, though this was not consistent throughout the patient cohort.

1.3.3. TRP channels and persistent pain

In healthy tissue, pain is primarily initiated by physical or chemical stimuli that are potentially damaging. In addition to the immediate withdrawal reflex, the mental perception of pain will teach us to stay away from the source of pain. Therefore, pain is utterly important for survival. Unfortunately, under certain conditions, pain can become chronic. The international association for the study of pain (IASP) defines chronic pain as 'pain without apparent biological value that has persisted beyond the normal tissue healing time'. Thus, pain loses its warning signal and becomes a burden of its own. Some prevalent examples are migraine, lower back pain, arthritis, (diabetes induced) neuropathies, chemotherapy-induced pain and inflammatory pain.

Chronic pain is a global health problem, with a high prevalence between 11 and 40% globally. The economic costs of medical care and productivity loss are outrageous, exceeding $600 billion annually in the USA alone [188]. Furthermore, the quality of life from patients is often beneath contempt. Therefore, chronic pain puts a growing pressure on the public health system and society. However, the biggest problem of chronic pain is the current lack of proper treatment [189]. As classic Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) are often not sufficient, patients are often shifted to a combination therapy with opioids. Even though this provides, at least temporarily, effective treatment for a group of patients, for some it is still inadequate (±10%). Furthermore, opioids have a great potential for side effects like central addiction and dependence. This mediates a true opioid epidemic, where patients sadly become victims [190]. Obviously, there is a high medical need for new pain medication, and preferably new targets to develop these novel analgesics. Increasing evidence is accumulating that TRP channels, at the frontline of the nociceptive pathway, can be such targets [191].

Indeed, several TRP channels involved in thermosensation and acute pain have also been implicated in the development of chronic pain, which can involve TRP channel malfunctioning due to mutations as well as increased functional expression at the plasma membrane or sensitized gating in the context of tissue injury or inflammation [192]. Inflammatory mediators are known to sensitize some somatosensory TRPs like TRPV1 and TRPA1. This includes nerve growth factor (NGF), bradykinin, prostaglandin, protons and ATP as well as proinflammatory cytokines released by immune cells like TNF and IL-1β. Hereby, those TRP channels play a key role in sensing peripheral inflammation [193]. Furthermore, activation of all somatosensory TRPs triggers the release of neuropeptides like substance P (SP) and calcitonin gene-related peptide (CGRP) into the vicinity of the peripheral sensory nerve terminal. These induce symptoms of neurogenic inflammation like vasodilatation and plasma extravasation and leukocyte migration, continuing the inflammatory process. This finally results in heightened responses of nociceptor neurons, which can lead to **hyperalgesia** [amplified pain response to noxious stimuli], **allodynia** [a pain response to stimuli that are normally not painful] or even spontaneous pain in the absence of stimuli [35].

Preclinical research in rodents has provided evidence that genetic ablation (using KO animals or antisense approaches) as well as pharmacological inhibition of all four temperature-sensitive TRP channels in sensory neurons (TRPV1, TRPA1, TRPM3 and TRPM8) can attenuate pain in a variety of persistent pain models, including inflammatory heat/cold/mechanical hyperalgesia as well as neuropathy-induced heat/cold/mechanical allodynia [194]. By far the most studied target is **TRPV1**, with

several specific blockers entered clinical trials [195]. Unfortunately, in initial human trials the pain-reducing effects were relatively limited and escorted with significant side effects. Several TRPV1 antagonists caused significant hyperthermia, which was found to be an 'on-target side effect' as TRPV1 is involved in thermoregulation. Furthermore, these antagonists also often blunted acute noxious heat sensation, increasing the risk of burn injuries during daily life, such as drinking coffee [196]. The cold-receptor TRPM8 demonstrates a rather whimsical role in modulating pain perception, without consensus if activation causing pain or analgesia. Consequently, effort in modulating the channel show the same contradictory effects, some groups report beneficial results when antagonizing the channel while others obtain analgesic effects using TRPM8 agonists [197]. A clinical trial using a potent TRPM8 antagonist developed by Pfizer did show successful results considering the suppression of cold-induced pain using a potent TRPM8 antagonist. However, subjects complained about hot sensations as adverse effect [198]. A long stretch of research went into finding and validating TRPA1 antagonist, since the channel is involved in numerous pain pathologies. While three antagonists already went to clinical trials, none was able to show optimal pharmacokinetics and fruitful results. Therefore, more investigation is necessary to unveil the full clinical potential of TRPA1 as a pain target [199]. TRPM3 is the least studied but arguably most promising TRP channel target to treat chronic pain so far. Several TRPM3 inhibitors were shown to alleviate inflammatory heat hyperalgesia. Moreover, in rats with chronic obstruction injury-induced neuropathic pain the TRPM3 antagonist isosakuranetin was effective against thermal, mechanical and cold hyperalgesia [200]. Importantly, TRPM3 is not involved in central regulation of body temperature, and TRPM3 antagonists do not cause hyperthermia, in contrast to what was observed with TRPV1 blockers. Finally, it is expected that TRPM3 antagonists will have only mild effects on acute noxious heat sensing, as long as TRPV1 functionality is preserved [201].

Therefore, optimization of TRPM3 blockers with the desired selectivity and pharmacokinetic profile for clinical use represents a promising route for the development of novel candidate analgesics. However, at the start of this research, little was known about TRPM3 expression and function in the human somatosensory system.

Chapter 2:
Aims & objectives

The members of the TRP channel family are important players in human physiology, contributing to a vast array of physiological processes in multiple cell types. Upon activation, most TRP channels permeate Ca^{2+}, and the subsequent rise in cytosolic Ca^{2+} triggers membrane depolarization and/or multiple cellular responses. Their pivotal role in human physiology goes hand in hand with the several diseases caused by malfunctioning of these channels. Many hereditary mutations are known to be the origin of their malfunctioning, prompted by changes in expression, permeation, gating or trafficking of the channel. On the other hand, acquired alterations in channel function are also a known cause for diverse channelopathies. However, the extensive TRP channel research of the last two decades has not yet resulted in novel TRP channel-based therapies, mainly due to a lack of understanding of basic disease mechanisms as well as poor translation from preclinical animal models to humans.

The general topic of this thesis is to obtain important novel insights into two TRP channel-related diseases, hereditary TRPV4-pathies and acquired chronic pain, with the aim of providing a basis for future therapy development.

Since decades, biomedical research is performed on non-human vertebrate species [202], for example the widely used laboratory mouse. However, despite their advantages, they are not completely predictive for human. Therefore, an underlying motif is to work on TRPs in a mammalian context by implementing novel experimental approaches like stem cell-derived sensory neurons and a mammalian-based *in vitro* screen for identifying protein interaction.

In **Chapter 3**, we focus on a remarkable example of TRP channelopathies, being the wide phenotypic spectrum of diseases caused by mutations in the TRPV4 gene. The long list of identified mutations results in a plethora of hereditary pathologies, ranging from diseases with mild symptoms to very severe or even lethal disorders. Furthermore, there is a remarkably diverse and variable list of symptoms associated with TRPV4-dependent pathologies, affecting distinct cell types and systems. Interestingly, the majority of the identified mutations is located in the ankyrin repeat domain (ARD), a region known for protein-protein interaction in the N-terminus of the channel. Therefore, we hypothesized a role for regulatory proteins interacting with the channel as potential players in the context of TRPV4-pathies. Although multiple earlier studies have put this hypothesis forward, not many interacting proteins were known. In this chapter, we describe our efforts to characterize novel interaction partners of human TRPV4, identified based on a screening platform in mammalian cells.

The specific objectives are:

- Discover novel proteins interacting with human TRPV4
- Unravel consequences of the interaction on TRPV4 expression, transport and/or function.

In **Chapter 4**, we investigate chronic pain, a second example of a human condition where TRP channels are involved. Malfunctioning of molecular detectors in sensory neurons, which include several members of the TRP channel superfamily, can results in *rebellious somatosensation*, resulting in pain and hypersensitivity after tissue healing, without useful purpose. To combat the growing epidemic of chronic pain, there is a high medical demand for improved analgesics and a desperate need for novel targets to develop them. Multiple rodent studies designated TRPM3 as a potential target for novel analgesics. The channel is expressed in sensory neurons as a receptor for noxious heat, and antagonizing TRPM3 has beneficial effect in multiple rodent pain models. However, a translational link to human (patho-)physiology was missing, mainly due to the difficulties to obtain human sensory neuronal tissue. In this chapter, we describe the first studies aimed at characterizing the expression and function of TRPM3 in

human sensory neurons. This includes measurements on human DRG neurons from donors, but since this access to such tissue is limited in quantity and quality, we also aimed at implementing a more sustainable and alternative cellular model to study TRPM3 in a relevant human cellular context. We therefore evaluated the use of sensory neurons derived from human pluripotent stem cells as a model system to study TRPM3 function and pharmacology.

The specific objectives are:

- Validate functional TRPM3 expression in human sensory neurons
- Implement an accessible *in vitro* system of stem cell-derived human sensory neurons to study TRPM3 function and pharmacology

Chapter 3:

The zinc-finger domain containing protein ZC4H2 interacts with TRPV4, enhancing channel activity and turnover at the plasma membrane

This chapter has been published in the following paper:

***Vangeel L**, Janssens A, Lemmens I, Lievens S, Tavernier J and Voets T.*
*The zinc-finger domain containing protein ZC4H2 interacts with TRPV4, enhancing channel activity and turnover at the plasma membrane, (2020) Int J Mol Sci.*3.1

3.1 Abstract

The Ca^{2+}-permeable cation channel TRPV4 is involved in a broad range of physiological processes, including the regulation of systemic osmotic pressure, bone resorption, vascular tone and bladder function. Mutations in the *TRPV4* gene are the cause of a spectrum of inherited diseases (or TRPV4-pathies), which include skeletal dysplasias, arthropathies and neuropathies. There is little understanding of the pathophysiological mechanisms underlying these variable disease phenotypes, but it has been hypothesized that disease-causing mutations affect interaction with regulatory proteins. Here, we performed a Mammalian Protein-Protein Interaction Trap (MAPPIT) screen to identify proteins that interact with the cytosolic N terminus of human TRPV4, a region containing the majority of disease-causing mutations. We discovered the zinc-finger domain containing protein ZC4H2 as a TRPV4 interacting protein. In heterologous expression experiments, we found that ZC4H2 increases both the basal activity of human TRPV4 as well as Ca^{2+} responses evoked by ligands or hypotonic cell swelling. Using total internal reflection fluorescence (TIRF) microscopy, we further show that ZC4H2 accelerates TRPV4 turnover at the plasma membrane. Overall, these data demonstrate that ZC4H2 is a positive modulator of TRPV4, and suggest a link between TRPV4 and ZC4H2-associated rare disorders, which have several neuromuscular symptoms in common with TRPV4-pathies.

3.2 Introduction

The Transient Receptor Potential (TRP) superfamily consists of polyvalent ion channels expressed throughout the whole body. The 27 mammalian members, which are subdivided into six families (TRPA, TRPC, TRPM, TRPML, TRPP, and TRPV) based on amino acid homology, play important roles in cellular signaling and a variety of physiological processes, including mineral and glucose homeostasis, cardiac rhythmicity, kidney function, taste and somatosensation. In line with their important physiological roles, mutations in several TRP channel genes are the cause of monogenic human diseases [45].

One particular member, TRPV4, shows a complex relationship between gene mutation and disease. TRPV4 is a mechanosensitive channel expressed in multiple tissues, including brain, bone and various epithelial/endothelial cell layers [203]. Moderate heat, cell swelling and several endogenous (e.g. arachidonic acid and 5',6'-epoxyeicosatrienoic acid) and synthetic (e.g. 4α-PDD, GSK1016790A) ligands activate this non-selective cation channel. The broad expression and polymodal gating of TRPV4 is reflected in a wide range of physiological functions, and in the complex phenotype of TRPV4 knockout in mice. Although viable and fertile, TRPV4 knockout mice suffer from compromised vascular endothelial function, deficits in osmosensation, a higher bone density, and alterations in bladder function [203]. Mutations in the human *TRPV4* gene give rise to a broad spectrum of disease phenotypes, known as TRPV4-pathies [117, 120, 122, 140, 141, 204]. At this point, more than 70 mutations in the *TRPV4* gene have been identified [203], and the resulting TRPV4-pathy disease spectrum can be classified in three different groups based on the key symptoms [117]. A first group includes the skeletal dysplasias (SD), which are characterized by abnormalities of bone and cartilage growth, malformations in the skeleton, platyspondyly and defects in bone ossification [120, 130, 148]. A second group are the neuropathies, including distal or scapuloperoneal spinal muscular atrophy (SMA) and Charcot-Marie-Tooth (CMT) type 2C, which mainly present with motor and sometimes sensory defects [117, 122, 140, 141]. Thirdly, TRPV4 mutations are the cause of a rare and aggressive osteoarthropathy of the fingers and toes, known as digital arthropathy-brachydactyly (FDAB) [145]. Note, however, that in clinical practice the classification is often less strict, and patients sometimes exhibit a variable mixture of skeletal, motor and neuronal symptoms [117, 133].

It is intriguing to note that some mutations affect bones and joints without signs of neuropathy, others lead to neuropathies without obvious skeletal pathology, and still others lead to a mixed phenotype. At this moment, there is no clear understanding of the link between specific mutations and the resulting pathology [117, 204]. Although there seem to be some mutation hotspots, in particular the N-terminal Ankyrin repeat domain (ARD) or in the transmembrane segments, the location or nature of the mutations do not allow to predict the actual disease phenotype. Most strikingly, mutation of residue at position 183 of TRPV4 can cause a skeletal dysplasia [130] or CMT type 2C [131] depending on the introduced amino acid, with no phenotypic overlap between the cases. Moreover, there are substantial interfamilial differences regarding onset, symptoms and severity of the disease. In some cases, TRPV4-pathies are accompanied with further symptoms such as hearing loss, disturbed temperature regulation and vocal cord paralysis [116, 133].

It has been suggested that the divergent pathological effects of the disease-causing TRPV4 mutations in various tissues may involve altered interaction with cell-specific regulatory proteins [117]. In this study, we made use of the Mammalian Protein Protein interaction trap (MAPPIT) [205] to search for proteins that interact with the cytosolic N-terminus of TRPV4. We identified the zinc-finger domain-containing protein ZC4H2 as a TRPV4 interaction partner, and provide evidence that it augments TRPV4-mediated Ca^{2+} signals and enhances channel turnover at the plasma membrane. Interestingly, hereditary, heterozygous mutations in the X-linked *ZC4H2* gene are the cause of so-called ZC4H2-associated rare disorders (ZARDs) [206]), formerly referred to as Wieacker-Wolff syndrome or Miles-Carpenter syndrome. ZARD patients suffer from a complex set of neurological problems, including several symptoms that overlap with typical features of TRPV4-pathies, such arthrogryposis, distal muscle weakness, club foot and camptodactyly. Our results thus describe a newly identified TRPV4 interactor, and suggest a potential link between ZARD and TRPV4-pathies.

3.3 Results

3.3.1 Identification ZC4H2 as a TRPV4 interactor

We hypothesized that cell-specific regulatory proteins binding to and shaping TRPV4 function could play a role in the pathophysiology of TRPV4-pathies. In order to discover proteins interacting with human TRPV4, we performed a MAPPIT screening [205]. MAPPIT is a two-hybrid technology based on the functional complementation of a human type 1 cytokine receptor. Only when bait and prey physically interact, a functional receptor is obtained. Ligand binding leads then to cross activation of associated Janus Kinases which in turn phosphorylate and activate STAT complexes. After migration to the nucleus, these complexes induce specific target gene transcription. In this experimental set-up, STAT complexes trigger expression of a bioluminescence enzyme luciferase. This simple read-out enables high-throughput screening in a human cellular context, for covalent as well as transient and indirect interactions.

Considering the mutational hot spot in the ARD, a protein domain susceptible for protein interaction [207], we used the N-terminus of TRPV4 (AA 1- 466) as bait. As possible preys, the hORFeome v8.1 and OC collection was used, containing 14.817 clones [208, 209]. After a primary screening of the full prey collection (Figure 1A), 17 top hits were retested in a double plasmid system (TRPV4 N-terminus in pSEL and pCLG-plasmid backbone) with multiple controls, to assure signal specificity (Figure 1B). Based on the amplitude and specificity of the luciferase signals (Figure 1B), as well as on a literature search for known functions and subcellular localization of confirmed hits, we initially focused on three cytosolic proteins: ZC4H2, abLIM3, and PNMA1.

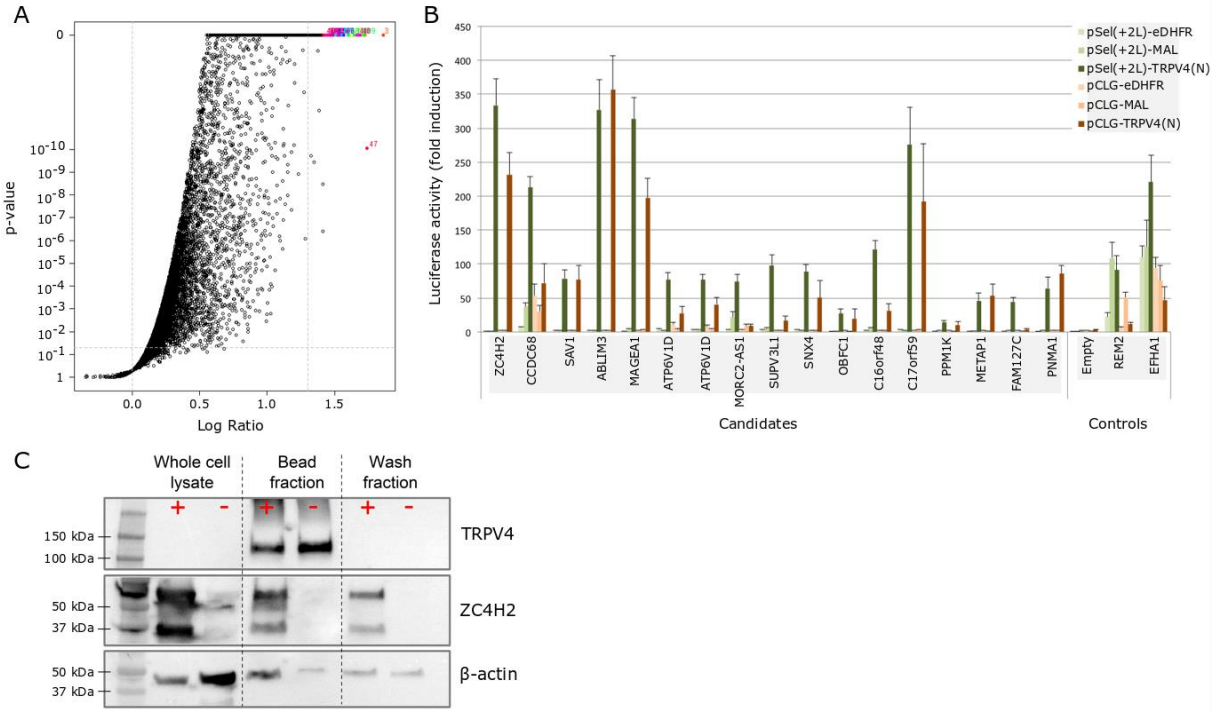


Figure 1: MAPPIT and co-IP show an interaction of ZC4H2 and TRPV4. (A) Vulcano plot of the whole protein library tested in an initial MAPPITscreen, with the p-value in function of the MAPPIT signal. Interesting 'hits' are located at the upper right corner. (B) Luciferase read-out of the top 17 hits identified in the MAPPIT screening. Baits cloned in pSEL and pCLG vector background were tested in parallel and contained either the human N-terminus TRPV4 or an irrelevant protein (eDHFR or MAL). Additionally, an empty prey plasmid was used as negative control. Positive assay controls are REM2 and EFHA1, two proteins known to bind the cytokine receptor complex, bait-independent. (C) HEK-293T cells were co-transfected with TRPV4-GFP and ZC4H2-mCherry (+) or TRPV4-GFP and mCherry (-). SDS-PAGE was performed for three conditions: the whole cell lysate, the fraction bound to GFP-Trap® beads, and the unbound (wash) fraction. Staining was done using specific antibodies for hTRPV4 (98 kDa), ZC4H2 (26 kDa) and β-actin (42 kDa). Note that in these experiments TRPV4 and ZC4H2 are coupled to GFP and mCherry, respectively, which increases the molecular weights of the detected proteins by 27kDa. In two independent experiments, we confirmed that there was negligible binding of mCherry and ZCH2-mCherry to the GFP-Trap® beads.

For these candidates, we performed qPCR-experiments to evaluate their expression in cell types relevant for TRPV4-pathies (Figure A1), including osteoclasts, osteoblasts, chondrocytes, sensory neurons and motorneurons. Levels of PNMA1 (paraneoplastic Ma antigen 1) were low or undetectable in all tested cell types. In osteoclasts, osteoblasts and chondrocytes, where TRPV4 expression was the highest (Figure A1), mRNA levels of actin binding LIM protein 3 (abLIM3) were low or below detection level, while higher levels were found in sensory neurons and motorneurons, where TRPV4 expression is low. Finally, the qPCR experiments showed substantial co-expression of TRPV4 and ZC4H2 in osteoblasts, osteoclasts and chondrocytes.

Interestingly, patients with mutations in the X-linked gene encoding the zinc-finger domain-containing protein ZC4H2 exhibit a highly variable clinical presentation, originally described as "Wieacker-Wolff" or "Miles-Carpenter syndrome" but recently confined into "ZC4H2-defiency" or ZC4H2-associated rare disorders (ZARDs) [206]. Generally, patients are diagnosed with intellectual disability accompanied by variable symptoms of central and peripheral nervous system involvement, including spasticity, hyperreflexia, muscle weakness, and arthrogryposis [206, 210], symptoms that are also present to a variable extent in patients with mutations in the *TRPV4* gene [116]. However, at this point, very little is known about the molecular expression, cellular function and (patho)physiological roles of ZC4H2. The 26-kDa protein, encoded on the X chromosome, contains a C-terminal zinc-finger domain, a coiled-coil

region and a nuclear localization signal. At the subcellular level, ZC4H2 partitions between nucleus and cytoplasm [210]. Expression of ZC4H2 is predominantly found in brain and CNS during embryonic development, where it is involved in neuronal development [210, 211], possibly via interaction with binding partners such as Smad3 and RNF220 [212, 213]. However, a clear molecular mechanism of ZC4H2 function or dysfunction has not yet be described. Considering the partial overlap of symptoms between ZARDs and TRPV4-pathies, and the substantial co-expression in relevant cell types, we focused our research on a potential functional interaction between ZC4H2 and TRPV4.

3.3.2 ZC4H2 binds to TRPV4 and enhances channel activity

As a first step, we confirmed the results from the MAPPIT screen by showing co-immunoprecipitation of TRPV4-GFP and ZC4H2-mCherry when co-expressed in HEK-293T cells (Figure 1C).

Next, we evaluated whether ZC4H2 influences TRPV4 function. We performed Fura-2-based imaging of the intracellular calcium concentration ($[Ca^{2+}]_i$) in HEK-293T cells expressing human TRPV4, which were co-transfected with either human ZC4H2 coupled to mCherry or with mCherry as control. At the mRNA level, expression of TRPV4 and ZC4H2 was very low in non-transfected HEK-293T, and increased by more than three orders of magnitude upon transfection (Figure A2). We observed a significant increase in baseline $[Ca^{2+}]_i$ levels in the cells co-expressing ZC4H2 compared to control (Figure 2A, B), indicating that basal TRPV4 activity is enhanced. Furthermore, the amplitude of the $[Ca^{2+}]_i$ rise in response to TRPV4 agonists was significantly larger in the presence of ZC4H2 (Figure 2A, C). This effect was stimulus-independent, as it was observed with different modes of activation, including the synthetic chemical agonists 4α-PDD, the endogenous ligand arachidonic acid and hypotonic cell swelling (Figure 2C, Figure A3). The decay of the $[Ca^{2+}]_i$ signal following TRPV4 activation by AA could be well fitted by a monoexponential function, and the resulting time constants were similar with or without ZC4H2 (243 ± 16 s for ZC4H2 *versus* 235 ± 7 s for control), which suggest that intracellular Ca^{2+} were not markedly affected. To investigate the specificity of the effect of ZC4H2, we performed similar experiments on TRPV3, the closest homologue of TRPV4. ZC4H2 was without effect on baseline $[Ca^{2+}]_i$ or on the response to the ligand 2-APB (Figure 2D, E). These results indicate that ZC4H2 does not affect functional expression of TRPV3, and argue against a general effect of ZC4H2 on intracellular Ca^{2+} handling.

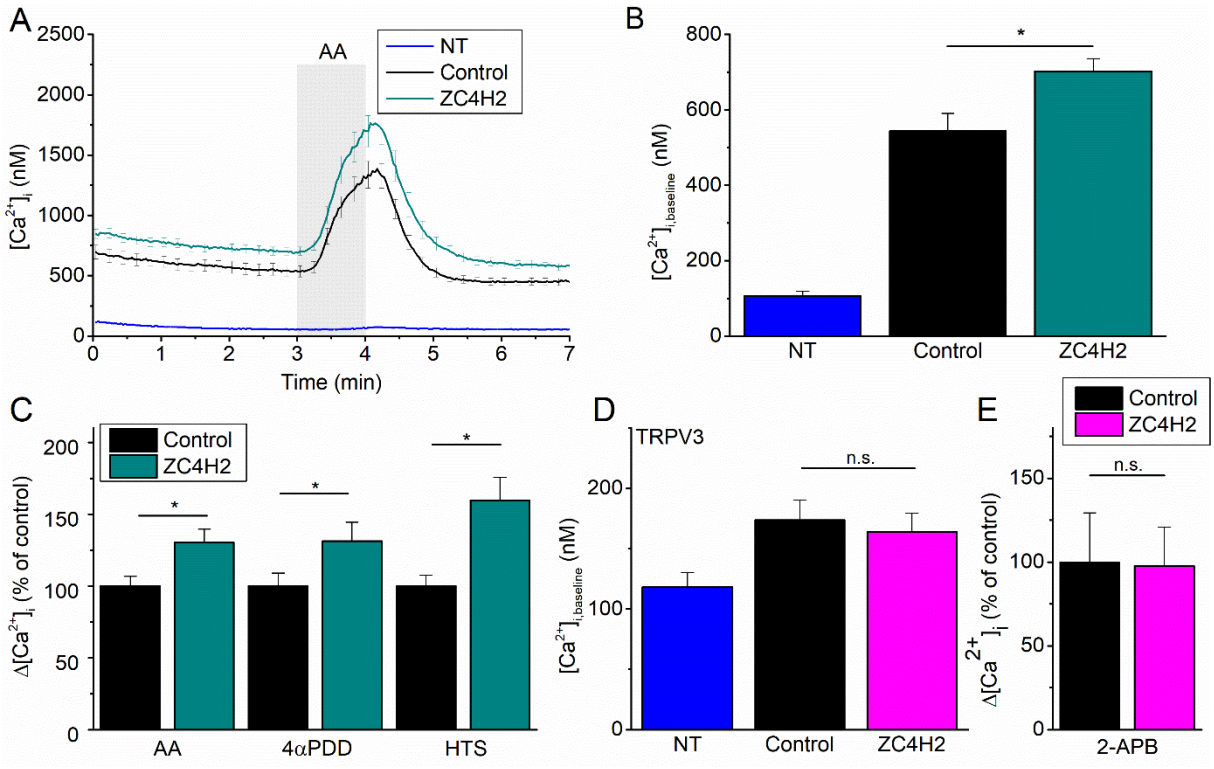


Figure 2: **Effect of ZC4H2 on TRPV4 channel activity.** (**A**) Time course of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (mean ± SEM) in non-transfected (NT) HEK-293T cells (n= 30), and cells co-transfected with TRPV4 and mCherry (control, n= 79) or ZC4H2-mCherry (n= 80), upon stimulation with Arachidonic acid (AA; 10µM) . (**B**) Mean baseline $[Ca^{2+}]_i$ in experiments as in (A). (**C**) Normalized $[Ca^{2+}]_i$ amplitudes in response to the TRPV4-activating stimuli AA (10µM), 4α-PDD (10mM) or hypotonic solution. Values are normalized to the response in cells expressing TRPV4 and mCherry (control). Representative traces are shown in Figure A3. (**D**) Co-expression of ZC4H2 is without effect on baseline $[Ca^{2+}]_i$ in cells expressing TRPV3 (n = 144 for ZC4H2 co-transfected, 183 for control, n = 26 for non-transfected). (**E**) Normalized $[Ca^{2+}]_i$ amplitudes in TRPV3 expressing cells in response to the agonist 2-APB (25µM). Values are normalized to the response of cells expressing TRPV3 and mCherry. P>0.05 (unpaired two-sampled t-test).

3.3.3 TIR-FRAP experiments unravels effect on TRPV4 turnover at the plasma membrane

When visualized using TIRF imaging, TRPV4 exhibited a typical plasma membrane staining, including expression in fine membrane protrusions, whereas the cytosolic protein ZC4H2 showed a uniform distribution in the cell, including but not limited to areas with high TRPV4 expression (Figure 3A). Consistent with the fact that ZC4H2 is a cytosolic protein and the observation that only a fraction of total ZC4H2 is bound to TRPV4 (Figure 1C), we did not observe specific colocalization of both proteins. There are several potential mechanisms that may explain the increased basal $[Ca^{2+}]_i$ and TRPV4-dependent stimulus responses in the presence of ZC4H2, including higher levels of channel expression, increased channel activation, or enhanced channel transport to the plasma membrane. First, we performed qPCR to address whether co-expression of ZC4H2 affects TRPV4 expression at the mRNA level. This analysis did not reveal any difference in mRNA levels between cells expressing ZC4H2 or the mCherry control (Figure 3B). Secondly, both the mean cellular GFP fluorescence (Figure 3C) as well as the total TRPV4 protein expression in whole cell lysates (Figure 3D, Figure A4) were similar in control and ZC4H2-expressing cells, indicating that ZC4H2 does not affect TRPV4 expression at the protein level. Third, biotinylation experiments to specifically probe for plasma membrane proteins did not reveal an effect of ZC4H2 on TRPV4 levels in the plasma membrane (Figure 3E, Figure A4). To further test this conclusion, we performed combined epifluorescence and TIRF imaging experiments to compare the

distribution of TRPV4 between the perimembrane area and the bulk cytosol. The ratio of GFP-fluorescence in the TIRF mode versus epifluorescence mode was unchanged in the absence or presence of ZC4H2 (Figure 3F, G). Taken together, these data indicate that ZC4H2 does not increase the expression levels of TRPV4, nor its steady-state presence at the plasma membrane.

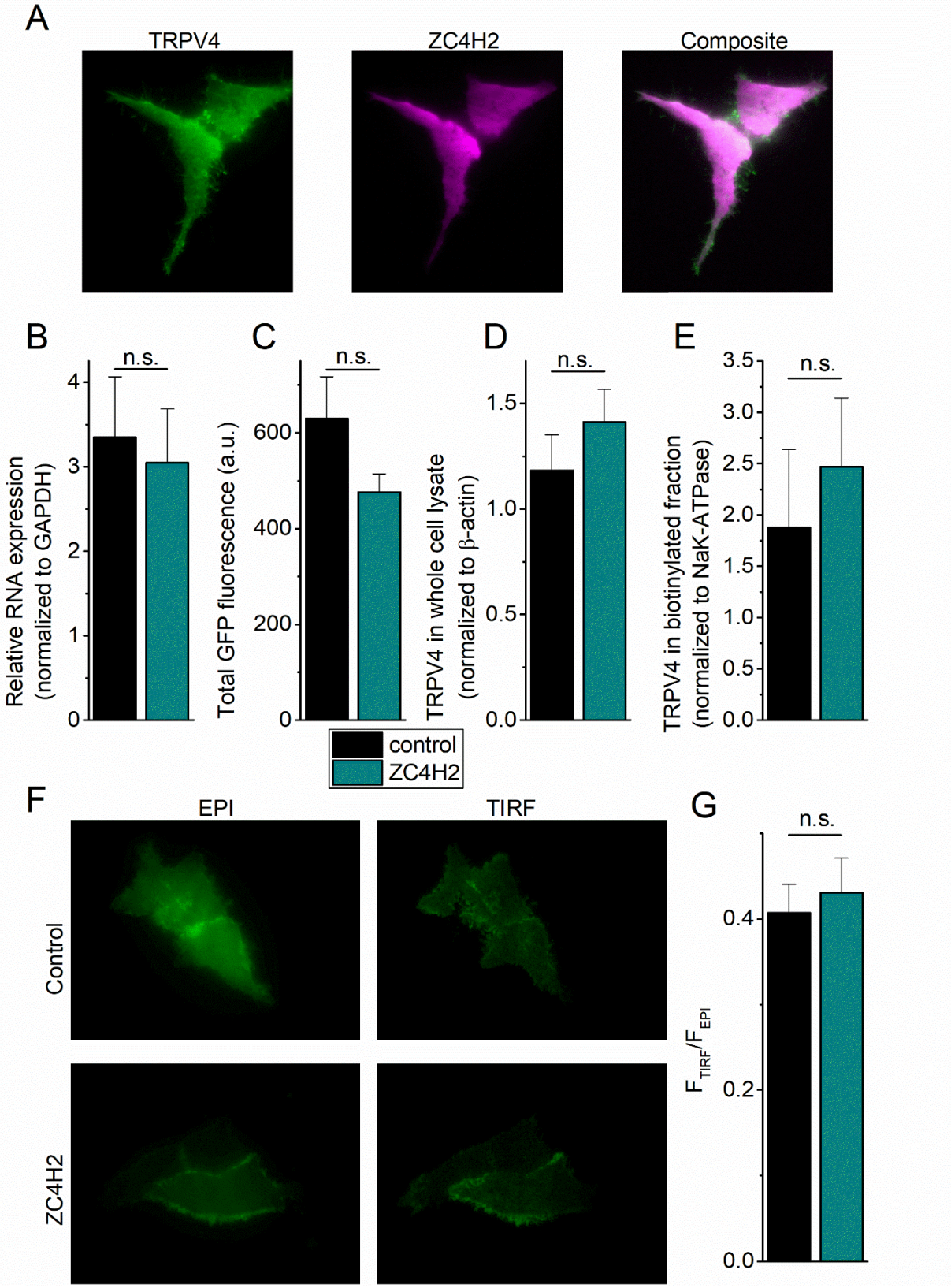


Figure 3: ZC4H2 does not affect expression or subcellular localization of TRPV4. (**A**) Representative TIRF images showing the localization of heterologously expressed TRPV4-GFP and ZC4H2-mCherry. (**B**) Relative mRNA expression of TRPV4 in cells co-transfected with ZC4H2 or mCherry control. Data are normalized to the housekeeping gene GAPDH. (n = 11) (**C**) Mean TRPV4-GFP fluorescence (EPI-mode). (n = 245 for ZC4H2, 220 for control) (**D**) Mean TRPV4 expression in whole cell lysate after SDS-PAGE. (n= 14) (**E**) Mean TRPV4 expression in the biotinylated fraction, normalized to the NaK-ATPase (plasma membrane marker). (n= 8) (**F**) Representative images showing TRPV4 GFP fluorescence measured in epifluorescence (EPI) mode and TIRF mode in cells co-transfected with ZC4H2 or mCherry control. (**G**) Ratio of TRPV4-GFP fluorescence in TIRF mode *versus* EPI mode, as an estimate of the distribution of TRPV4-GFP between the bulk cell and the perimembrane area in close vicinity of the coverslip. (n= 24 for ZC4H2, n= 21 control). Values are mean ± SEM.

Dynamic changes in TRP channel availability at the membrane may constitute an important regulatory mechanism for TRP channel function *in vivo* [44]. Yet, detailed knowledge regarding cellular trafficking kinetics or mechanisms of membrane incorporation and retrieval is available for only a limited number of TRP channels [39, 43]. To characterize the perimembrane dynamics of TRPV4 in the absence or presence of ZC4H2, we performed Total Internal Reflection – Fluorescence Recovery After Photo-bleaching (FRAP) experiments (Figure 4A). In these experiments, we used TIRF laser to selectively bleach the fluorescently labeled TRPV4 in close proximity of the glass-plasma membrane interface (Figure 4B), and followed the recovery of the GFP fluorescence in the evanescent field as a measure of the transport of new, unbleached TRPV4-GFP from the cell interior to the plasma membrane (Figure 4C). Intriguingly, whereas the pre-bleaching (baseline) TRPV4-GFP fluorescence was not different between control and ZC4H2-expressing cells, (Figure 4D), we observed significantly more extensive bleaching in the presence of ZC4H2 (Figure 4E). Following bleaching, fluorescence recovery was also clearly enhanced in the ZC4H2-expressing cells, such that the final recovered fluorescence at the end of the experiment was similar as in control cells (Figure 4E). To quantify the recovery process in more detail, we fitted the time-dependent fluorescence signal with exponential functions (Figure 4C). In the absence of ZC4H2, the recovery phase was generally well described using a monoexponential function, with an exponential time constant of 371 ± 83 s (Figure 4C,F). In contrast, two exponentials were required to describe the recovery of TRPV4-GFP fluorescence in cells expressing ZC4H2. The fast kinetic component was characterized by an exponential time constant of 23.7 ± 4.9 s, compared to 457 ± 71 s for the slow kinetic component (Figure 4C,F), the latter value being similar to the monoexponential time constant in control cells. The estimated amplitudes of the fast kinetic component amounted to approximately 40 % of the total recovery in the ZC4H2-expressing cells (Figure 4G). Note that we monitored the recovery phase for 800 s following bleaching, at which time point the recovery phase did not reach steady-state in a significant fraction of the cells. Therefore, a potential effect of ZC4H2 on slower components of the recovery process cannot be excluded at this point.

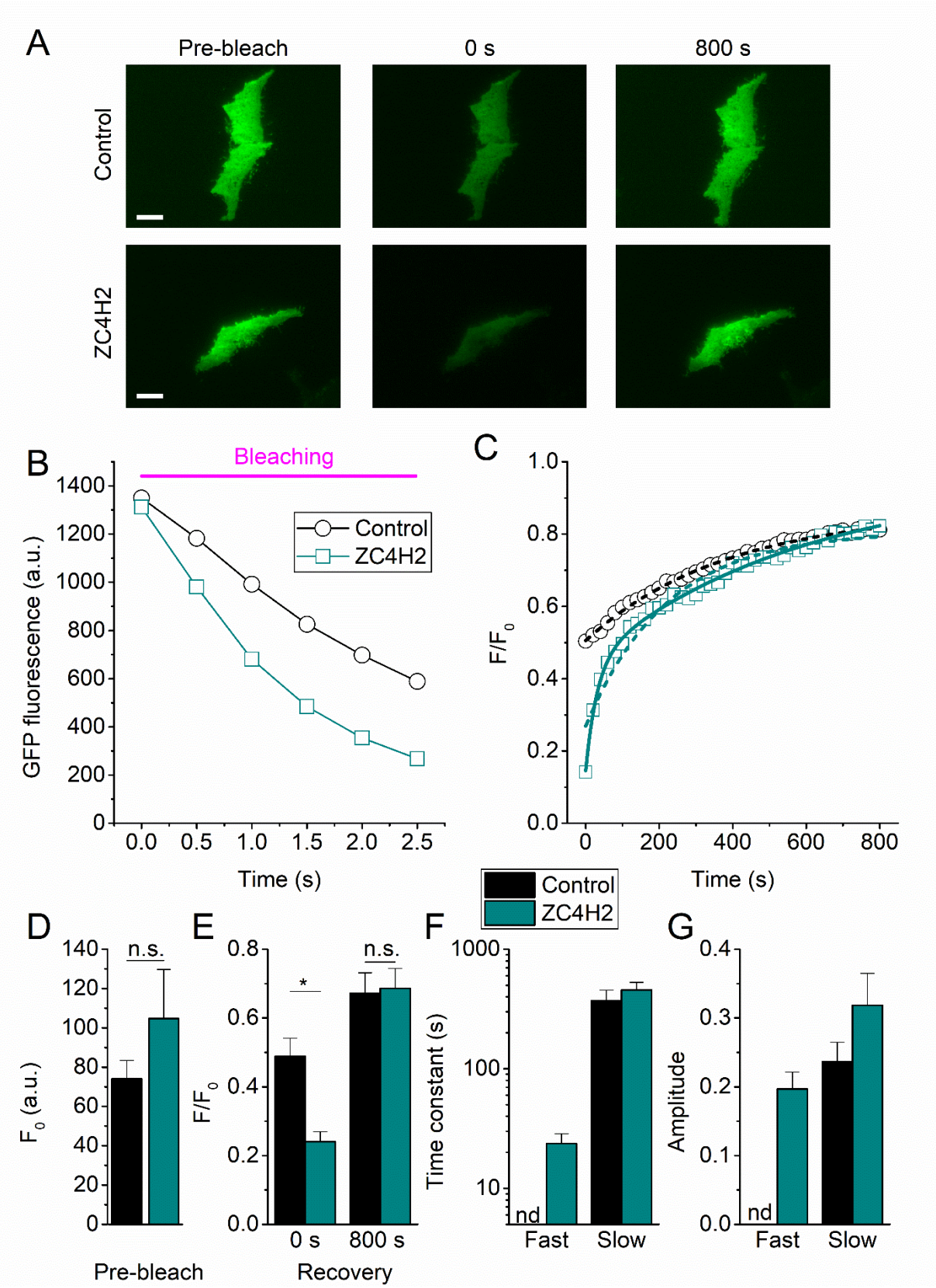


Figure 4: Effect of ZC4H2 on TRPV4 turnover at the plasma membrane assayed using TIR-FRAP. (A) TIRF images of TRPV4-GFP in HEK-293T cells co-expressing mCherry (control) or ZC4H2-mCherry. Images were taken before bleaching, and at 0 and 800s after bleaching. Scale bar = 10 µm. (B,C) Time course of the decay of TRPV4-GFP fluorescence during the bleaching process (B) and the recovery of the fluorescence following bleaching (C) in representative cells expressing ZC4H2 or control. Dotted and solid lines in C represent mono- and bi-exponential

fits, respectively. **(D)** Mean basal TRPV4-GFP fluorescence before bleaching, in control and ZC4H2-expressing cells. **(E)** Mean TRPV4-GFP fluorescence at 0 and 800s after bleaching. (**F,G**) Time constants and corresponding relative amplitudes obtained from exponential fits to recovery time courses as in (**C**). In control cells, a mono-exponential fit was generally sufficient to describe the recovery process. In ZC4H2-expressing cells, adequate fitting required a second, faster kinetic component. Number of cells in (**D-G**): control: n=16; ZC4H2: n=14.

3.4 Conclusion & discussion

In this study, we provide molecular and functional evidence for an interaction between the ion channel TRPV4 and the zinc-finger domain-containing protein ZC4H2. Based on a MAPPIT-screen, ZC4H2 was identified as one of the strongest interactors with the cytosolic N terminus of TRPV4, and this physical interaction was confirmed using co-immunoprecipitation in a heterologous expression system. Using $[Ca^{2+}]_i$ imaging, we further provide evidence that ZC4H2 enhances basal TRPV4 activity and potentiates responses to chemical ligands and osmotic cell swelling. ZC4H2 was without effect in cells expressing the closest homologue TRPV3, excluding a general effect on TRP channel activity or on cellular Ca^{2+} handling. Using TIR-FRAP experiments, we further demonstrate that ZC4H2 has a pronounced effect on the perimembrane dynamics of TRPV4. Indeed, we found that ZC4H2 accelerates both the bleaching and the recovery from bleaching of TRPV4-GFP, which can indicate a faster delivery to and retrieval from the plasma membrane. These findings may point toward a larger pool of rapidly recycling, TRPV4-containing periplasmic vesicles in cells expressing ZC4H2, potentially affecting the number of functional TRPV4 channels at the plasma membrane. Our biochemical and imaging experiments seem to argue against a pronounced effect of ZC4H2 on the number of functional channels, as they did not reveal any significant difference in the steady-state levels of TRPV4-GFP at the plasma membrane. However, we acknowledge that these approaches may not be sensitive enough to pick up changes in protein levels in the range of 20%, which would nevertheless be sufficient to explain the observed effects on $[Ca^{2+}]_i$. Moreover, there is evidence that TRPV4-mediated responses to mechanical and chemical stimuli not only depend on the activation of channels that were already present in the membrane, but also on the rapid recruitment of additional channels from a pool of rapidly cycling perimembrane vesicles [214-216]. Finally, we cannot exclude at this point that the altered trafficking and increased functionality represent two distinct actions of ZC4H2 on the cellular behavior of TRPV4. Taken together, we conclude that ZC4H2 represents a newly identified regulator of TRPV4 that interacts with the N-terminus, and thereby regulates channel function and turnover at the plasma membrane.

In earlier work, several other cytosolic proteins interacting with the N terminus of TRPV4 have been identified [203] (http://trpchannel.org/summaries/TRPV4), including Os-9, Pacsin3 and AIP4, but their effects on TRPV4 differed from what we found for ZC4H2. Binding of Os-9 to the N terminus of TRPV4 lowers the levels of TRPV4 in the plasma membrane by inhibiting the release of the channel from the ER [217]. In contrast, the interaction of the SH3 domain of Pacsin3 with a proline-rich domain in the N terminus of TRPV4 increases the levels of the channel in the plasma membrane, but inhibits basal activity as well as responses to heat and cell swelling [79, 80]. Finally, the HECT-family ubiquitin ligase AIP4 reduces plasma membrane levels of TRPV4 by facilitating its endocytosis [218, 219]. Moreover, evidence has already been presented in earlier work that channel activating stimuli such as shear stress or the agonist GSK 1016790A can induce recruitment of intracellular pools of TRPV4 to the plasma membrane [214-216]. It remains to be elucidated how these signals and interacting partners of TRPV4 contribute to functional channel expression at the plasma membrane in health and disease. Further research into the ZC4H2-TRPV4 interaction may provide further insights into TRPV4 channel trafficking and turnover at the membrane.

At this point, the cellular function of ZC4H2 is not well understood, and, as for TRPV4, the link between specific mutations in the *ZC4H2* gene and the resulting disease symptoms is elusive [206, 211, 213]. ZC4H2 contains a C-terminal zinc-finger domain characterized by four cysteine and two histidine residues. Expression is predominately found in brain and spinal cord in the embryonic stage [210]. ZC4H2 also contains a nuclear localization signal, and shuttles between the nucleus and the cytosol [210, 220]. ZC4H2 has been shown to interact with Smad signaling proteins [213] and with the ubiquitin E3 ligase Rnf220 [212, 221], and disease causing mutations may influence the transcriptional regulation of specific genes by these targets, thereby affecting neuronal development. Of note, although zinc-finger containing proteins are best known in the context of transcription in the nucleus, it is not uncommon that they affect protein function via direct interaction [222]. Notably, many disease-causing mutations in the *ZC4H2* gene affect the nuclear localization signal, leading to a more abundant presence in the cytosol [220, 223]. It will be of great interest to further investigate whether such cytosolic mutants are more prone to enhance TRPV4 function, and thereby provoke disease symptoms that are common to TRPV4-pathies and ZARDs.

3.5 Material & Methods

Cell culture

Human embryonic kidney cells (HEK-293T) were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, 2 U/ml penicillin and 2 mg/ml streptomycin (Gibco/Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidity controlled incubator with 10% CO_2. Regularly, potential cell contamination with mycoplasma species was tested using PlasmoTest - Mycoplasma Detection kit (InvivoGen, Toulouse, France). Cells were transiently transfected with 1µg cDNA encoding either ZC4H2-mCherry or mCherry cloned in the pcDNA3.1 vector together with 1µg of human TRPV4-GFP cloned in the PCIneo-vector, using TransIT®-293 Transfection Reagent (Mirus Corporation, Madison, WI, USA). On the next day, cells were reseeded on poly-L-lysine-coated (0.1 mg/ml) 25-mm glass coverslips with thickness of 0.16–0.19 mm (Gerhard Menzel GmbH, Braunschweig, Germany) for TIRF experiments, or 18-mm glass coverslips with thickness of 0.13–0.16 mm (Gerhard Menzel GmbH) for Ca^{2+}-imaging.

Protein expression analysis

The whole cell lysis buffer consisted of (in mM) 50 HEPES, 150 NaCl, 1.5 $MgCl_2$, 1 EDTA, 1 PMSF, 10% glycerol, 1% Triton X-100, supplemented with protease inhibitor cocktail (Sigma-Aldrich, Bornem, Belgium). For immunoprecipitation, ChromoTek's GFP-Trap® coupled to magnetic agarose beads (ChromoTek, Planegg – Germany) was used according to the manufacturer's protocol to pull down GFP-fused hTRPV4. For purification of the membrane protein fraction, biotinylation assays were performed on co-transfected HEK-293T cells using EZ-Link™ Sulfo-NHS-SS-Biotin (1mg/ml for 30min, Thermofisher Scientific, Massachusetts - USA) before whole cell lysis. Thereafter, biotin-bound membrane proteins were isolated using Pierce™ Streptavidin Agarose Resin beads (Thermofisher Scientific, Massachusetts – USA) according to the manufacturer's protocol. Next, whole cell lysate, bead-bound fractions, wash fractions and biotinylated fractions were prepared for SDS-page by adding 4-fold concentrated Laemmli sample buffer (Biorad, California – USA) substituted with 2-β mercaptoethanol (99%, Sigma-Aldrich, Bornem - Belgium) and heating to 95° for 5min. Samples were evaluated by SDS-PAGE using NuPAGE Novex Bis-Tris 4–12% Gels (Life Technologies) according to the manufacturer's protocol. Separated proteins were transferred to a PVDF membrane (Millipore, Billerica, MA - USA) and immersed for 1 hour in blocking solution (5% w/v nonfat dry milk in TBS containing 0.1% Tween-20). The membranes were probed with anti-hTRPV4 (in-house), ZC4H2 (ab100924, Abcam, 1/1000), β-actin (A1978, Sigma,

1/4000) and Na/K-ATPase (ab7671, Abcam, 1/2000) antibodies overnight at 4°C. Next, the membranes were washed in TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1/5000; Cell Signaling Technology Inc, Beverly, MA, USA) for 1 hour at room temperature. Immunoreactive complexes were visualized using ECL Western blotting detection reagent (GE Healthcare, Buckinghamshire, UK) and ChemiDoc MP Imaging System (version 5.01 Beta, Bio-rad Laboratories, Hercules, CA, USA). Results were analyzed using Image Lab Software (version 5.01 Beta, Bio-Rad Laboratories). After visualization, membranes were stripped (Re-blot plus mild solution, Merck-Millipore, Massachusetts - USA), washed, blocked and reblotted using the above described procedure. Quantification of protein bands was performed using Fiji analysis software.

MAPPIT

The mammalian-based screening tool MAPPIT (MAmmalian Protein-Protein Interaction Trap) [205], was used to identify interactors of the TRPV4 protein. The screening was performed using the hORFeome v8.1 library [208] and ORFeome Collaboration clones [209] as preys in HEK-293T cells. The bait consisted of the full intracellular N-terminus of TRPV4 (AA 1 – 466).

qPCR

RNA extraction from samples was done using RNeasy kits (Qiagen, Hilden - Germany) according to the manufacturer's instructions, followed by reverse transcription into cDNA using Ready-To-Go Youprime First-Strand beads (GE healthcare Life Sciences, Buckinghamshire - UK). Quantitative Real-time PCR was carried out using specific TaqMan™ gene expression assays accompanied with TaqMan universal mastermix (Applied Biosystems® Foster City – USA). Experiments were performed using a 7500 Real Time PCR system and software (Applied biosystems, Lennik – Belgium) using a 40-replication cycles protocol. Sample threshold were analyzed relative to the housekeeping gene HPRT or GAPDH as control (ΔC_t), and data are presented as ($2^{-\Delta Ct}$). Statistical analysis was performed on the average ΔC_t from three technical replicates for each sample. Tissues used were Dorsal Root Ganglia (DRG), embryonic spinal motor neuron tissue, and primary osteoclasts, chondrocytes and osteoblasts.

Ca^{2+} imaging

For intracellular Ca^{2+} measurements, cells were incubated for 30min with 2µM Fura-2 acetoxymethyl (AM) ester (Biotium, Hayward, CA, USA) in cell culture medium. Fluorescent signals were evoked during alternating illumination at 340 and 380 nm using a MT-10 illumination system (Tokyo, Japan) and CellM software from Olympus. Absolute Ca^{2+} concentrations were calculated from the ratio of these fluorescent signals as described before [224]. Experiments were performed at room temperature with standard extracellular perfusion buffer containing (in mM) 150 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to pH 7.4 using NaOH. For stimulation of the cells, the following modulators were used: GSK1016790A (10nM), 4αPDD (10µM), arachidonic acid (10µM) and ionomycin (1µM), all from Sigma-Aldrich (Bornem, Belgium). In experiments where osmotic responses were analysed, we used a modified isotonic solution containing (in mM): 105 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 90 mannitol, adjusted to pH 7.4 using NaOH. A hypotonic solution was obtained by omitting mannitol from the modified isotonic solution.

TIRF (Total Internal Reflection Fluorescence) microscopy

For TIRF experiments, cells were maintained in an extracellular solution containing (in mM): 150 NaCl, 6 KCl, 2 $CaCl_2$, 1,5 $MgCl_2$, 10 HEPES, adjusted to pH 7.4 with NaOH. Images were obtained at room temperature through an inverted Zeiss Axio Observer.Z1 microscope with 100X oil objective (numerical aperture of 1.45) and a Hamamatsu Orca-R2 camera, using Axiovision software. Fluorescence excitation

was performed with multidimensional acquisition using 488-nm (GFP) and 561-nm (mCherry) lasers at 2% of their maximal power. Incident fluorescent excitation angle in TIRF mode was adjusted based on the laser wavelength (between 65° and 68°) to evoke an evanescent wave with an expected decay length constant of 120 nm. Time series were recorded at intervals of 500 ms and constant focus was guaranteed by use of Zeiss Definite Focus module. Microscopy images were analyzed using AxioVision 4.8 digital image processing software (Zeiss), ImageJ and Origin 7.0 (OriginLab Corporation, Northampton, MA, USA).

For TIR – Fluorescence Recovery After Photo-bleaching (FRAP) experiments, bleaching of the samples was performed using full laser power (100%) at 400 ms exposure for 5 frames with an interval of 500ms. The recovery phase was measured at 3% laser power, with an exposure of 50 ms for 40 frames with an interval of 20s.

3.6 Supplementary information

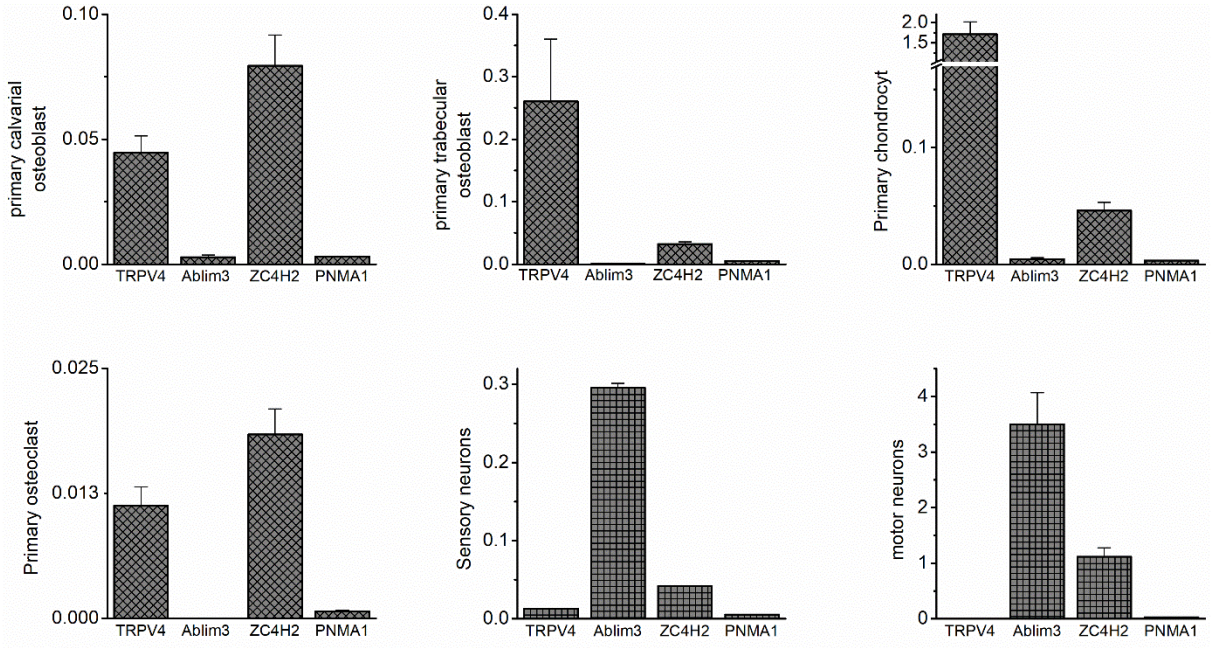


Figure A1: Initial evaluation of other MAPPIT hits. mRNA expression of TRPV4 and the indicated MAPPIT hits in the indicated mouse tissues. Values indicate relative expression normalized to the internal control HPRT. PNMA1 expression was low in all samples, while abLIM3 was primarily found in tissues with low TRPV4 expression.

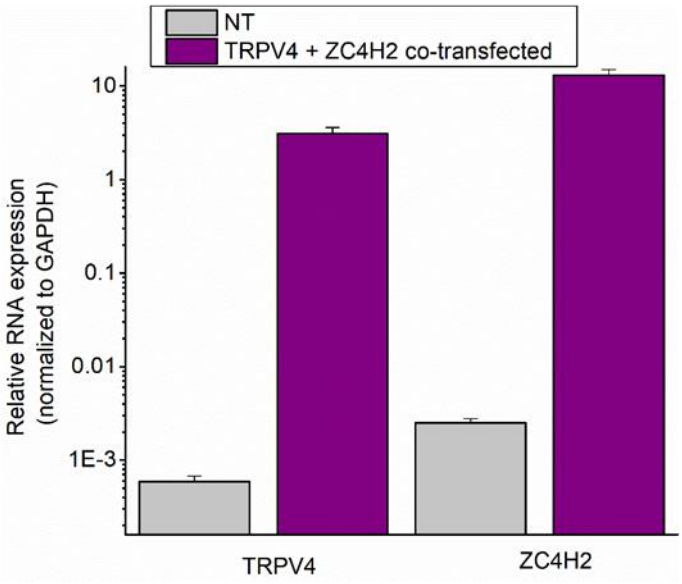


Figure A2: Expression levels of TRPV4 and ZC4H2 in HEK-293T cells. mRNA expression of TRPV4 and ZC4H2 in non-transfected (NT) cells and cell co-transfected with TRPV4-GFP and ZC4H2-mCherry.

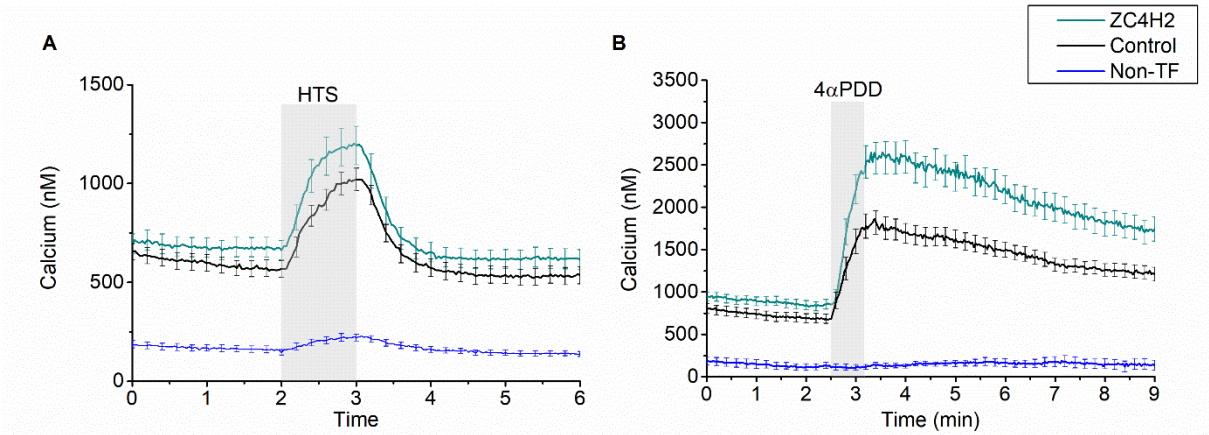


Figure A3: Effect of ZC4H2 on TRPV4 function is not stimulus specific. Representative time course traces of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (mean ± SEM) in non-transfected (NT) HEK-293T cells (n= 30), and cells co-transfected with TRPV4 and mCherry or TRPV4 and ZC4H2-mCherry, upon stimulation with (**A**) HTS and (**B**) 4αPDD (10µM).

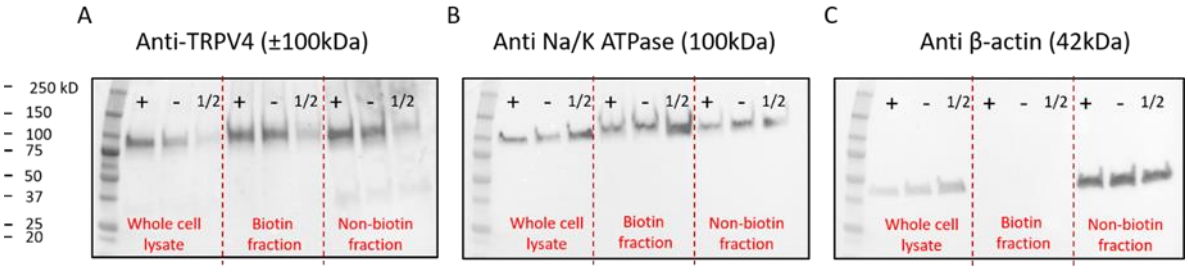


Figure A4: Biotinylation experiments do not reveal differences in TRPV4 membrane expression. Example blot probed for (**A**) TRPV4, (**B**) the membrane marker Na/K ATPase and (C) β-actin. The protein ladder (left) is followed by three lanes of whole-cell lysis samples, middle three lanes demonstrate the biotinylated membrane fraction, while the last three lanes consist of the non-biotinylated fraction. Samples are, from left to right; ('+') HEK-293T cells co-transfected with TRPV4 and ZC4H2, ('-') HEK-293T cells co-transfected with TRPV4 and control, ('1/2') HEK-293T cells transfected with only half the quantity of TRPV4 (0.5 µg).

Chapter 4:
Functional expression and pharmacological modulation of TRPM3 in human sensory neurons

This chapter has been published in the following paper:

***Vangeel L**, Benoit M, Miron Y, Miller PE, De Clercq K, Chaltin P, Verfaillie C, Vriens J and Voets T. Functional expression and pharmacological modulation of TRPM3 in human sensory neurons, (2020) Br J Pharmacol.*

4.1 Abstract

The transient receptor potential (TRP) ion channel TRPM3 functions as a noxious heat sensor and plays a key role in acute pain sensation and inflammatory hyperalgesia in rodents. Despite its potential as novel analgesic drug target, little is known about the expression, function and modulation of TRPM3 in the human somatosensory system. We studied TRPM3 in freshly isolated human dorsal root ganglion (hDRG) neurons and in human stem cell-derived sensory (hSCDS) neurons. Expression was analyzed at the mRNA level using RT-qPCR, and channel function was assessed using Fura 2-based calcium imaging and whole-cell patch-clamp recordings. TRPM3 was detected at the mRNA level in both hDRG and hSCDS neurons. The TRPM3 agonists pregnenolone sulphate (PS) and CIM0216 evoked robust intracellular Ca^{2+} responses in 52% of hDRG and 58% of hSCDS neurons. Whole-cell patch-clamp recordings in hSCDS neurons revealed PS- and CIM0216-evoked currents exhibiting the characteristic current-voltage relation of TRPM3. PS-induced calcium responses in hSCDS neurons were reversed in a dose-dependent manner by the flavonoid isosakuranetin and by antiseizure drug primidone. Finally, the μ-opioid receptor agonist DAMGO and the $GABA_B$ receptor agonist baclofen inhibited PS-evoked TRPM3 responses in a subset of hSCDS neurons. These results provide the first direct evidence of functional expression of the pain receptor TRPM3 in human sensory neurons, largely recapitulating the channel's properties observed in mouse sensory neurons. hSCDS neurons represent a valuable and readily accessible *in vitro* model to study TRPM3 regulation and pharmacology in a relevant human cellular context.

4.2 Introduction

Pain is a global problem, being the most common symptom why patients seek medical attention [225]. Contradictory to this high medical need, the available therapies to treat pain conditions are often inadequate. Indeed, about half of the chronic pain sufferers report insufficient pain relief with the prescribed medication, and opioid-based pain therapies are associated with serious side-effects, tolerance and addiction. In order to resolve this unmet need, preclinical pain research is exploring new analgesic strategies since decades [226], and there is a continuous quest for new pain targets [227, 228].

Transient Receptor Potential (TRP) channels are a superfamily of cationic channels with diverse properties and a broad repertoire of physiological functions [19, 229]. A subset of these TRP channels are expressed in sensory neurons, where they function as molecular chemo- and thermosensors in somatosensation and pain [192, 230]. As such, they represent promising therapeutic targets to treat pain at the periphery [191]. In particular, extensive preclinical and clinical research has been performed in the last two decades modulating the activity of the sensory neuron-specific TRP channels TRPV1, TRPA1 and TRPM8 to treat different forms of pain [195]. Whereas significant progress has been made in targeting these channels to treat pain in a variety of preclinical animal models, translation of these results to humans remains limited and challenging [231]. In particular, the species-specific expression profile and pharmacological modulation of several TRP channels turned out to be a significant hurdle in drug development [232-234]. Therefore, it is of utmost importance to investigate the expression and pharmacological modulation of targeted TRP channels in the context of human sensory neurons.

Recently, TRPM3 has been identified as a novel key player in somatosensation and pain [174]. TRPM3 is a calcium-permeable cation channel that is activated by heat and by chemical ligands such as the neurosteroid pregnenolone sulphate (PS) [235] and the potent synthetic ligand CIM0216 [236]. In mouse, TRPM3 is expressed in approximately 60% of somatosensory neurons, and plays a central role in the detection of noxious heat [183]. Strikingly, $TRPM3^{-/-}$ mice do not develop inflammatory

hyperalgesia in response to injection of complete Freud's adjuvant [183], and systemic TRPM3 antagonists were shown to alleviate mechanical and thermal hyperalgesia in mouse and rat models of inflammatory and neuropathic pain [200, 237, 238]. Moreover, TRPM3 activity in sensory neurons is strongly suppressed by μ-opioid receptor activation in sensory neurons, suggesting that TRPM3 may contribute to peripheral analgesic effects of opioids [185, 239, 240].

These data highlight the role of TRPM3 in nociception in acute and persistent pain models, emphasizing its significance as a novel target in the development of new analgesics [231]. However, at this point, very little is known about TRPM3 expression, function or modulation in human sensory neurons. Here, we describe functional expression of TRPM3 in a large subset of human dorsal root ganglion (hDRG) neurons, and show that human stem cell-derived sensory (hSCDS) neurons can be used as a valuable alternative to donor material to study the pharmacological properties and modulation of TRPM3 in a relevant human cellular context.

4.3 Results

4.3.1 Functional TRPM3 expression in human DRGs

We used RT-qPCR to evaluate mRNA expression of *TRPM3* and all other members of the TRP superfamily in DRG tissue from two human donors. These exploratory experiments indicate expression of *TRPM3* at levels comparable to those of other TRP channels known to be involved in somatosensation and pain, including *TRPA1*, *TRPV1* and *TRPM8* (Figure 1A). Next, we used Fluo-8-based intracellular Ca^{2+} imaging to probe functional TRPM3 expression in hDRG neurons from two additional donors. We found that a large subset of hDRG neurons (107 out of 206; 52%) displayed a robust Ca^{2+} response to two TRPM3 agonists: the neurosteroid PS (50 μM) and the synthetic TRPM3 agonist CIM0216 (1 μM) (Figure 1B,C; Supplementary Figure 1A). In these experiments, we also used capsaicin to probe for functional expression of TRPV1, an established marker of nociceptor neurons [15], and found that the majority of PS-sensitive neurons (77 out of 107; 72%) also responded to capsaicin (Figure 1B,C). Next, we tested the effect of the flavonoid isosakuranetin, a selective TRPM3 antagonist [241], on PS responses in hDRG neurons. Neurons were stimulated three times with PS, and the second application occurred in the presence of either isosakuranetin (10 μM) or vehicle control (Figure 1C). Responses to the second PS application were almost completely suppressed in the presence of isosakuranetin, and partially recovered upon washout (Figure 1D,E; Supplementary Figure 1B). The range of response amplitudes to the first PS application were similar in hDRGs from the two different donors (Supplementary Figure 1C). Taken together, these results demonstrate molecular and functional expression of TRPM3 in hDRG neurons.

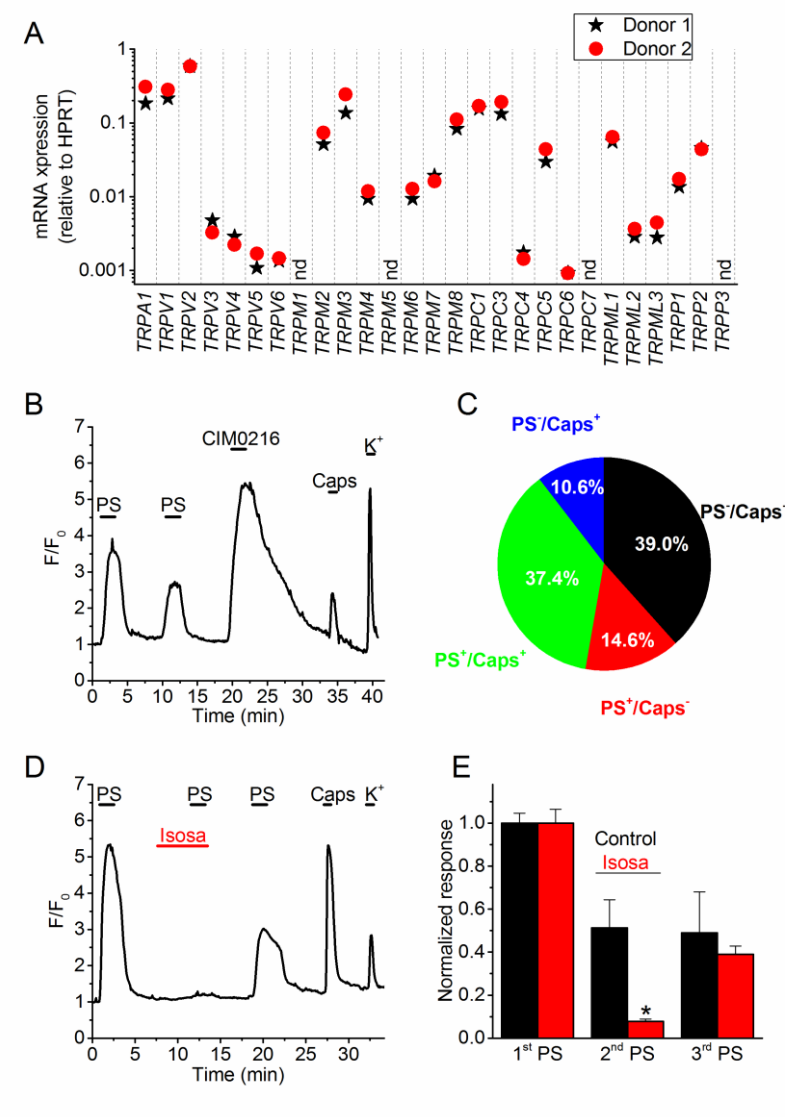


Figure 1: Functional TRPM3 expression in human DRG neurons. (A) Expression levels of TRP channel mRNA relative to *HPRT* in DRG neurons from two donors, determined using RT-qPCR. Access to fresh human DRG tissue is scarce, hence the limited number of samples in this exploratory experiment. nd: not detected. (B) Representative example of changes in Fluo-8-fluorescence in human DRG neurons in response to the TRPM3 agonists PS (50 μM) and CIM0216 (10 μM) and to the TRPV1 agonist capsaicin (200 nM). A high K^+ solution was applied at the end of the experiment to confirm neuronal excitability. A trace showing the mean ± SEM of the changes in Fluo-8-fluorescence for all cells in this particular experiment is provided in Supplementary Figure 1A. (C) Pie chart showing the distribution of neurons responding to PS, capsaicin or both (n=206 hDRG neurons). (D) Representative example showing the reversible inhibition of PS-induced responses in hDRG neurons by the selective TRPM3 antagonist isosakuranetin (20 μM). A trace showing the mean ± SEM of the changes in Fluo-8-fluorescence for all cells in this particular experiment is provided in Supplementary Figure 1B. (E) Normalized responses to repeated PS applications. Neurons were stimulated three times with PS, and the second application occurred in the presence of either isosakuranetin (10 μM; *n*=50) or vehicle control (*n*=39). *, P<0.05 (Two-Way Repeated Measures ANOVA with Tukey's post hoc test).

4.3.2 hESC-derived nociceptors represent a somatosensory phenotype

Access to fresh human DRG tissue for functional studies is restricted in most research laboratories, thus limiting their use as a model to study the pharmacology and modulation of TRPM3 in the context of human sensory neuron. As an alternative, we examined TRPM3 expression and function in sensory

neurons derived from human embryonic stem cells (hESC). We used a previously described 3-phased protocol, which results in the differentiation towards neurons that exhibit several hallmarks of sensory neurons, in particular small-diameter nociceptors [242, 243]. The resulting cultures of hSCDS neurons had the expected cellular morphology, consisting of clusters of small diameter cell bodies connecting through neurites. (Figure 2A). In whole-cell current-clamp experiments, differentiated hSCDS neurons had a resting potential of -52 ± 5 mV, and fired action potentials upon current injection with a threshold at -32 ± 8 mV (*n*=5; Figure 2B).

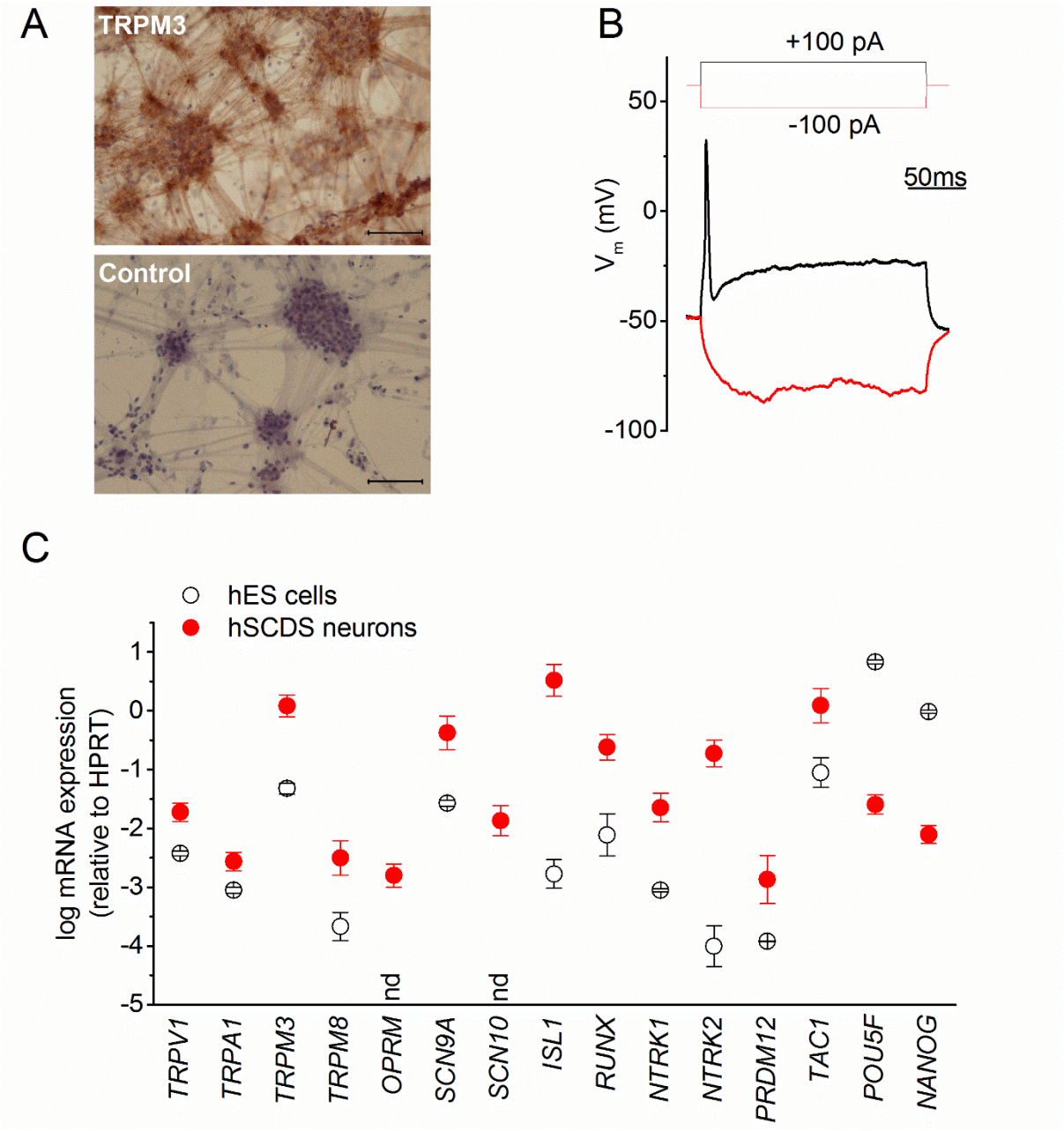


Figure 2: **Properties of hSCDS neurons.** (**A**) Brightfield image of immunohistochemically stained hSCDS neurons with anti-TRPM3 antibody and negative control (only secondary antibody) showing a neuronal morphology with cell body clusters interconnected by neurites. Brown peroxidase-based TRPM3 staining is visible in cell bodies as well as neurites. Scale bar: 500 µm. (**B**) Whole-cell current-clamp recordings on hSCDS neurons demonstrates the ability to fire action potentials upon current injection. (**C**) RT-qPCR comparing relative expression of sensory TRP channels, nociceptor marker genes and pluripotency genes between hES cells (black) and hSCDS neurons (red). Results represent mean ± SEM from 5 independent experiments with 3 technical replicates each. nd: not detected.

We used RT-qPCR to compare mRNA expression of relevant genes between hESC and differentiated hSCDS neurons (Figure 2C), based on markers used elsewhere [242, 243]. Differentiation resulted in a downregulation of pluripotency genes *POU5F1* and *NANOG*, whereas several sensory neuronal markers were significantly upregulated. These included the genes encoding the transcription factors RUNX1 and ISL-1, the neurotrophin receptors TrkA (*NTRK1*) and TrkB (*NTRK2*), the voltage-gated Na^+ channels Na_V 1.7 (*SNC9A*) and Na_V1.8 (*SNC10A*), the μ-opioid receptor (*OPRM1*) and the somatosensory TRP channels *TRPA1*, *TRPM8*, *TRPV1* and *TRPM3* (Figure 2C). Using an anti-TRPM3 antibody [244], we detected extensive immunostaining of cell bodies and neurites of hSCDS neurons (Figure 2A).

Using Fura-2-based intracellular Ca^{2+} imaging, we tested the functional expression of somatosensory TRP channels by examining responses to the chemical ligands menthol (a TRPM8 agonist and weak TRPA1 agonist), mustard oil (MO, a potent TRPA1 agonist), capsaicin (a potent TRPV1 agonist) and PS (Figure 3A). Pluripotent stem cells did not respond to high potassium solution nor to any TRP channel agonist. In hSCDS neurons, we observed responses to all four agonists in different, partly overlapping subsets (Figure 3A, B). Most prominently, 58% of all hSCDS neurons responded to PS, indicating widespread TRPM3 expression (Figure 3B). Overall, the neuronal responsiveness to TRP channel agonists corresponded well with the relative TRP channel expression at the mRNA levels determined by RT-qPCR (*TRPM3* > *TRPV1* > *TRPM8* > *TRPA1*). PS-induced calcium responses were fully and reversible inhibited by isosakuranetin, and all PS-responsive neurons also responded to CIM0216 (Figure 3C,D). Taken together, these results indicate that a large fraction of hSCDS neurons express functional TRPM3.

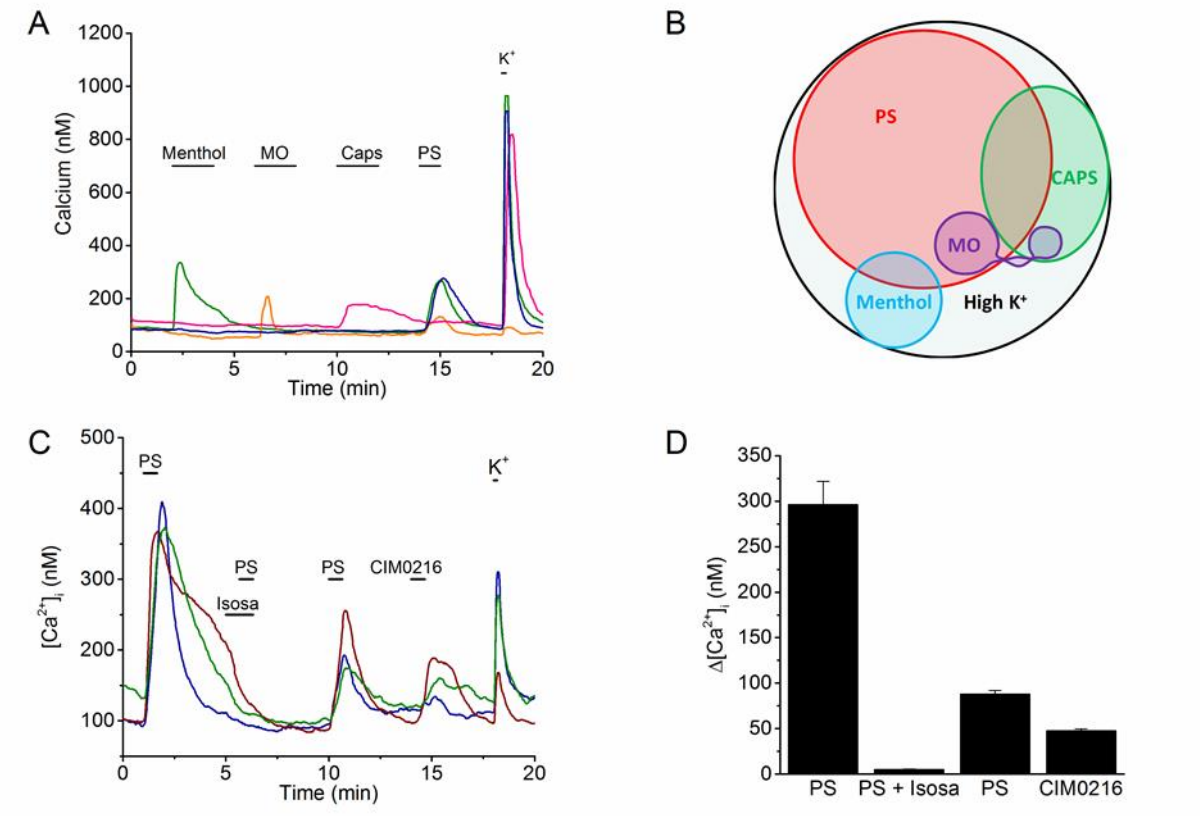


Figure 3: Functional expression profile of somatosensory TRP channels in hSCDS neurons. (**A**) Changes in intracellular calcium (>50nM) in single hSCDS neurons stimulated with the TRP channel agonists Menthol (100 μM), MO (100 μM), Capsaicin (1 μM) and PS (40 μM), and with a high K^+ solution to probe excitability. (**B**) Venn diagram showing the pattern of responses to TRP channel agonists in hSCDS neurons (*n*=1180 cells in 5 independent differentiations). (**C**) Intracellular calcium measurements showing reversible inhibition of PS-evoked

responses by isosakuranetin (5 µM). PS-responsive hSCDS neurons also responded to the synthetic TRPM3 agonist CIM0216 (1 µM). (**D**) Quantification of calcium responses for experiments as in panel C (*n*=72).

4.3.3 Electrophysiological properties of TRPM3 in hSCDS

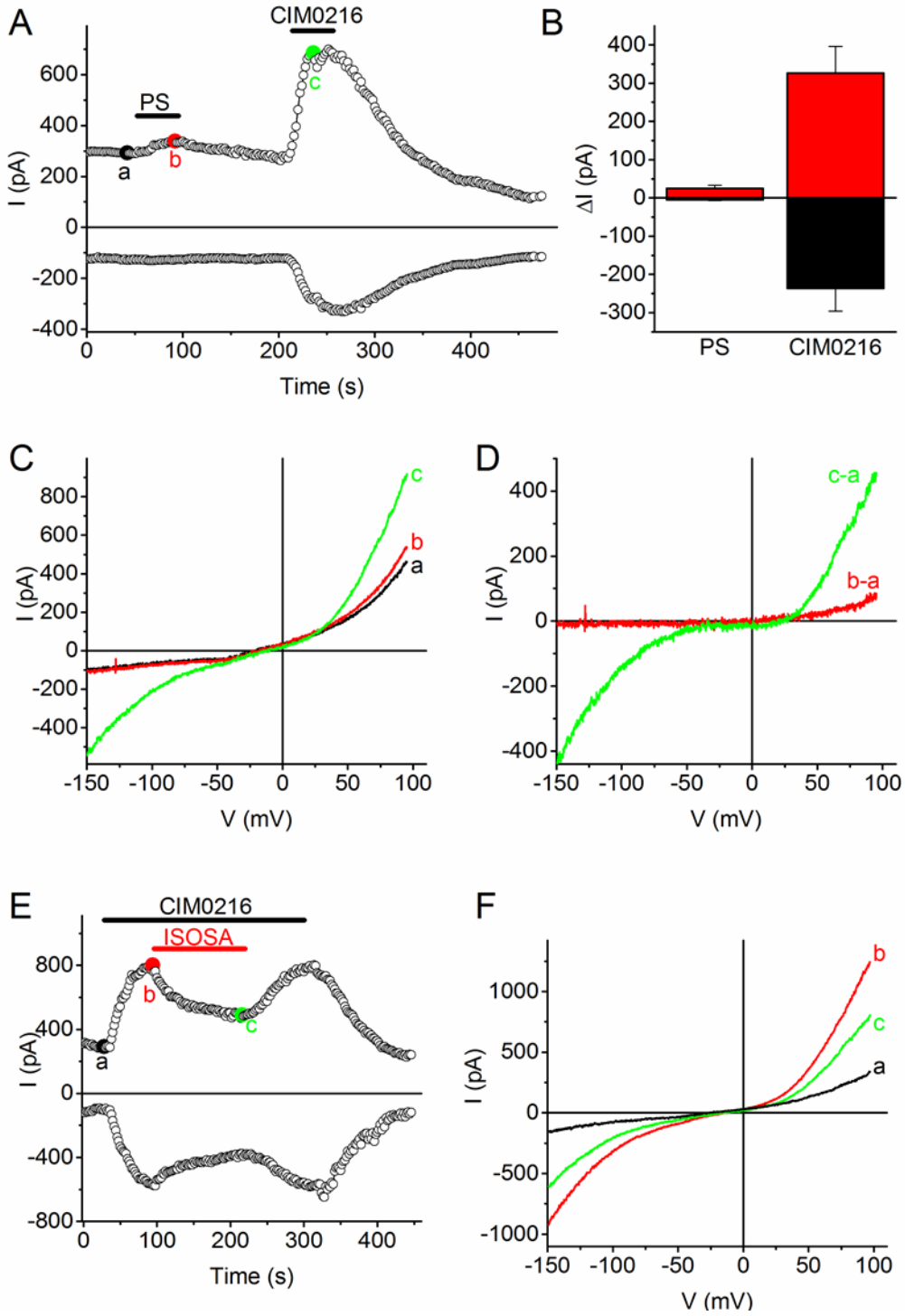


Figure 4: **Electrophysiological properties of TRPM3 in hSCDS neurons.** (**A**) Time course of inward (at -120 mV) and outward (at +80 mV) whole-cell currents in hSCDS neurons showing the effects of the TRPM3 agonists PS (40 µM) and CIM0216 (1 µM). (**B**) Quantification of the amplitude of inward and outward currents activated by PS and CIM0216 (*n*=6). (**C**) IV-relationships corresponding to the indicated time points in panel A; black (a): baseline, Red (b): PS-activated, green (c): CIM0216 activated current. (**D**) Difference currents illustrate the typical outward rectification of PS-activated currents and double rectification of CIM0216-activated currents. (**E,F**) CIM0216-

activated whole-cell currents were partially and reversibly inhibited by Isosakuranetin (5 µM). Colored traces represent; black (a): baseline, red (b): CIM0216 activated, green (c): current blocked by Isosakuranetin.

Next, we performed whole-cell patch-clamp recordings to characterize the electrophysiological properties of TRPM3-mediated currents in hSCDS neurons. In these experiments, extracellular Ca^{2+} was omitted to reduce channel desensitization [181]. The agonists PS and CIM0216 reversibly activated whole-cell currents in hSCDS neurons (Figure 4A, B). PS-activated currents were relatively small and outwardly rectifying, and therefore primarily detectable at positive potentials (Figure 4A-D). Currents activated by CIM0216 were much more robust, and showed characteristic double rectification (Figure 4A-D). Moreover, these currents were reversibly inhibited by isosakuranetin (5 µM; Figure 4E, F). The shape of the current-voltage relations (outwardly rectifying for PS *versus* doubly rectifying in the presence of CIM0216; Figure 4C,D,F) are in accordance with the properties of heterologously expressed mouse TRPM3, and of endogenous TRPM3-mediated currents in mouse sensory neurons and beta cells [181, 235, 236].

4.3.4 Pharmacological modulation of TRPM3 in hSCDS neurons

The high levels of functional TRPM3 expression in the hSCDS neurons render them an excellent *in vitro* model to study human TRPM3 in a relevant cellular environment, and to compare its pharmacological properties with those of heterologously expressed and/or rodent TRPM3. To evaluate the concentration-dependence of the agonistic effect PS, we used Fura-2-based intracellular Ca^{2+} imaging and measured responses to PS concentrations increasing from 1 to 300 µM. This assay yielded a concentration-dependent increase in intracellular Ca^{2+} with an EC_{50} of 48 ± 3 µM and a Hill coefficient (n_H) of 2.1 ± 0.1 (Figure 5A, B). In comparison, PS activates heterologously expressed mouse TRPM3 tested using calcium imaging or voltage-clamp with EC_{50} values of 1 µM and 23 µM, respectively [235, 245].

To determine the concentration dependence of the TRPM3 antagonists isosakuranetin and primidone [238, 241], we stimulated hSCDS neurons with PS and then applied increasing concentrations of the inhibitory compound (Figure 5C, D). The percentage of antagonist-induced inhibition was calculated after correction for rundown of the response to a sustained PS application in the absence of antagonists (Figure 5C). This approach yielded IC_{50} values of 659 ± 18 nM for isosakuranetin (n_H = 1.9 ± 0.1) and 705 ± 28 nM for primidone (n_H = 1.7 ± 0.1; Figure 5D).

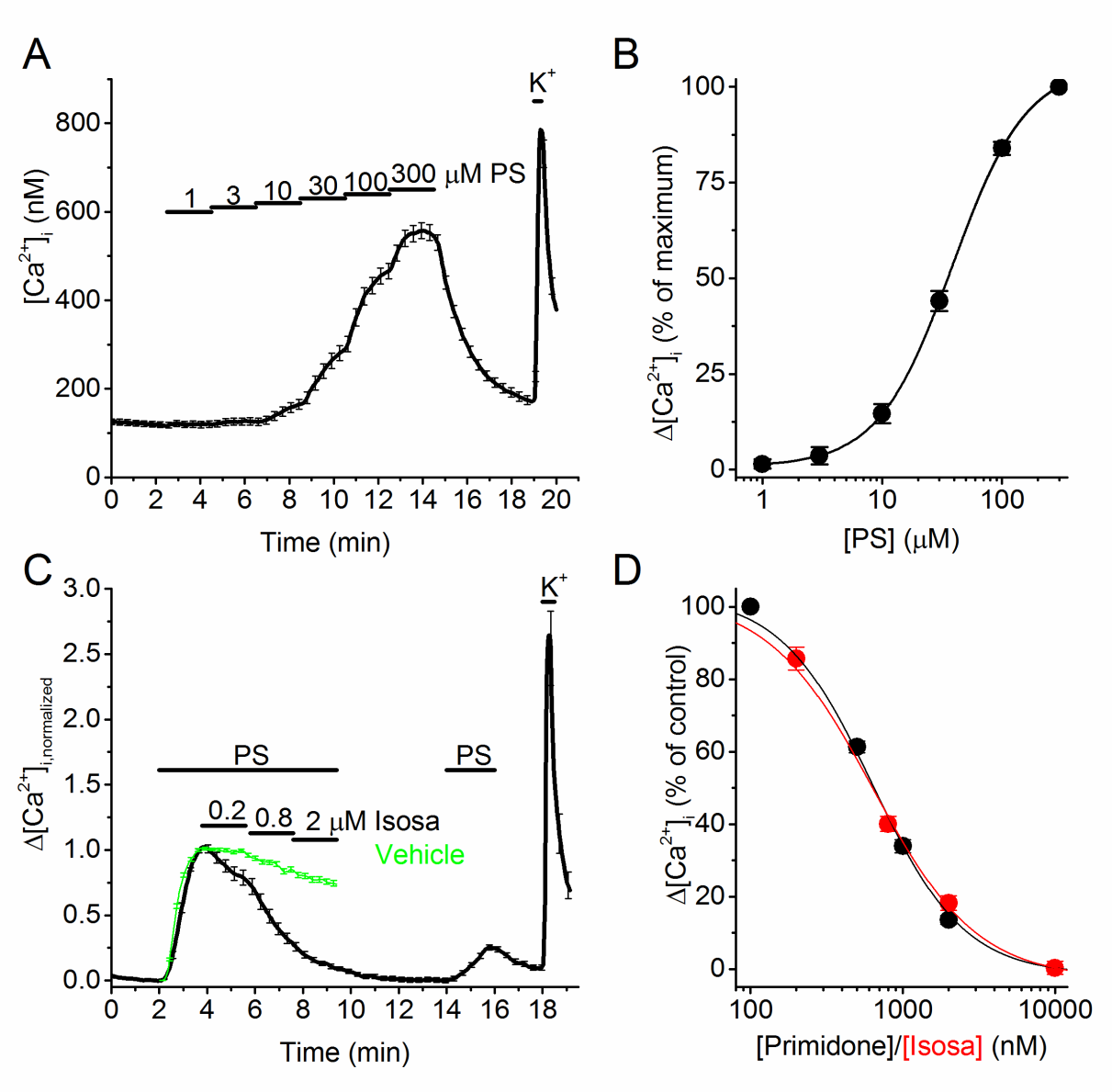


Figure 5: **Pharmacological characterization of TRPM3 in hSCDS neurons.** (**A**) Time course of the intracellular Ca^{2+} concentration in PS responding hSCDS neurons upon increasing the concentration of PS from 1 to 300µM. (**B**) Concentration dependence of the PS-induced calcium response in hSCDS neurons. Responses were normalized to the maximal response (*n*=117). Solid line represents a fit using a Hill function. (**C**) Normalized calcium traces showing the effect of increasing concentrations of the TRPM3 antagonist isosakuranetin on the response to PS (40 µM). Green trace represents the vehicle control, showing mild rundown of the signal in the absence of antagonist. (**D**) Concentration-response curve for the inhibition of PS-evoked calcium responses by isosakuranetin (*n*=93; red squares) and primidone (*n*=61; black dots). The mean normalized calcium response of vehicle-treated hSCDS neurons (green trace in panel C) was used as control. Solid line represents a fit using a Hill function.

In rodents, it has been previously shown that TRPM3 activity is suppressed following activation of a variety of G protein-coupled receptors, including the µ-opioid receptor, via a mechanism involving the $G_{\beta\gamma}$ subunit of trimeric G proteins [185, 186, 239, 240]. Considering the role of TRPM3 in nociception, it has been proposed that this pathway could contribute to the analgesic effects of peripheral opioids. To investigate whether µ-opioid-dependent regulation is conserved in hSCDS neurons, we performed Fura-2-based intracellular Ca^{2+} imaging and tested the effect of DAMGO, a selective µ-opioid agonist [246], on the response to PS (Figure 6A). DAMGO caused a rapid and reversible inhibition of the PS response in hSCDS neurons, with a mean inhibition of 24%. Notably, the extent of the DAMGO-induced inhibition

varied substantially between neurons (Figure 6A, B). Indeed, in some neurons the PS response was suppressed by more than 80% by DAMGO, whereas in other neurons the inhibition was more moderate or absent (Figure 6A, B). Overall, DAMGO inhibited the PS response by at least 20% in 50% (115 out of 230) of hSCDS neurons. In contrast, subsequent addition of isosakuranetin inhibited the PS response in all tested neurons (Figure 6A). In a similar approach, we also tested the effect of baclofen, an agonist of the $GABA_B$ receptor [185, 186]. Like DAMGO, baclofen evoked a rapid and reversible inhibition of the PS response (Figure 6C). Notably, the inhibitory effect of baclofen was more pronounced than that of DAMGO neurons, with a mean inhibition of 61 % (Figure 6D). Overall, baclofen inhibited the PS response by at least 20% in 98% of the hSCDS neurons (Figure 6D).

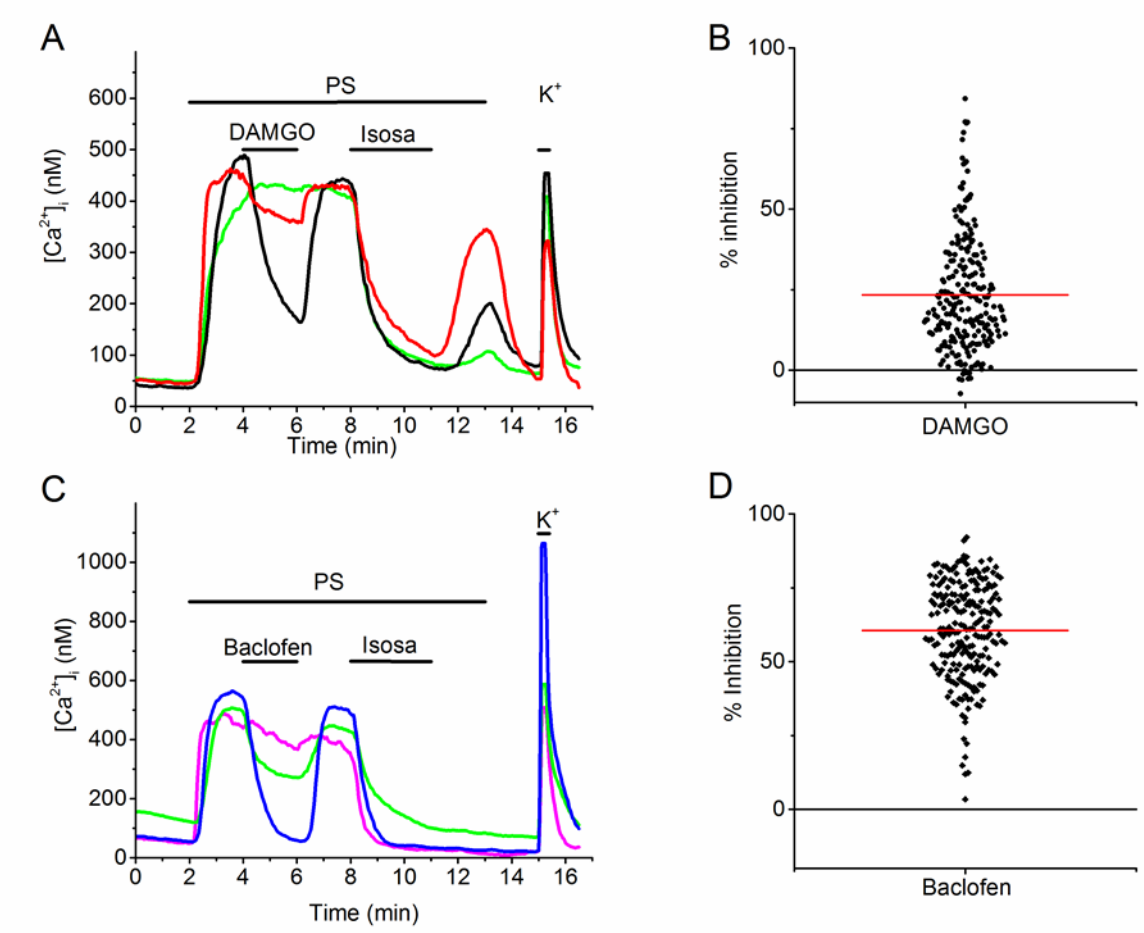


Figure 6: **Modulation of TRPM3 in hSCDS neurons by G protein-coupled receptors.** (**A**) Example intracellular calcium measurements showing variable degrees of inhibition of PS-induced responses upon activation of endogenously expressed μ-opioid receptors using DAMGO (300nM). (**B**) Scatter plot showing the range of DAMGO-induced inhibition of PS responses in hSCDS neurons. Red line represents the mean percentage of TRPM3 inhibition caused by DAMGO (data from 6 experiments, with hSCDS neurons from 3 different differentiations). (**C**) Example intracellular calcium traces showing inhibition of PS-induced responses by the $GABA_B$ receptor agonist baclofen (25 μM). (**D**) Scatter plot showing the range of baclofen-induced inhibition of PS. Red line represents the mean percentage of inhibition caused by baclofen (data from 6 experiments, with hSCDS neurons from 2 different differentiations).

4.4 Conclusion & discussion

In recent years, research in rodents has revealed that the cation channel TRPM3 plays a central role in the somatosensory pathway, where it is involved in acute noxious heat sensing and in the development

of inflammatory hyperalgesia [183, 247]. Moreover, pharmacological inhibition of TRPM3 alleviates pain in a variety of preclinical models in mice and rats [237, 238, 241], highlighting the potential of TRPM3 as a target for novel analgesic drugs [248]. However, whether TRPM3 is implicated in somatosensation and pain in humans remains unknown. In this study, we have evaluated for the first time the functional expression and pharmacological properties of TRPM3 in the context of human sensory neurons. In the first part, we demonstrated that TRPM3 is functionally expressed in a large subset of human DRG neurons. The majority of these TRPM3-expressing human DRG neurons also responded to capsaicin, indicating that they represent TRPV1-expressing nociceptors. In the second part, to overcome the limited availability of sensory neurons from human donors [249], we examined TRPM3 in nociceptor-like neurons derived from human embryonic stem cells. We found that hSCDS neurons obtained following a 3-phased small molecule inhibitor protocol [243] can be used to study the properties and pharmacological modulation of human TRPM3. Our results demonstrate that the majority of hSCDS neurons express functional TRPM3, which can be readily assayed using whole-cell patch-clamp recordings and intracellular calcium measurements. Therefore, hSCDS represent as an accessible and unlimited model to study the pharmacological properties and modulation of TRPM3 in the context of human sensory neurons.

It is now well established that at least four TRP channels (TRPV1, TRPM8, TRPA1 and TRPM3) function as thermo- and chemoreceptors in the somatosensory system of rodents [174, 175, 177, 183, 192, 230, 250-253]. We found that these four channels are also expressed at similar levels in bulk mRNA isolated from DRG neurons from human donors. Importantly, we here provide the first direct evidence for functional TRPM3 expression in human somatosensory neurons, by showing responses to the agonists PS and CIM0216 in 52% of human DRG neurons, which were reversibly inhibited by the flavonoid TRPM3 antagonist isosakuranetin. In line with earlier findings in mice [174, 183], our results also indicate significant coexpression of TRPM3 with TRPV1, as 75% of PS-responsive neurons also responded to capsaicin. These results indicate that TRPM3 is abundantly expressed in human nociceptors, supporting a role for the channel in human somatosensation and pain.

We also investigated the molecular and functional expression of TRPM3 in hSCDS neurons. These *in vitro* derived neurons exhibit the defining properties of primary nociceptive neurons, including the mRNA expression of the nociceptor-specific neurotrophin receptor TrkA, the voltage-gated sodium channels $Na_V1.7$ and $Na_V1.8$, and the capsaicin receptor TRPV1 [243]. Moreover, in line with earlier work using human stem cell-derived sensory neurons [243, 254, 255], agonists of TRPV1, TRPA1 and TRPM8 evoked calcium responses in different subsets of neurons. It should be noted, however, that the percentage of TRPV1 and TRPA1 positive neurons (21% and 4%, respectively), is substantially lower than what has been described in mouse and human DRGs, where typically 30 - 50% of the neurons respond to capsaicin or mustard oil (Supplementary Figure 2). In contrast, we found that 58% of hSCDS neurons exhibited functional expression of TRPM3, which is comparable to the fraction of TRPM3-positive DRG neurons found in human or mouse DRG [183]. Overall, these results indicated that hSCDS functionally express four sensory TRP channels implicated in thermosensation and pain, but also indicated quantitative differences in the expression patterns of these channels compared to other *in vitro* models.

Given the abundant functional expression of TRPM3, we used hSCDS neurons to further study the properties of human TRPM3 in the context of nociceptor-like neurons. In whole-cell patch-clamp recordings, PS activated small, outwardly rectifying currents, in line with earlier findings in heterologous expression systems showing that TRPM3 is a voltage-dependent channel whose open probability increases upon depolarization [181, 183, 256]. Notably, TRPM3 currents were strongly potentiated in the presence of CIM0216, and exhibited a characteristic double rectification pattern. Earlier work using heterologously expressed mouse TRPM3 has shown that the CIM0216-induced inwardly rectifying

current component is mediated by an alternative permeation pathway in TRPM3, distinct from the central pore and located in the voltage sensing domain [181, 236]. Taken together, endogenously expressed TRPM3 in hSCDS largely recapitulates the electrophysiological characteristics of heterologously expressed mouse TRPM3 [181].

Currently, there are only few pharmacological tools that can be used to inhibit TRPM3 function *in vitro* and *in vivo*. The most widely used experimental TRPM3 antagonists are on the one hand isosakuranetin and related plant-derived flavonoids [241], and on the other hand the anticonvulsant primidone [238]. These compounds inhibit rodent TRPM3 at submicromolar concentrations, and suppress TRPM3-mediated pain behavior in mice [238, 241]. Using Fura-2-based calcium imaging, we measured the concentration dependence of the inhibition of TRPM3 by isosakuranetin and primidone in hSCDS neurons. Both compounds were able to reverse PS-induced calcium responses in a concentration-dependent manner, and the IC_{50} value for primidone (705 nM) closely matches the published IC_{50} value obtained for mouse TRPM3 expressed in HEK293 cells [238]. For isosakuranetin we obtained an approximately 10-fold higher IC_{50} value in comparison to the value obtained for heterologously expressed mouse TRPM3 [241]. This difference in IC_{50} value may reflect variation in isosakuranetin affinity between species or may be related to the expression of different TRPM3 isoforms. Alternatively, the differences may reflect the distinct experimental approaches used to determine the concentration dependence. Indeed, Straub et al. pretreated their cells with isosakuranetin for 2.5 minutes before applying PS [241], whereas isosakuranetin was applied after full development of the PS response in our experiments.

Interestingly, it was recently shown that activation of the μ-opioid receptor inhibits TRPM3-mediated responses in mouse sensory neurons, via a mechanism that involved direct interaction of $G_{\beta\gamma}$ with the channel. Accordingly, peripherally applied opioids such as morphine or DAMGO were shown to inhibit TRPM3-dependent pain responses in mice [185, 239, 240], and it has been suggested that this mechanism may contribute to the peripheral analgesic effect of opioids [185, 239]. We observed that PS responses were rapidly and reversibly inhibited by DAMGO in about 50% of the hSCDS neurons. Such a variable inhibition of TRPM3 by μ-opioid receptor activation was also reported in isolated mouse sensory neurons, where PS responses were insensitive to DAMGO or morphine in between 5 and 30% of the neurons [185, 239, 240]. The variable inhibition of the TRPM3 responses may reflect the unequal expression of the μ-opioid receptor or its downstream signaling pathway in sensory neurons. In this respect, we observed a more profound less variable inhibition of TRPM3-dependent responses in hSCDS neurons upon activation of the $GABA_B$ receptor using baclofen [185, 186]. Overall, our data indicate that modulation of TRPM3 via the GPCR-$G_{\beta\gamma}$ pathway is conserved in human sensory neurons.

There is a high unmet need for effective and safe analgesic treatments, fueling continuous research into novel molecular players in the pain pathway [226-228]. In this respect, sensory TRP channels have been intensively pursued as pain targets ever since the identification of the capsaicin receptor TRPV1 more than two decades ago [228, 231]. However, despite promising preclinical results with antagonists of TRPV1, TRPA1 and TRPM8 in various animal models of acute and chronic pain, effective treatments in humans are still outstanding [231]. In particular, translating findings in rodent models to humans has been challenging, in part due to species-specific differences in TRP channel expression, function and pharmacology [232-234]. Analyzing channel regulation and pharmacology in a relevant human cellular context therefore represents an important step in the development of novel TRP channel-based pharmacotherapies. Our present results provide the first characterization of TRPM3, a more recently identified pain target, in human DRG neurons, and reveal hSCDS neurons as an unlimited resource to study its pharmacological modulation. Thus, hSCDS neurons represent an instrumental human nociceptor-like cellular model that can be used in the development of TRPM3-specific antagonists for

the treatment of various forms of pain. Recently, a *de novo* mutation in TRPM3's S4–S5 linker region was identified in seven probands with developmental and epileptic encephalopathy, which in some patients was associated with markedly altered pain and heat thresholds [49]. As such, hSCDS neurons represent an attractive cellular model to investigate the consequences of such mutations on TRPM3 function.

To conclude, we have demonstrated functional expression of TRPM3 in a large subset of human DRG neurons and in human nociceptor-like neurons derived from embryonic stem cells. In this cellular context, human TRPM3 exhibits similar electrophysiological and pharmacological properties as have been described earlier in rodents, including reversible inhibition upon activation of μ-opioid and $GABA_B$ receptors. These results support the hypothesis that TRPM3 is an important molecular player in the human pain pathway. Our findings demonstrate that hSCDS neurons represent an accessible human cellular model to study TRPM3 pharmacology and regulation, which may be highly instrumental for the development of TRPM3-targeting therapies to treat various forms of pain.

4.5 Material & Methods

Human DRG neurons isolation and culture

All human DRGs used for this study were obtained from brain-dead organ donors in the United States after obtaining informed consent in accordance to state and federal regulations, and United Network for Organ Sharing (UNOS) policies. All donor tissue collections for the DRGs used in the present study were performed in accordance to Federal and State regulations using protocols approved by the Organ Procurement Organization. DRGs from the first thoracic vertebra (T1) through the first sacral vertebra (S1) from 4 donors were used in the present study. The DRGs used for RT-PCR analysis were from two male donors aged 19 and 47 years, respectively. The DRGs used for calcium imaging were from one 49-years-old male and one 33-years-old female. These latter DRGs were stripped of connective tissue and enzymatically digested at 37°C for 2 hrs using the methods described elsewhere [257]. Dissociated cells were seeded on 96-well plastic bottom plates (Corning) that had been pre-coated with poly-D-lysine. Cells were maintained in culture at 37°C with 5% CO2 in DMEM/F12 supplemented with 10% horse serum (Thermo Fisher Scientific), 2 mM glutamine, 10 ng/mL hNGF (CellSignaling Technology), 10 ng/mL GDNF (Peprotech), and penicillin/streptomycin (ThermoFisher Scientific). Half of the culture medium was replaced with fresh medium every 3 days, and experiments were performed between 3 and 6 days after isolation [257].

Embryonic stem cell culture and differentiation to sensory neurons

Human Embryonic stem cells (WA09, WiCell®, Madison - USA) were grown on 6-well plates coated with hESC-qualified Matrigel® Matrix (corning®, Massachusetts - USA) and incubated at 37°C with 5% CO_2. Medium (½ Essential 8™ Flex medium (Gibco Life Technologies, Grand Island NY - USA) and ½ StemFlex (Gibco)) was changed every two days and fortified with RevitCell™ (Gibco). Cells were passaged every 5-7 days. Briefly, cells were washed with phosphate buffered saline (PBS) and treated with Versene® EDTA 0.02% (Lonza, Verviers - Belgium) for 1 min at room temperature. Thereafter, cells were collected from the bottom of the well using a cell scraper, and the mixed cell suspension was plated onto freshly coated plates.

To differentiate hESC cells to sensory neurons, we used a previously described protocol [242, 243, 258]. Briefly, neuronal differentiation protocol is executed in different phases, starting with the formation of neurectoderm [259]. Confluent hESC were dissociated using 5 min incubation with Accutase™ (Sigma-Aldrich, Missouri - USA), and replated at a high density on Matrigel® matrix covered 6 well plates. Two

days later, fully confluent cells were ready for neuronal induction (day 0). Neural induction medium was prepared as a 1:1 mixture of N2 medium (DMEM/F12, 1X N2 supplement (Gibco), 5 µg/ml Insulin, 1mM L-Glutamine, 1X MEM Non-essential Amino Acid Solution, 90µM β-mercaptoethanol and 50U/ml Pen + 50mg/ml Strep) and B27 medium (neurobasal medium with 1X B27 supplement (Gibco), 1mM L-glutamine, 50U/ml Pen + 50mg/ml Strep), and spiked with small molecule inhibitors LDN193189 (1µM, STEMCELL technologies, USA) and SB431542 (10µM, Tocris bioscience, Bristol - UK). Medium was changed every other day until day 5. After, neuronal precursors were driven into a sensory neuron phenotype using a tailored differentiation and maturation protocol [242, 243]. From day 5 until day 10, specification was performed using N2B27 neuronal induction medium supplemented with CHIR99021 (5µM), DAPT (5µM; both from STEMCELL technologies), SU5402 (5µM; Tocris bioscience), LDN193189 (1µM; STEMCELL technologies) and SB431542 (10µM; Tocris bioscience) with medium changes every other day. On day 6 of the protocol, cells were transferred into 24-well plates using a 5 min incubation with Accutase™ (Sigma-Aldrich) at 37°C and replated into Matrigel® matrix-coated 24-well plates with or without glass coverslips (see below) for continuation of the protocol. Sensory neuron maturation into mature nociceptors (day 15 – day 30) consisted of medium changes with DMEM/F12 (10% FBS) (Gibco), supplemented with neurotropins BDNF (10ng/ml, STEMCELL technologies), GDNF (10ng/ml, STEMCELL technologies), NT3 (10ng/ml, STEMCELL technologies), NGF (10ng/ml, peproTech, London - UK) and Ascorbic acid (200µM, Sigma-Aldrich) three times a week. An in-depth characterization of the derived neurons, including an expression profile of several neuronal and nociceptive markers was performed in [243].

RNA extraction and RT-qPCR

RNA isolation from hDRGs and hSCDS neurons was done using the RNeasy Mini Kit (Qiagen, Hilden - Germany) according to manufacturer's instructions. Quantity and quality of the obtained RNA was checked using DropSense 16 (Trinean, Gent – Belgium). cDNA was synthesized with Ready-To-Go YouPrime First-Strand Beads (GE Healthcare Life Sciences, Buckinghamshire - UK) and used for quantitative RT-PCR using specific TaqMan™ gene expression assays (see table) together with the accompanying TaqMan universal mastermix (Applied biosystems® Foster city, USA). Experiments were carried out using a 7500 Real time PCR system and software (Applied biosystems, Lennik - Belgium), using a protocol of 40 replication cycles. Sample threshold cycles were analyzed relative to housekeeping gene HPRT as endogenous control (ΔC_t), and data are shown as $2^{-\Delta Ct}$ (mean ± SEM).

TRPA1:	Hs00175798_m1
TRPM3:	Hs00257553_m1
TRPM8:	Hs01066596_m1
TRPV1:	Hs00218912_m1
SCN9A (Nav1.7):	Hs01076699_m1
SCN10A (Nav1.8):	Hs01045137_m1
GAPDH:	Hs99999905_m1
OPRM1 (μ-opioid R):	Hs01053957_m1
HPRT1:	Hs01003267_m1
Isl-1:	Hs00158126_m1
NTRK1 (TrkA):	Hs01021011_m1
NTRK2 (TrkB):	Hs00178811_m1
PRDM12:	Hs00964106_m1
NANOG:	Hs02387400_g1
CALCA (CGRP):	Hs01100741_m1
POU5F1B:	Hs04995079_g1
RUNX1:	Hs01021970_m1
TAC1:	Hs00243225_m1
TRPV2	Hs00901640_m1

TRPV3	Hs00376854_m1
TRPV4	Hs01099348_m1
TRPV5	Hs00219765_m1
TRPV6	Hs00367960_m1
TRPM1	Hs00170127_m1
TRPM2	Hs01066071_m1
TRPM4	Hs00214167_m1
TRPM5	Hs00175822_m1
TRPM6	Hs00214306_m1
TRPM7	Hs00292383_m1
TRPC1	Hs00608195_m1
TRPC2	Hs03453918_m1
TRPC3	Hs00162985_m1
TRPC4	Hs01077392_m1
TRPC5	Hs00202960_m1
TRPC6	Hs00175753_m1
TRPC7	Hs00220638_m1
TRPML1	Hs01100653_m1
TRPML2	Hs00401920_m1
TRPML3	Hs00217663_m1
TRPP1	Hs00165517_m1
TRPP2	Hs00175850_m1
TRPP3	Hs00950467_m1

Functional assays

For intracellular calcium imaging, human dorsal root ganglion neurons were loaded with 3μM Fluo-8-AM (AAT Bioquest) containing 0.1% Pluronic F-127 (Sigma-Aldrich) for 20 min. at room temperature. Extracellular solution contained (in mM): 145 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose adjusted to pH 7.4 with NaOH. Fluo-8-loaded cells were excited at 480nm and emission was collected at 520nm with a pcoEDGE sCMOS camera (PCO) mounted on an inverted microscope (Olympus IX71). Images were acquired at 0.2 Hz for 10 min in constant perfusion of the extracellular solution while adding the agonist and/or antagonist in the perfusion. Image acquisition and data analysis were performed using MetaMorph software (Molecular Devices). At the end of the experiment, a high-K^+ solution was applied, which contained (in mM) 100 NaCl, 50 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose adjusted to pH 7.4 with NaOH. Cells that did not respond to this depolarizing solution were considered to be either unhealthy or non-neuronal, and omitted from analysis.

Intracellular calcium measurements in hSCDS neurons were performed after incubation with 2μM ratiometric calcium indicator FURA 2-acetoxymethyl (AM) for 30 min. Fluorescence was measured during alternating illumination at 340 and 380 nm using an Eclipse Ti (Nikon) fluorescence microscopy system, and absolute calcium concentration was calculated from the ratio of the fluorescence signals at these two wavelengths ($R = F_{340}/F_{380}$) as $[Ca^{2+}] = K_m \times (R-R_{min})/(R_{max}-R)$, where K_m, R_{min} and R_{max} were estimated from *in vitro* calibration experiments with known calcium concentrations [224]. The standard bath solution contained (in mM) 150 NaCl, 6 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, pH 7.4 with NaOH. Mustard oil (MO), pregnenolone sulphate (PS), capsaicin, menthol, primidone, DAMGO ([D-Ala^2, *N*-$MePhe^4$, Gly-

ol]-enkephalin) and Baclofen were obtained from Sigma-Aldrich, CIM0216 from Tocris Bioscience, and isosakuranetin from CarlRoth. At the end of each experiment, cells were subjected to a depolarizing high-K^+ solution containing 100 NaCl, 50 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, pH 7.4 with NaOH, and non-responding cells were considered to be either unhealthy or non-neuronal and hence omitted from analysis.

Whole-cell patch-clamp experiments were performed using an EPC-10 amplifier and associated PatchMasterPro software version V2x73.5 (HEKA Elektronik). In current clamp recordings, the pipette solution contained (in mM): 140 aspartic acid, 10 NaCl, 10 EGTA, 1 $MgCl_2$, 10 HEPES, pH 7.2 with KOH. In voltage-clamp recordings, the pipette solution contained 100 aspartic acid, 45 CsCl, 10 EGTA, 1 $MgCl_2$, 10 HEPES, pH 7.2 with CsOH. The extracellular solution contained (in mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4 with NaOH. Note that Ca^{2+} was omitted from the external solution to attenuate rundown of TRPM3 currents [181]. Pipettes had resistances between 2.5 and 6 MΩ when filled with pipette solutions. Approximately 50-70% of the series resistance was compensated before starting the measurements.

In current-clamp experiments, steps of current injections of increasing amplitude ranging between -200 and +200 pA were applied, until an action potential was evoked. The threshold voltage was then defined as the voltage at the inflection point, i.e. where the second derivative of the voltage trace changes from negative to positive.

The voltage protocol in voltage-clamp experiments consisted of 200-ms inverted voltage ramps from +100 mV to -150 mV, applied at 0.5 Hz from a holding potential of -70 mV. The use of the inverted ramp resulted in an effective suppression of voltage-gated Na^+ currents through voltage-dependent inactivation, whereas voltage-gated K^+ currents were minimized by the use of a Cs^+-based intracellular solution and voltage-gated Ca^{2+} currents by the omission of extracellular Ca^{2+}.

Immunohistochemical staining

hSCDS neurons on glass coverslip were fixated for 1 min in ethanol/formol (4%) and subsequently washed with TBS (0.01M) and TBS-T (0.1% Tween 80). Cells were permeablized using 0.25% Triton X-100. After blocking, TRPM3 antibody (Alomone Labs, Cat# ACC-050, RRID:AB_10918820; 0.2µg/ml) was incubated overnight. Later, coverslips were washed with TBS-T and blocked in 1% milk before using secondary antibody (PO conjugated AffiniPure Goat anti-Rabbit IgG, Jackson immunoresearch). Brown reaction product was obtained by ImmPACT DAB (Vector laboratories) and nuclei were stained with Mayer's Haematoxylin. Finally, coverslips were mounted using fluorescence mounting medium (Depex mounting medium, BDH Prolabo). Immunohistochemistry has been conducted conform to BJP Guidelines [260]. We note that the specificity of the anti-TRPM3 antibody for TRPM3 has not been fully established in human cells.

Data analyses

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology [261]. Group data are shown as mean ± SEM. In this study, *n* refers to the number of analyzed hDRG neurons or hSCDS neurons. Since the experiments were aimed at describing the functional properties of TRPM3 in human sensory neurons, and do not include direct statistical comparisons between experimental groups, no blinding or randomizing was performed. Off-line analysis of all cellular experiments was performed in an automated

manner using home-written routines in Igor Pro 8 (www.wavemetrics.com), thereby limiting operator bias.

The concentration dependence of ligand-induced calcium responses from individual cells were fitted using a Hill function of the form:

$$\Delta[Ca^{2+}]_{i,normalized} = \frac{100\%}{1+\left(\frac{EC_{50}}{[A]}\right)^{n_H}},$$

where $\Delta[Ca^{2+}]_{i,normalized}$ represent the ligand-induced increase in intracellular calcium normalized to the maximal increase in the same cell, EC_{50} the concentration for half-maximal activation, [A] the concentration of ligand and n_H the Hill coefficient. The concentration-dependent inhibition curves were fitted using a Hill function of the form:

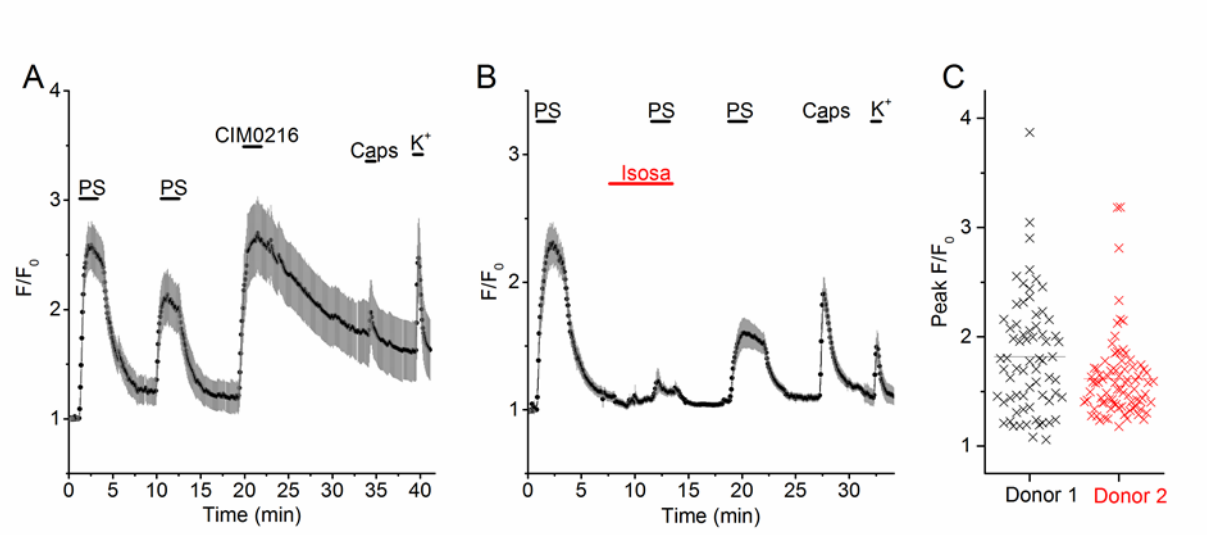
$$\Delta[Ca^{2+}]_{i,normalized} = \frac{100\%}{1+\left(\frac{[B]}{IC_{50}}\right)^{n_H}},$$

where [B] is the concentration of channel blocker, and IC_{50} the concentration for half-maximal inhibition. Here, the increase in intracellular calcium in the presence of blocker was normalized to that in vehicle-treated cells. The normalizations reduced variation due to cell-cell differences in intracellular calcium buffering and extrusion, affecting maximal calcium increases. OriginPro 9.0 was used for statistical analyses and data display.

Nomenclature of targets and ligands

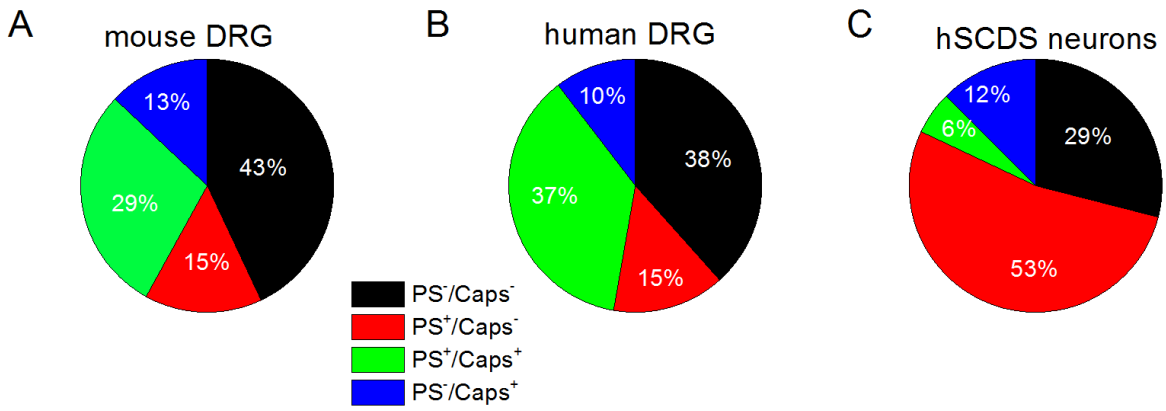
Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to Pharmacology [262], and are permanently archived in the Concise Guide to Pharmacology 2017/18 [18].

4.6 Supplementary information



Supplementary Figure 1: **Human data.** (**A,B**) Mean ± SEM of all traces in the experiment shown in Figure 1B (*n*=23) and Figure 1D (n=27), respectively. (**C**) Amplitude of the first PS response of all neurons, separated by

donor. Solid line indicates the mean.



Supplementary Figure 2: **Distribution of TRPV1 and TRPM3 responders in different DRG models.** (**A-C**) Piecharts showing the percentage of TRPV1 and/or TRPM3 responders in (A) mouse DRG [174], (**B**) human DRG and (**C**) hSCDS neurons.

Chapter 5:
Concluding discussion & perspectives

TRP channels are indispensable for human physiology. Due to their intimate relationship with ion transport and calcium signaling, they are involved in countless physiological processes [19]. However, sometimes channel gating or permeation fails, and TRP channels end up to be responsible for pathophysiological processes [45]. The aim of this thesis was to focus on two of these TRP channelopathies. Firstly, the remarkably broad spectrum of hereditary diseases caused by TRPV4 mutations. Secondly, acquired hyperactivity of the somatosensory pathway, which leads to chronic pain. TRP channels are molecular detectors at the frontline of somatosensation, play a role in the development of chronic pain and can be regarded as important targets for the development of novel analgesics. The result of these investigations are discussed below.

5.1 TRPV4 and the remarkable amount of diseases

Over sixty disease-causing mutations have been identified in the human *trpv4* gene (see table in chapter 1.2.5), mostly single nucleotide, missense alterations. Although they are spread over the whole gene, there is an accumulation near the ankyrin repeat domains (ARD) and the pore region. They are causative of a myriad of hereditary pathologies, with a remarkably wide phenotypic spectrum. Based on the key symptoms, TRPV4-pathies are divided in three categories; skeletal dysplasias (SD), digital arthropathy-brachydactyly (FDAB) and neuropathies affecting mainly the motor neurons [117]. This cell-type specific effect of the mutations made us speculate about the possible role of regulatory interactions between TRPV4 with other proteins or cellular components. This was supported by the cluster of mutations in the ARD, a domain known for protein binding. Furthermore, some structural studies also predict that differential effects on regulatory protein-protein interactions cause phenotypic variability [263].

In our quest to identify novel interaction partners of TRPV4, we used the Mammalian Protein Protein interaction trap (MAPPIT). One of the potential candidates was zinc-finger containing protein ZC4H2, a protein that, when mutated, causes inherited forms of intellectual disability accompanied by variable symptoms of central and peripheral nervous system involvement, such as spasticity, hyperreflexia, muscle weakness, and arthrogryposis. The resemblance of this phenotype with several symptoms of TRPV4-pathies made us examine this protein and its effect on TRPV4 in more detail. Using Fura-2 ratiometric calcium imaging, we observed an increase in basal calcium levels in cells co-expressing human TRPV4 and ZC4H2 when compared to control. Furthermore, the amplitude of calcium responses upon activation of the channel with various stimuli was also significantly increased in the presence of ZC4H2. The effect showed specificity for TRPV4, as its closest homologue, TRPV3, was unaffected.

Initially, we hypothesized that this effect would be caused by increased expression of the channel. However, RNA as well as protein levels were unchanged by ZC4H2. Similarly, the subcellular TRPV4 distribution was comparable with or without ZC4H2, and the fraction of membrane-associated TRPV4 was unaltered. Similarly, no obvious differences in the steady-state presence TRPV4 were detected using TIRF microscopy, a technique to selectively visualize fluorophores in the close vicinity of the glass-plasma membrane interface. However, by implementing the TIR-FRAP technique, we revealed some important differences. TIR-FRAP is a method to measure near-membrane trafficking events as it tracks recovery of fluorescent molecules in the TIRF imaging field (membrane and near-membrane) following selective bleaching of the perimembrane area. Using this approach, we observed a significantly faster bleaching of TRPV4 molecules when ZC4H2 was co-expressed, associated with a faster recovery of fluorescence, indicating a faster transport of new TRPV4 molecules towards the plasma membrane. These findings may point toward a larger pool of rapidly recycling TRPV4 vesicles near the plasma membrane in cells expressing ZC4H2. We speculate that the enhanced peri-membrane dynamics may underlie the observed increase in TRPV4-mediated responses, since the transport of channels to and from the membrane represents an important determinant of cellular response. However, we cannot

exclude at this point that the altered trafficking and increased function represent two distinct actions of ZC4H2 on the cellular behavior of TRPV4. Taken together, we conclude that ZC4H2 represents a novel regulator of TRPV4 that interacts with the N-terminus to regulate channel function and turnover at the plasma membrane.

ZC4H2 is a relatively small protein (224 amino acids) containing a coiled coil region and a C-terminal zinc-finger domain characterized by four cysteine and two histidine residues. It represents a rather unexplored protein, with primarily reports about patients containing a mutation in the X-linked *ZC4H2* gene. Mutations included missense mutations as well as chromosomal inversions or *de novo* deletions, of which the majority is predicted to destabilize the protein. Patients were initially diagnosed with 'Wieacker-Wolf syndrome' [210, 264] or Miles-Carpenter syndrome [211] and suffer from variable levels of intellectual disability (ID) associated with symptoms of central and peripheral nervous system involvement. Symptoms have an *in utero* onset (fetal akinesia), and patients are born with severe contractions grouped under the name Arthrogryposis Multiplex Congenita (AMC), multiple skeletal abnormalities (hip dislocation, scoliosis, pes equinovaris), progressive distal muscular atrophy (muscle weakness), oculomotor apraxia and dysarthria. However, much more and highly variable symptoms exist, recently gathered under the name ZC4H2-associated rare disorders (ZARD) [206].

The exact cellular function(s) of ZC4H2 are poorly understood, and current research results aimed at unraveling its role in disease are rather incoherent. In mouse and zebrafish, ZC4H2 is highly expressed throughout the brain and spinal cord. Expression is highest during embryonic development but drops postnatal, suggesting an important role in brain development [210]. In primary hippocampal neurons, transiently expressed ZC4H2 localizes in excitatory neurons. Some disease-causing mutations encode proteins that have a similar localization, but cause a significant decrease in synapse number and density [210]. Knockdown of the protein in zebrafish results in abnormal swimming due to impaired motor neuron development, more specifically a reduced number of motor neurons and less neuronal branching associated with disorganized neuromuscular endplates. In affected humans, muscle fibers were smaller when measured at six weeks of age while they normalized after, suggesting a delayed innervation. Myelination of the motor neuron was unaffected [210]. The formation of disorganized endplates was speculated to be due to dysregulation of axonal-guidance. This is known to cause permanent contractures and arthrogryposis, both key symptoms in ZC4H2-linked diseases as well as in some TRPV4-pathies. However, May *et al*. did not detect any sensory or motor neuron defects in zebrafish expressing mutant ZC4H2, though mutant larvae did exhibit abnormal eye position and abnormal flexion of pectoral fins. In brain and spinal cord, they observed a specific reduction in V2 GABAergic interneurons, suggesting a role of ZC4H2 in interneuron development [211]. For one of the tested mutants, the effect was attributed to the fact that mutations disrupted the nuclear localization signal (NLS , aa 201 – 224) and thus that the mutant ZC4H2 cannot exert whatever function it normally exerts in the nucleus. Impaired nuclear trafficking was also observed in two other mutations in the NLS, but the functional consequences of these mutations was not studied in more detail [265, 266]. Later, ZC4H2 was linked to RNF220, an E3 ubiquitin ligase that plays a crucial role in the development of ventral interneurons and motor neurons [212]. Other studies propose a stabilizing effect of ZC4H2 on Smads in order to enhance BMP signaling, a pathway involved in neuronal patterning and development [213].

Interestingly, the particular function of the zinc-finger in ZC4H2 was never addressed. Zinc fingers, which can adopt a variety of structures, are best known for their DNA-binding properties in transcription factors. However, they have a wide variety of molecular functions including regulation of several cellular processes [267]. Structural studies can provide more information about different protein partners, binding modes and affinities [222]. In previous studies, expression of ZC4H2 was found in both cytosol and nucleus [210], suggesting at least a dual function instead of solely DNA binding. Also in our study,

we detected ZC4H2 expression in cytosol. Furthermore, direct interaction was confirmed with the TRPV4 protein and expression of TRPV4 was unchanged, arguing against a transcriptional effect of the zinc-finger as the cause of altered TRPV4 function. Note that, using TIRF imaging, we found a uniform distribution of ZC4H2, without obvious co-localization with TRPV4. One explanation could be that the critical interaction between the proteins occurs in the cytosol away from the plasma membrane and beyond the TIRF-field. Furthermore, the MAPPIT assay can pick up direct as well as transient or indirect interactions [205], so it is possible that ZC4H2 exerts its function on TRPV4 through transient and short-lived interactions.

In case of ZC4H2 co-expression, steady-state subcellular distribution of TRPV4 remained unchanged, but we detected a more rapid laser-induced bleaching as well as faster recovery of TRPV4-GFP at the membrane. This suggests a swifter turnover of TRPV4 in the membrane, with new channels from vesicular structures rapidly replacing old internalizing channels in the membrane. The balance of such vesicles fusing with and disappearing from the membrane is crucial to determine the number of channels in the membrane, and thus the cellular response. It is based on the concept of recycling vesicles residing 'on hold' underneath the plasma membrane, which allows a fast reaction from the cell if more channels are required. Not only can this mechanism increase responses on short notice, constant replacement can also prevent complete desensitization of available channels. Such a mechanism may be of particular importance for a channel like TRPV4 that is constitutively active at body temperature.

Dynamic changes in availability at the membrane have already been observed for multiple TRP channels, including TRPV4. For example, shear stress increases exocytosis of functional TRPV4 channels [214], leading to sensitization of certain tissues to agonist activation. The results seems to be tissue dependent, as it was more pronounced in HUVEC cells compared to HEK293 cells. These authors found that calcium release from intracellular stores is necessary to trigger the process, and that an intact actin cytoskeleton is required. The obtained increase in Ca^{2+} influx is important to fine-tune vasoregulatory pathways by activating NO synthase and Ca^{2+}-dependent K^{+} channels. Later, the same group published a similar recruitment of intracellular pools of TRPV4 to the plasma membrane after stimulation with agonist GSK 1016790A [215]. In this study, they detect that the temporal increase in channel density is limited to 20 minutes. An opposing method to increasing surface expression has been described for Pacsin3, a protein that interacts with the proline-rich region in the N-terminus of TRPV4 and is known to be involved in endocytosis. Binding of TRPV4 to Pacsin3 inhibits dynamin-mediated endocytosis, which results in an increased relative amount of TRPV4 channels in the membrane compared to cytosol [79]. However, this does not result in an overall increase in channel function, as Pacsin3 antagonizes PIP_2 binding and thus attenuates basal activity of TRPV4 as well as responses to heat and cell swelling [80, 81]. The physiological meaning behind these opposing regulatory effects has yet to be determined. In fact, for most of these studies, it remains to be elucidated how these effects contribute to channel function in health and disease.

In our TIR-FRAP experiments, we observed a generally homogenous recovery of TRPV4 in the membrane, pointing to a recovery occurring mainly from inside the cell towards the plasma membrane. This is in contrast with TRPV2, where in the same time-frame recovery mainly occurred through lateral diffusion [43]. In control settings, recovery could be described by a monoexponential fit. However, in the presence of ZC4H2, recovery was better described using a double exponential function consisting of an initial fast phase and a slower phase that was similar to the monoexponential function in control cells. This shows that ZC4H2 adds a fast component to TRPV4-integration in the membrane. The mechanisms whereby ZC4H2 affects dynamic TRPV4 transport remain elusive. The two phases of recovery might represent the two distinct fusion arrangements of recycling vesicles observed in [215].

A complete fusion of vesicles to steadily release all intravesicle structures to the plasma membrane, accompagnied by the more transient kiss-and-run principle of partial fusion [268]. In the study of Baratchi *et al*., they observed that increase in TRPV4 density at the membrane is dependent on intracellular calcium release [214]. Therefore, it may be of interest to address whether ZC4H2 affects ER calcium stores, or whether depletion of intracellular calcium stores (e.g. using thapsigargin) affects the effect of ZC4H2 on TRPV4 dynamics. In the case of TRPM8, it was shown that vesicles containing lysosomal-associated membrane protein 1 (LAMP1) and vesicle-associated membrane protein 7 (VAMP7) mediate the transport of the channel to the plasma membrane [39]. These and related proteins can be examined for their presence with TRPV4 in transport vesicles, to obtain better insights into the mechanism of trafficking and membrane fusion of TRPV4.

We can only speculate how increased perimembrane trafficking affects TRPV4 function in physiological or pathophysiological conditions. As a calcium permeable channel, TRPV4 is involved in many processes, and its malfunction can lead to problems in bone formation as well as in the nervous system. Likewise, the function of ZC4H2 has not been elucidated so far, but given its high expression in brain and CNS during embryonic development, it is presumably involved in the formation and spouting of new neurons. Accordingly, mutations identified in patients lead to fetal akinesia-induced contractures and muscular atrophy. Ca^{2+} can be involved in various ways, and is a known factor during neurogenesis [269]. However, a role for TRPV4 in neuronal development has not been established so far, though expression in the brain has been reported [74, 270]. Interestingly, one study reported that ectopic expression of TRPV4 into zebrafish embryos leads to severe developmental abnormalities, with a curved and shortened body axis and malformation of eyes and brain [82]. Furthermore, TRPV4 may interact with and modulate the cytoskeleton, which is in turn important in neuronal guidance and development [271]. Collectively, it is not unlikely that the reciprocal relationship between TRPV4 and ZC4H2 is critical to fine-tune Ca^{2+} signals during embryonic development and neurogenesis, and that mutation-induced alterations in either protein can lead to various developmental abnormalities.

5.2 TRPM3 in human sensory neurons

One in five adults suffer from chronic pain, a condition in which pain sensation persist longer than three months after injury, without any clear reason. Unfortunately, available analgesic treatment is often inadequate or outshined by side effects (e.g. opioid addiction). In this research, we studied TRPM3, a novel potential analgesic target, in human sensory neurons. We demonstrate its expression and functionality in isolated endogenous human DRGs as well as in a stem-cell derived model for human sensory neurons. The accessibility of such model allowed in-depth characterization of this pain-related channel, showing similar pharmacokinetics as observed in animal models. Furthermore, we observed inhibition of TRPM3 upon activation of the $GABA_B$ and μ-opioid G protein-coupled receptors, similar to earlier findings in rodent sensory neurons. Our study provided the first evidence of functional TRPM3 expression in the human sensory pathway, opening doors for further research to develop novel analgesics.

Somatosensory TRP channels are indisputably involved in the pathway that translates physical and chemical stimuli into sensations and pain [272]. As molecular receptors at the frontline of sensation, they are susceptible candidates for modulation [273]. Therefore, several of these TRP channels have a long history as targets for pain relief. Research in mice has identified TRPM3 as a novel key player in the pain pathway [183]. The cation channel is expressed in mouse sensory neurons, where it acts as one of the sensors of acute noxious heat [174] and plays a central role in the development of inflammatory hyperalgesia [201]. TRPM3 KO animals do not develop heat hyperalgesia, and similarly, TRPM3 blockers attenuates heat hyperalgesia and other forms of pain in animal models. Therefore, TRPM3 represents

a promising target for the development of novel analgesic drugs [248]. However, before this study, information on the role of TRPM3 in human somatosensation and pain was lacking, making it unsure whether the results form rodents could be translated to humans.

We were able to collaborate with the company Anabios, allowing limited access to fresh human DRGs from deceased patients. Apart from RNA expression, Fura-2 Ca^{2+} imaging revealed functional expression of TRPM3 in 52% of the neurons. This is similar to rodent models, were TRPM3 is functional in about 60% of DRG and TG neurons. The TRPV1 agonist capsaicin evoked responses in 47% of neurons. As this research is obviously limited by the availability of human DRG tissue, we explored a more sustainable model. Using small molecules that mimic neuronal development *in utero*, we derived human stem cell-derived sensory (hSCDS) neurons starting from pluripotent embryonic stem cells [242, 258]. In this model, functional expression of TRPM3 was observed in 59% of the neurons, highly comparable to the findings in human and animal DRGs. However, we detected less co-expression with TRPV1 neurons, mainly because TRPV1 expression was in general in lower than in primary neurons from human or rodents.

The prominent detection of TRPM3 in this model enabled us to characterize the channel in more detail, including the pharmacology of some known agonists and antagonists. A more recent, interesting finding about TRPM3, is the negative modulation by G-protein-coupled receptors (GPCR; μ-opioid, GABA-B and neuropeptide Y receptors). The $G_{\beta\gamma}$-subunit of the trimeric G proteins that are activated by these GPCRs can block TRPM3 effectively and transiently, which suggests that TRPM3 is at least partially responsible for the peripheral pain-reducing effect of opioids [185, 186, 239]. Also in our hSCDS neurons, we could obtain this effect with the synthetic opioid DAMGO and GABA agonist baclofen. Taken together, these results provide the first, encouraging evidence that TRPM3 is a valuable target in human for novel analgesics. The value of this approach in analgesic therapy should be assessed in clinical trials, when a clinically appropriate TRPM3 antagonist can be developed.

Furthermore, we consider this model an optimal tool for further research. The use of human stem cell-derived sensory neurons can be a first step to close the gap between rodent and human science. This translation to human is necessary to create credible and effective drugs. The protocol we used is based on an established method for the formation of neurectoderm [259] followed with a further differentiation to sensory neurons with a nociceptor phenotype [242, 243]. These neurons were thoroughly characterized by Young *et al*. and express different canonical markers (for example TAC1, SLC17A6 (VGLUT2), SCN9A ($Nav_{1.7}$), SCN10A ($Nav_{1.8}$) and RunX1) and functional properties of human nociceptors, including voltage-gated currents and response to capsaicin and ATP. The similarity in expression profile between endogenous human DRG and *in vitro* differentiated DRG is striking [243]. Further efforts are being done to fully define the transcriptome of SC-derived sensory neurons [274]. Obviously, hSCDS neurons exhibit limitations, and differences in differentiation are frequently reported [275]. Therefore, it remains crucial to evaluate the purity of the obtained cells.

Mouse and human nociceptors exhibit several similarities [234], but the observed differences can be relevant for the efficacy of developed pain medications. Even though rodents will remain important model organism for pain research, hSCDS neurons can facilitate its translation into relevant therapeutics. An additional advantage is the relatively high efficiency and rather short time of the protocol, enabling a constant availability with potential upscaling for high-throughput drug testing. Future perspectives for using stem cell-derived neurons are very broad. Firstly, induced pluripotent stem cells (iPSC) instead of embryonic stem cells can be used to obtain a patient-specific background. Although this will increase variability, it provides the opportunity to investigate the pathophysiology of some pain-syndromes, for example the familial episodic pain syndrome cause by mutated TRPA1 [50]. Furthermore, it creates possibilities for the development of personalized treatments [276].

Alternatively, CRISPR-CAS technology can be used to create variations in stem cells and examine the results concerning sensory neuron development and function. Transiently transfecting genomic content can be useful for examining the effects of overexpression or knock down of some proteins known to affect neuronal function. Similarly, for our project it can be interesting to create knock out (using CRISP-CAS technique) or knock-down (using small intereference RNA's) of several TRP channels, given their importance in neuronal function and maybe neuronal development. Furthermore, hSCDS neurons can also be co-cultured with other relevant neuronal cell types, for example glial cells, to allow them to make complex networks and make them more authentic.

To conclude, our data provide the first direct evidence that TRPM3 is functional in human nociceptors, which represents a crucial step in the evaluation of the channel as a potential drug target for analgesic drug development. Secondly, we show that stem cell-derived sensory neurons are an accessible model to study TRPM3 function and modulation in a relevant human cellular context. Considering that clinical development efforts for other TRP channels have been severely hampered by species-specific differences in pharmacology and modulation, this human cellular model will be highly instrumental for translational studies and drug development.

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Chapter 1

This introduction was written by LV and corrected by TV. Part of the introduction was published as review in Cold Spring Harbor Perspectives (2019) by LV and TV.

Chapter 3

The results reported in this chapter is integrally submitted and accepted as paper in International Journal of Molecular Sciences (2020), were LV is first author.

LV, JT and TV conceived and designed the experiments; LV, AJ, IL and SL performed experiments; LV and TV analyzed the data and wrote the paper.

Chapter 4

The results reported in this chapter are integrally published in British Journal of Pharmacology (2020), were LV is first author.

LV, MB, YM and KDC conducted the experiments. LV, PEM, PC, CV, JV and TV participated in the research design, data analysis and interpretation. TV supervised the project. LV and TV wrote the manuscript. All authors reviewed and revised the final version of this manuscript and approved its submission.

Conflict of interest

JV and TV are co-inventors on patents entitled “Treatment of pain” derived from WO2012149614, and their labs have received research funding for pain-related research from industrial parties. Otherwise, the authors and co-authors declare no conflict of interest.

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Curriculum Vitae

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Employment

July 2020 - present: Project manager
Laboratory of virology & chemotherapy, Prof. Johan Neyts - **Rega Institute**, KU Leuven

Sept 2013 – June 2020: Doctoral researcher
Transient Receptor Potential (TRP) channels in human disease: From sensors to suppressors.

Laboratory of Ion Channel Research LICR , Prof. Thomas Voets - **KU Leuven** – Cellular & Molecular medicine, **VIB** – Center for brain & disease

Additional courses

- Pluripotent stem cell culture maintenance & quality control – SCIL Leuven, JUN 2017
- Presentation skills for biomedical scientists – Leuven, OCT 2015
- Start to teach – Leuven, OCT 2015
- Advanced RT-PCR training course, Life technologies – Brussels, FEB 2014
- Basic RT- PCR training, Life technologies – Leuven, SEPT 2013

Education

2013: Master Biomedical Sciences, KU Leuven

Course laboratory animal science (FELASA B)

Master Thesis: *Laboratory of neurobiology and gene therapy*, Prof. Veerle Baekelandt
"Translational research on Parkinson's disease using viral vectors *in vitro* and in rodent models"

Scientific output

Manuscripts

Vangeel L, Janssens A, Lemmens I, Lievens S, Tavernier J and Voets T. The zinc-finger domain containing protein ZC4H2 interacts with TRPV4, enhancing channel activity and turnover at the plasma membrane. *Int J mol sci* **2020** DOI: 10.3390/ijms21103556

Vangeel L, Benoit M, Miron Y, Miller P E, De Clercq K, Chaltin P, Verfaillie C, Vriens J & Voets T. Functional expression and pharmacological modulation of TRPM3 in human sensory neurons. Br J Pharmacol **2020** DOI: 10.1111/bph.14994

Vangeel L, Voets T. Transient Receptor potential channels and calcium signaling (2019) Cold Spring Harb Perspect Biol. **2019** DOI: 10.1101/cshperspect.a035048.

Buntinx L, Voets T, Morlion B, **Vangeel L**, Janssen M, Cornelissen E, Vriens J, de Hoon J, Levtchenko E. TRPV1 dysfunction in cystinosis patients harboring the homozygous 57 kb deletion. *Sci Rep* **2016** DOI: 10.1038/srep35395.

Conference oral presentations

2020, Brussels | 1st Young Researchers Day of the Belgian Pain Society

2018, Dubai | ZC4H2 Think Tank

International poster presentations

2017, New Orleans | 61th annual meeting of the biophysical society

2016, Munich | Regulation of cell functions by TRP channels

2016, Los Angeles | 60th annual meeting of the biophysical society