Identification and functional characterization of thermosensitive ion channels (TRPV1 and TRPM8) in neuronal and non-neuronal cells

By
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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by **Rakesh Kumar Majhi** entitled **"Identification and functional characterization of thermosensitive ion channels (TRPV1 and TRPM8) in neuronal and non-neuronal cells"** and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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5. Kumar S, **Majhi RK**, Sanyasi S, Goswami C, Goswami L. (2018) Novel polysaccharide-based hydrogel for bone tissue engineering in absence of any growth factors. *Connective Tissue Research*. (Accepted). doi: 10.1080/03008207.2018.1442444.

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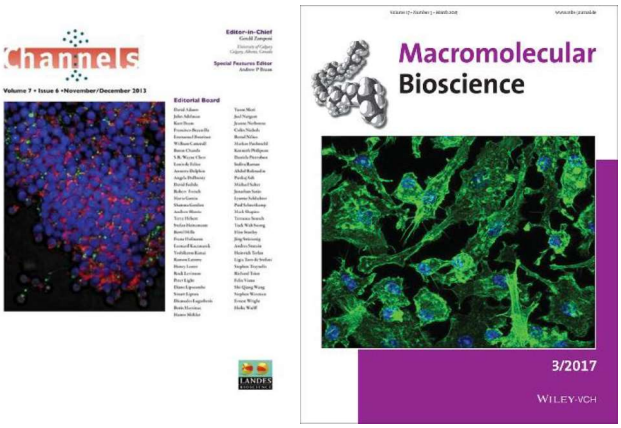

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2. Kumar A, Kumari S, **Majhi RK**, Swain N, Yadav M, Goswami C. (2015) Regulation of TRP channels by steroids: Implications in physiology and diseases. *Gen Comp Endocrinol*. 220:23-32.

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at Homi Bhabha National Institute (HBNI) and is deposited in the Library to be made available to borrowers under rules of the HBNI.

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3. Kumari S, Kumar A, Sardar P, Yadav M, **Majhi RK**, Kumar A, Goswami C. (2015) Influence of membrane cholesterol in the molecular evolution and functional regulation of TRPV4. *Biochem Biophys Res Commun*. 456(1):312-319.

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T cell activation markers CD25 and CD69, and decreased release of IFNγ by T cells pre-treated with TRPV1 inhibitor, even after incubation with ConA. These results indicate that TRPV1 regulates T cell activation and TRPV1 expression is upregulated in activated T cells to carry out immunological effector functions by activated T cells.

In this study, TRPV1 and TRPM8 were found to be endogenously expressed in vertebrate sperm (Piscean, amphibian, reptilian, avian and mammalian) [33,34]. However their localization pattern differs from species to species indicating that they may play different roles in the sperm of different species. The mitochondrial region of sperm from most of the vertebrates have enhanced expression of TRPV1 and TRPM8 indicating their possible role in regulating energy homeostasis of sperm cells, which is vital to enable their motility. Non-motile human sperm have reduced percentage of cells expressing TRPV1 and TRPM8. Even the abundance of these two channels is decreased in non-motile sperm. This indicates that these two channels are essential for sperm motility. This is supported by the fact that in fish sperm and bull sperm, TRPV1 activation leads to enhanced motility, while TRPV1 inhibition leads to decrease in motility. TRPV1 and TRPM8 activation and inhibition regulate sperm motility without affecting capacitation or acrosomal reaction of sperm. This indicates that modulators of TRPV1 and TRPM8 can be potential drugs for contraception as well as for motility-related infertility cases.

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distribution of intracellular Ca^{2+} in the leading edges. On the other hand, TRPM8 activation leads to shrinking of macrophages, resulting in decrease in cell spreading while TRPM8 inhibition leads to more circular and enlarged macrophages. These phenotypic changes indicate that TRPV1 and TRPM8 modulation can alter rate of vesicular fission-fusion at the macrophage membrane thereby affecting the shape of the macrophages.

TRPV1 activation leads to decrease in rates of macrophage migration, while TRPV1 inhibition leads to enhanced macrophage migration. However, TRPM8 activation increases macrophage migration while TRPM8 inhibition reduces macrophage migration. Migration of cells is dependent upon increased rate of microtubule polymerization at the leading edge, and increased depolymerization at the trailing edge. Hence it is likely that TRPV1 and TRPM8 affect macrophage migration by altering rates of cytoskeletal dynamics. In fact, intense TRPV1 activation has been shown to induce microtubule depolymerization [31]. However, the effect of TRPM8 on microtubule dynamics is not yet known. TRPV1 activation promotes bacterial phagocytosis, while TRPM8 inhibition reduces bacterial phagocytosis, which again indicate that both these channels can be modulating cytoskeletal dynamics in macrophages.

In case of resting T cells, TRPV1 is localizes mainly in the intracellular pool, while TRPM8 is primarily present at the membrane [32]. Upon T cell activation, accumulation of TRPV1 and TRPM8 at the surface increases, indicating that both these channels are essential for T cell effector functions. Inhibition of TRPV1 prevented T cell activation by ConA, which supports that TRPV1 needs to be in the on-state during T cell activation process. This was further confirmed by reduced surface expression of

results in increased rate of vesicular exocytosis (vesicle fusing to membrane and contributing additional membrane fusion for neurite extension). Besides pharmacological agents, TRPV1 and TRPM8 can also be modulated by endogenous lipids and cholesterol. Few reports have shown that TRP channel activity is dependent on whether it is present within or outside the cholesterol enriched lipid rafts at the cell membrane [26,27]. TRPM8 was found to co-localize with lipid raft components. This strong association was not disrupted even after depleting cellular cholesterol, indicating that TRPM8 clusters are strongly associated with lipid rafts.

TRPV1 and TRPM8 channels have been relatively under-explored in non-neuronal cells like immune cells. Few TRP channels, namely, TRPV2, TRPV4, and TRPM8 have been reported to regulate phagocytosis in macrophages [28-30]. In this study, nearly 100% of the RAW264.7 macrophages and peritoneal macrophages were found to be positive for TRPV1 and TRPM8 expression. However, the major TRPV1 pool was intracellularly localized in resting and LPS activated conditions, while the majority of TRPM8 was localized at the membrane. This indicates that a small percentage of TRPV1 at the macrophage membrane is sufficient for immunological function in macrophages, while larger population of TRPM8 has to be present at the surface for effective immunological response. The surface accumulation of both TRPV1 and TRPM8 increase upon LPS mediated macrophage activation which again indicates that surface expression of these two channels is essential for immune response. TRPM8 inhibition significantly reduces macrophage adhesion indicating that TRPM8 activation helps to form focal adhesion points during macrophage adhesion. TRPV1 activation leads to elongated macrophages, while TRPV1 inhibition leads to more circular and enlarged macrophages. TRPV1 activation also induces higher and inhomogeneous

TRPV1 inhibitor. In bull sperm, TRPV1 activation appears to increase the percentage of rapidly moving cells, while TRPV1 inhibition appears to increase the percentage of static cells. TRPM8 activation appears to decrease sperm motility while TRPM8 inhibition doesn't appear to affect motility rates of bull sperm. Neither TRPV1 nor TRPM8 modulation affect the capacitation and acrosomal reaction of bull sperm.

Discussion and conclusion:

TRPV1 and TRPM8 channels have been reported to regulate sensory functions in primary sensory neurons like Trigeminal and DRG neurons [10-12]. Most of the research with TRPV1 and TRPM8 channels have been focused on their role as pain-receptors [13]. However, over the last decade there is increasing evidence that TRP channels are also present in several non-neuronal cells like keratinocytes [16], muscle cells [17], endothelial cells [18], sperm cells [19,20], immune cells [21,22], etc. However the exact function and regulation of TRPV1 and TRPM8 channels in these neuronal and non-neuronal cells are still poorly understood. Using F11 cells (cloned initially by the fusion of embryonic DRG neurons with mouse neuroblastoma cells [23]) our group has previously shown that TRPV1 overexpression leads to enhanced nuritogenesis [24] and TRPV1 acts as a synaptic protein [25]. However endogenous levels of TRPV1 were undetectable in F11 cells, while the expression profile and role of endogenous TRPM8 in F11 neurons was not explored.

In this study TRPM8 was found to be endogenously expressed in F11 neurons. TRPM8 activation increased rate of Transferrin uptake while TRPM8 inhibition resulted in elongated neurites, thereby suggesting that TRPM8 activation results in rapid rate of endocytosis (vesicle pinching off from membrane) and long term TRPM8 inhibition

with TRPV1 inhibitor 5'-IRTX. TRPV1 is involved in the cytokine secretion as ConA-mediated Interferon-gamma secretion by activated T cells was also prevented by TRPV1 inhibitor 5'-IRTX. ConA mediated Ca^{2+}-influx into T cells was blocked by TRPV1 inhibitor 5'-IRTX, but couldn't be blocked by TRPM8 inhibitor AMTB.

4. Expression and functional significance of TRPV1 and TRPM8 in sperm cells.

Sperm cells are a group of thermosensitive cells that are capable of swimming. Expression profiling and functional analysis of TRPV1 and TRPM8 in vertebrate sperm of fish, amphibian, reptilian, avian and mammalian origin were performed by Immunofluorescence and Flow cytometric analysis. Nearly 100% of the Piscean, Avian, Bull and Human sperm were positive for TRPV1 and TRPM8. However there are differences in localization pattern of TRPV1 and TRPM8 in sperm from different species. In Duck sperm, TRPV1 is primarily enriched in the neck region, while TRPM8 is specifically absent in the neck region. The post-acrosomal region of Bull sperm is enriched with TRPV1 while this region is devoid of TRPM8. Super Resolution imaging revealed distinct localization of TRPM8 clusters along the mitochondrial coiling at the neck of Bull sperm. TRPV1 is primarily enriched at the tail of un-capacitated Murine sperm and its localization remains unaltered even after capacitation and/or acrosomal reaction. TRPM8 is primarily enriched at the acrosome of un-capacitated Murine sperm and it gets relocated from acrosome in capacitated sperm, followed by enrichment at post acrosomal region in acrosome reacted sperm. In human sperm, percentage of $TRPV1^{+ve}$ or $TRPM8^{+ve}$ cells in immotile sperm is lower than in motile sperm.

However, TRPV1 intensity is lower while TRPM8 intensity is higher in immotile human sperm. TRPV1 activation by its endogenous activator NADA lead to higher rates and longer duration of motility of Rohu fish sperm, an effect which can be blocked by

in peritoneal macrophages, thereby confirming the functional presence of these channels in macrophages. Bacterial Lipopolysaccharide (LPS) mediated macrophage activation significantly increased the levels of TRPV1 and TRPM8 localization on the macrophage surface. TRPV1 activation appeared to increase macrophage adhesion to glass surface while TRPM8 inhibition reduced the macrophage adhesion. TRPV1 activation increased macrophage elongation while TRPM8 activation decreased macrophage elongation. TRPV1 activation blocked LPS-mediated elongation of peritoneal macrophages while TRPM8 inhibition prevented LPS-mediated macrophage elongation. TRPV1 activation decreased rate of macrophage migration, while TRPV1 inhibition increased the rate of migration. TRPM8 modulation showed exactly opposite effect on macrophage migration. Both TRPV1 activation and TRPM8 activation were able to increase bacterial phagocytosis, but only TRPM8 inhibition was able to decrease bacterial phagocytosis by macrophages. TRPV1 inhibition enhanced bacterial killing by macrophages, while neither TRPV1 activation, nor TRPM8 modulation appeared to affect bacterial killing by macrophages.

3.Expression and functional significance of TRPV1 and TRPM8 in T cells.

Confocal imaging and Flow Cytometry analysis revealed that nearly 100% of the T cells (Jurkat T cell-line, primary human T cells, and primary murine T cells) express TRPV1 and TRPM8. TRPV1 intensity increased in Concanavalin-A activated T cells. Pharmacological activation of T cells by TRPV1 or TRPM8 activator increased intracellular Ca^{2+}-levels in T cells showing that T cells express functional TRPV1 and TRPM8 channels. Flow cytometric evaluation of CD25 and CD69 at surface levels indicated that ConA mediated T cell activation was effectively blocked by co-incubation

pharmacological activator WS12 induces significant increase in intracellular Ca^{2+}-level in F11 cells, while TRPM8 inhibitor AMTB (N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)-benzamide hydrochloride (1:1)) was unable to change intracellular Ca^{2+} level in F11 neurons. Bioinformatics analysis revealed that TRPM8 has 33 CRAC or CRAC-like motifs which may potentially bind cholesterol. These motifs are evolutionarily conserved throughout vertebrate evolution. In agreement with the prediction, TRPM8-GFP clusters co-localize with Cholesterol (detected by Fillipin dye) and other lipid raft markers like Flotillin and Caveolin. Even upon cholesterol depletion by Pravastatin (cholesterol biosynthesis inhibitor) and/or by βMCD (cholesterol sequestering agent), TRPM8 clusters retain cholesterol, flotillin and caveolin. TRPM8 activation led to significant increase in endocytosis, while TRPM8 inhibition doesn't affect the rate of endocytosis in F11 cells. Neither TRPM8 activation nor TRPM8 inhibition affects rate of exocytosis in F11 neurons. TRPM8-GFP clusters exhibit very fast rates of vesicle recycling as detected by fast bleaching and FRAP experiments. Pharmacological modulation of TRPM8 alters various parameters of neuritogenesis events to different extent.

2.Expression and functional significance of TRPV1 and TRPM8 in Macrophages.

Both TRPV1 and TRPM8 were detected by immunofluorescence analysis in murine macrophage cell line RAW264.7 and primary murine peritoneal macrophage cells. 100% of the RAW cells were positive for TRPV1 and TRPM8 at the intracellular level. On the other hand, only 40% of the RAW264.7 macrophages showed surface expression for TRPV1 while nearly 100% of RAW cells were positive for TRPM8 at their surface. TRPV1 and TRPM8 modulation could increase intracellular Ca^{2+} levels

information relevant to concerned work and aims of the study. The Chapter 2 comprises of all the results obtained in this study to achieve the above-mentioned objectives. The Chapter 3 comprises of detailed discussion on the results obtained during this work and its analysis with reference to pre-existing literature. The Chapter 4 summarizes the results, conclusions and future direction in the light of the current study. The Chapter 5 includes materials and methodologies utilized in this study. The Chapter 6 includes all the literature references mentioned in this study.

Results:

1.Expression and functional significance of TRPM8 in Neuronal cells.

In spite of several attempts by our group and several other groups world-wide, endogenous expression of TRPV1 was not possible to detect in Dorsal Root Ganglion (DRG) neuron derived F11 cell line. Recently it has also been reported that F11 cells have undetectable levels of TRPV1 mRNA [14] and TRPV1 protein [15]. F11 cells even don't respond to TRPV1 activator Capsaicin [14]. Therefore this chapter deals with the expression, localization and functional aspects of only TRPM8 using F11 cells as a model system.

TRPM8 has been reported to be functionally expressed in primary DRG neurons and thereby regulates low temperature-induced responses [11-12]. However, so far there has been no report on the endogenous expression of TRPM8 in F11 cells. Immunofluorescence analysis showed TRPM8 expression throughout the cytoplasm, and almost evenly distributed all over the cell body and neurites. Western blot analysis revealed a TRPM8 band at 105kDa. The TRPM8 signal was lost upon pre-blocking the TRPM8 antibody with its antigenic peptide. TRPM8 activation by its specific

TRPV1 and TRPM8 was largely unexplored in each of these four cell types, hence this study aimed to characterize these aspects through expression analysis and functional analysis specific to these cell types.

In this work, relevance of TRPV1 and TRPM8 have been explored in peripheral neurons, different immune cells like macrophages and T cells and also in mature sperm cells. In all these cellular systems, endogenous expression, localization, molecular organization and diverse cellular functions have been investigated. This work suggests that functional TRPV1 and TRPM8 channels are present in different thermosensitive cells and regulate functions that involve vesicular recycling, cytoskeletal reorganization and Ca^{2+}-homeostasis. This in turn regulates plethora of different cell lineage-specific functions such as neuritogenesis, immune modulation and sperm motility. Interestingly in most cases TRPV1 and TRPM8 have differential or precise opposite role in regulating all these cellular functions. This work establishes the involvement of these hot- and cold-sensitive ion channels as key regulators for complex cell signaling events that have immense importance in biological systems.

Objectives of this Study:

i) Analysis of endogenous expression and localization of TRPV1 and TRPM8 in different cellular systems which are extremely thermosensitive in nature.

ii) Characterization of different cellular functions regulated by TRPV1 and TRPM8 in different specialized cells.

Organization of the thesis:

This Thesis work is distributed into four chapters. The Chapter 1 comprises of general introduction to TRP channels and review of the literature with current scientific

different environmental cues and survive. These ion channels also offer excellent drug targets relevant in several pathophysiological conditions.

TRPV1 and TRPM8 have been primarily characterized in sensory neurons [10-12] and have been under intensive study in pain research [13]. Being thermosensitive in nature, the expression of these two channels were initially thought to be restricted to neuronal systems only. However recently TRPV1 and TRPM8 have been also reported to be present in several non-neuronal cells and regulate different cellular functions. So far, available information is fragmented and it is not clear if these channels are expressed endogenously and the range of cellular activities in which these channels are involved. Therefore expression profile and functional importance of these channels in the neuronal and non-neuronal systems needs better understanding.

In this work, four model systems have been used, each of them is highly thermosensitive, yet with their own unique cellular features, environmental conditions and physiological challenges. Therefore the specific functions carried out by these systems are used as read-out systems in this work. The neuronal cells are adherent cells and can extend their neurites to great lengths and nonlinear pathways for their sensory/motor functions. The macrophage cells are highly migratory, and can migrate to sites of infection/inflammation, adhere there, invade tissues and carry out their functions. T cells are floating cells and attach only to antigen presenting cells, other T cells and B cells to form immunological synapse and relay information. Sperm cells are swimmers, they sense thermal and chemical gradient in the female reproductive tract, travel great lengths to finally fuse with the oocyte. In addition, vertebrate sperm carry out external or internal fertilization, thereby sperm of each species has its own unique range of requirements and challenges. The expression and functional relevance of

Introduction:

Environmental as well as internal body temperatures have profound effect on different biological processes such as animal physiology, feeding and mating behavior, circannual and seasonal rhythms [1]. The ability to detect temperature is vital for avoiding harmful levels of heat and cold and to maintain thermal homeostasis [2]. Adequate response to thermal stimuli is essential for survivability [3]. Therefore all organisms have developed molecular mechanisms to detect temperature changes and to initiate responses to adapt/counter adverse temperature environment not only at the individual level, but also at the tissue and cellular levels [4]. In vertebrates, the environmental temperature is sensed by somatosensory neuronal endings present in the skin, which express a group of ion channels that are specialized to detect specific temperatures [5-8].

Transient receptor Transient Receptor Potential (TRP) channels represent a group of non-selective cation channels that can be modulated by several endogenous and exogenous factors [9]. Selected few members of this group, especially TRPV members are uniquely thermosensitive in nature and are activated by diverse physical stimuli, chemical stimuli such as endogenous molecules as well as external stimuli including synthetic compounds. TRPV1 is known as “Capsaicin receptor” and can be activated at temperatures above 42°C, and is therefore termed as the “heat-sensitive receptor”. Similarly, TRPM8 is known as the “Menthol receptor” and it can be activated by low temperatures below 25°C and therefore is termed as “cold-sensitive receptor”. Besides temperature, both these channels can be modulated by other physical stimuli, such as pH, mechanical pressure, osmolality, etc. These channels also regulate a battery of important cellular and physiological functions which are essential for organisms to sense

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SYNOPSIS OF Ph. D. THESIS

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5. **Board of Studies: Life Sciences**

SYNOPSIS

NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium Hydroxide
NOS	Nitric oxide synthase
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PI	Complete protease inhibitor
PIPES	1,4-Piperazinediethanesulfonic acid
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospho Lipase C
PMSF	Phenylmethanesulfonyl fluoride
PVDF membrane	Polyvinylidene difluoride membrane
ROS	Reactive oxygen species
RPM	Revolution-Per-Minute
RT	Room temperature
RTX	Resiniferatoxin
Sd	Swim-down
SDS	Sodium Dodecyl Sulphate
Su	Swim-up
TAE	Tris-Acetic Acid-EDTA
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TG	Trigeminal
TM	Transmembrane
TNF	Tumor Necrosis Factor
Tris	Tris Hydroxymethylaminoethane
TRP	Transient Receptor Potential
v/v	Volume per volume
w/v	Weight per volume

List of Abbreviations

2-APB	Aminoethoxydiphenyl borate
5'-IRTX	5'-iodoresiniferatoxin
AM	Acetoxymethyl ester
Amp	Ampicillin
AMTB	N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)-benzamide hydrochloride (1:1)
APS	Ammonium persulphate
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue G250
CCM	Cholesterol Consensus Motif
CD	Cluster of Differentiation
CRAC	Cholesterol recognition amino acid consensus
Cyt C	Cytochrome C
DAG	Di acyl glycerol
DAPI	4',6-Diamidino-2-phenylindole
DMSO	Dimethyl Sulfoxide
DRG	Dorsal root ganglion
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl ether)tetraacetic acid
EM	Electron Microscopy
ER	Endoplasmic Reticulam
FBS	Fetal calf serum
GFP	Green Fluorescence Protein
H	hour (Time unit)
HCl	Hydrogen Chloride
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HRP	Horseradish Peroxidase
Hz	Hertz
IFN	Interferon
IL	Interleukin
InsP3	Inositol-1,4,5-trisphosphate
Kan	Kanamycin
Kb	Kilo base
kDa	Kilo Dalton
KO	Knock out
KOH	Potassium Hydroxide
L	Litre (volume unit)
LB	Luria-Bertani
MFI	Mean Fluorescence Intensity
Min	Minutes (Time unit)
NA	Numerical aperture

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all vertebrates and both expression as well as localization of TRPV4 differs in motile vs immotile human sperm indicating that it could be an important regulator of sperm motility [267]. Although functional role of calcium selective TRPV5 has not been reported, TRPV5 is also expressed at low levels in rat spermatogenic cells and in mature sperm [268]. In a similar context, TRPV6 null as well as TRPV6 pore-mutant male mice have been shown to be infertile [269,270].

TRPM4 and TRPM7 are highly expressed in spermatogenic cells and spermatozoa of rat testis [268]. TRPM8 has been shown to be present in the head and flagellum of human sperm. Activation of TRPM8 either by low temperature or menthol increases intracellular Ca^{2+} levels in human sperm and triggers acrosome reaction [271,272,273]. The discovery that TRPM8 is the testosterone receptor has made TRPM8 a much more interesting channel to probe for in the context of sperm development and function [274,275].

[254]. Interestingly, the TRPP2 mutant sperm in spite of being motile, were unable to reach the sperm storage organ in the females (seminal receptacles and spermatheca) [253]. Necrospermia and predominance of immotile sperm has been reported in human with autosomal polycystic kidney disease due to defects in polycystin (PKD) genes [255].

The ability of TRPV1 to be activated by an endocannabinoids like anandamide (arachidonoylethanolamide, AEA) has been used to unravel its role in reproduction. Although TRPV1 knockout mice are normal and fertile [256,257], in larger mammals, TRPV1 appears to be very important for fertilization. In uncapacitated boar spermatozoa, TRPV1 co-localizes with cannabinoid receptor CB1R and fatty acid amide hydrolase (FAAH) (anandamide hydrolyzing enzyme) at the post-acrosomal head and mid-piece. Post capacitation, TRPV1 is re-localized to the anterior part of the sperm head [256,257]. Later, TRPV1 was reported to be present on the post-acrosomal region of human spermatozoa, and was shown to be important for the progesterone-induced sperm oocyte fusion [259]. Anandamide was shown to capacitate bull sperm via TRPV1 [260] and bicarbonate was shown to influence shift in TRPV1 localization during capacitation [261]. TRPV1 apparently also plays a role in stabilization of the plasma membranes in capacitated spermatozoa before the sperm-zona pellucida interaction and the zona pellucida mediated true acrosome reaction [262]. Further, Anandamide has been shown to induce sperm release from female oviduct epithelia, a critical aspect in final sperm-oocyte fusion [263,264]. Recently, TRPV1 has also been demonstrated to mediate thermotaxis of human spermatozoa [265]. Interestingly TRPV4 has been also shown to mediate thermotaxis in mouse spermatozoa [266]. Our group has recently reported that the expression of TRPV4 is conserved in the sperm of

proliferation and secretion of pro-inflammatory cytokines [244]. TRPM4 differentially regulates Th1 and Th2 function by altering calcium signaling and NFAT localization [245]. Mice with conditional deletion of TRPM7 showed blockage of Thymocyte differentiation from the $CD4^{-}CD8^{-}$ to $CD4^{+}CD8^{+}$ stage in the thymus, resulting in decreased number of circulating T cells [246].

1.6.3. TRP channels in sperm cells

TRP channels have emerged as important players in sperm physiology and function [247]. TRPC1 is localized in the mid-piece and flagellum of mouse sperm [248] and human sperm [249] implying its role in sperm motility. TRPC1 is also present at the acrosomal region of mouse sperm [250]. TRPC2 is present at the anterior head of mouse spermatozoa and takes part in Zonapellucida induced acrosome reaction [251]. In the, TRPC3 is present at the post acrosomal head and tail in mouse [248] and human spermatozoa [249]. Pharmacological inhibition of TRPC3 inhibits sperm motility, reduces mitochondrial potential and induces capacitation by changing the intracellular Ca^{2+} levels [252]. Although TRPC4 protein has not been detected in mice sperm, human spermatozoa has weak TRPC4 expression at the head and strong expression at the mid piece and tail [249] suggesting its role in acrosome reaction as well as motility. TRPC6 is present at the post-acrosomal head region and sperm flagellum in mouse sperm [248] and mid-piece and tail in human sperm [249].

Limited information is available about the role of TRPP channels in sperm physiology. PKD2 (Drosophila homolog of TRPP2) is present in the head and the tail of the *Drosophila* sperm and targeted disruption of pkd2 gene results in male infertility without affecting spermatogenesis [253]. Later it was shown that *Drosophila* sperm swims backwards in female reproductive tract in a TRPP2 activation dependent manner

cannabinoid induced Ca^{2+}-influx into human T cells [234] while TRPC3 mutation reduces TCR stimulation induced Ca^{2+} influx in T cells [235]. TRPC3 mRNA is upregulated in $CD4^+$ T cells after TCR stimulation [236] while TRPC5 is upregulated in activated $CD4^+$ and $CD8^+$ human T cells [237]. TRPC5 is believed to contribute to TCR-mediated suppression of T cell effector functions in experimental autoimmune encephalomyelitis (EAE) [237] and in the NOD (non-obese diabetic) mouse model of type 1 diabetes [238]. TRPC6 downregulation alters TCD induced Ca^{2+}-influx in Jurkat cells. TRPC3 and TRPC6 levels are upregulated in T cells and induce apoptosis of T cells in mouse having sepsis [239].

Indeed, mRNA and protein level expression of TRPV1 has been shown in primary mouse and human T cells and in mouse and rat thymocytes [231]. Capsaicin induced Ca^{2+} levels increase and apoptosis was observed in human peripheral T cells and in Jurkat cells (44). Using both Ca^{2+} imaging and electrophysiology the functional presence of TRPV1 has been shown in Jurkat cells [240]. Using the TRPV1 antagonist capsazepine, TRPV1 has been suggested to be important for TCR-induced Ca^{2+} influx and T cell activation [241]. TRPV1 mediated regulation of TCR induced Ca^{2+}-influx, T cell activation, release of effector cytokines and colitis severity has also been shown recently [242]. TRPV2 expression in Jurkat cells and primary human T cells and impairment of TCR and Thapsigargin-induced Ca^{2+} levels increase upon TRPV2 down-regulation has been reported [243]. TRPV3 mRNA at very low levels and TRPV4 mRNA at higher levels was detected in human T cells [242]. Functional TRPV5 have been reported to be and expressed in mouse and human primary T cells and in Jurkat cells TRPV5 regulates cell cycle progression. TRPM2 is endogenously present in mouse primary $CD4^+$ T cells and contributes to TCR stimulation induced T cell

intracellular calcium, superoxide production, and NO production in alveolar macrophages and protected against lung injury [226]. TRPML1 is important for phagosome maturation [227], lysosomal calcium release, lysosomal trafficking, and large particle phagocytosis [228] by macrophages.

1.6.2 TRP channels in T cells

Antigen Presenting Cells present the antigen to the T cell receptor (TCR) and trigger downstream signaling that involves phosphorylation of phospholipase Cγ1 (PLCγ1), an enzyme that hydrolyses phosphatidylinositol (4,5) bisphosphate (PIP2) into diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP3). In turn, DAG and IP3 activate protein kinase C (PKC) which trigger release of Ca^{2+} from intracellular stores. This is followed by an influx of Ca^{2+} from the extracellular environment, and the process is called store-operated Ca^{2+} entry (SOCE) [229]. Although the Ca^{2+} release-activated Ca^{2+} (CRAC) channel is believed to be the major player in SOCE in T cells [230], the presence of several other ion channels including TRP channels, have opened the possibility that TRP channels could be also involved in this process.

Expression of TRPA1, TRPC1, TRPC2, TRPC3. TRPC5, TRPC6, TRPV1, TRPV2, TRPV3, TRPV4, TRPV5, TRPV6, TRPM1, TRPM2, TRPM4, TRPM5, TRPM6, and TRPM7 was reported by different investigators [231]. TRPA1 mRNA and protein levels were initially detected in human T cell line Jurkat, in lymph nodes and in spleen [232]. Recently TRPA1 has been shown to be functionally present in $CD4^+$ T cells and there it regulates the activity of TRPV1 channel in mediating inflammatory responses especially in the context of colitis [233]. TRPC1, TRPC3, TRPC5, and TRPC6 have been found to be expressed in T cells [234]. TRPC1 contributes to

getting immunologically activated, have reduced levels of iNOS, IFN-γ and IL-12 expression, which is reflected from the increased susceptibility of $Trpm2^{-/-}$ mice to *Listeria monocytogenes* infection [214]. This impaired immunity can be rescued by transferring macrophages from wild-type mice, thereby indicating vital role of TRPM2 in macrophage mediated immunity [215]. TRPM2 promotes phagosomal acidification, and is important for bactericidal activity of macrophages [216]. $TRPM4^{-/-}$ mice show defective phagocytosis, higher levels of circulating monocytes, increased mortality due to sepsis [217]. TRPM7 regulates polarization of macrophages towards the M2 phenotype and promotes proliferation of M2 macrophages [218]. TRPM8 in macrophages has been shown to determine pro- or anti-inflammatory actions of macrophage by controlling tumor necrosis factor-α and interleukin-10 production [219].

TRPV2 has been shown to accumulate at the phagosomes upon encounter between bacteria and macrophages and regulates the early phases of phagocytosis by causing membrane depolarization, Na^+-influx, PIP_2 synthesis and sub-membranous actin remodeling [220].

TRPC1 has been shown to be important for host defense against bacterial infections via the TLR4-TRPC1-PKCα signaling circuit [221]. $TRPC1^{-/-}$ macrophages have decreased Ca^{2+} entry, lesser proinflammatory cytokines, and reduced bacterial clearance [221]. Enhancing vesicular fusion mediated insertion of TRPC6 to alveolar macrophage plasma membrane helps in restoring phagosome activity in cystic fibrosis, where phagosomal acidification is impaired [222]. TRPV1 protects against sepsis [223] and autoimmune diabetes [224] by its positive regulation of macrophage functions. TRPV4 has been shown to mediate LPS induced phagocytosis of bacteria [225]. TRPV4 activation by 4-alpha-phorbol didecanoate (4αPDD) significantly increases

Filopodial structures are formed by rearrangement of the actin cytoskeleton in specialized areas such as in the growth cones. So far several TRP channels have been detected to be specially localized at the filopodial tips and activation of TRP channels leads to changes in filopodial dynamics. However, the regulation of cytoskeleton by TRP channels has close relationship with myosins [208]. It has been shown that TRPM7 regulates the cytoskeleton, its contractility and cell adhesion by phosphorylation of myosin IIa. Myosins determine the net growth of filopodial length by regulating retrograde F-actin flow [209]. On the other hand, transport of TRP channels is dependent on myosin motors. Besides that, other TRP channels have been shown to interact with cytoskeletal proteins. TRPC5 interacts with stathmin 2 and regulates filopodial length [210].

Biochemical evidence for direct interaction of TRPV1 with microtubules [207] and TRPV4 with microtubules [196] have been supported by cell biological experiments showing functional relevance for TRPV1-microtubule interaction for channel activity [211,212] and TRPV4-microtubule interaction in regulating mechanosensation in osteoblasts [213].

1.6 Role of TRP channels in non-neuronal cells

Although TRP channels have been extensively studied in neuronal cells, there is increasing evidence that TRP channels are not only present, but also play functionally important roles in non-neuronal cells.

1.6.1 TRP channels in Macrophages

The role of TRP channels in innate immunity has been best studied in macrophages and monocytes. TRPM2 null mice macrophages have impaired ability in

TRP channels are expected to be involved in various modes of vesicle endocytosis, as these are associated with many vesicular proteins. Besides endocytosis, TRP channels have been largely studied with respect to vesicle mediated exocytosis.

TRP channels are typically engaged in ion influx and efflux. For example, Ca^{2+} influx through TRPV1 may result in exocytosis as demonstrated by unloading of FM4-64 [194]. However, accumulating reports of TRPV5 indicate a more general role of TRPV channels in vesicle fusion [199]. TRPM7 channels have been largely reported to be involved in cholinergic vesicle fusion to the plasma membrane [200] and neurotransmitter release [201]. TRPM7 is located in the membrane of synaptic vesicles and interacts with proteins namely synapsin I, synaptotagmin I and snapin that are known to be involved in exocytosis of synaptic vesicle resulting in neurotransmitter release [202-205]. In general, several TRP channels may be involved in regulating vesicle mediated exocytosis and produce a wide range of effects like membrane expansion, retraction, membrane architecture regulation, cell migration and filopodia (neurite) formation/ elongation etc.

1.5.3 Role of TRP channels in cytoskeletal dynamics

Most of the cytoskeletal changes are regulated by local Ca^{2+} concentrations and therefore cellular Ca^{2+} dynamics in general. In that context, TRP channels play important roles in regulating the cytoskeletal dynamics. Activation of TRPV1 results in rapid retraction of growth cones along axons due to rapid disassembly of microtubules [198,206]. However, in resting stage, the implication is different. TRPV1 interacts with different tubulin dimers and also with the microtubule filaments. Such interactions through the C-terminal domain of TRPV1 even stabilizes microtubules under certain conditions, mainly in resting conditions [198,207].

induces endogenous expression of non-conventional Myosin II and Myosin III [196]. Second, the elongation or retraction of filopodia is intimately associated with the "fusion" or "pinching off" of vesicles respectively. This is logical as filopodial elongation requires insertion of more membrane in a short duration. In reverse, filopodial retraction must coordinate with the shrinking amount of membranes. In agreement with this hypothesis, intra-filopodial movements of vesicles containing TRPV1 and synaptic proteins have been detected [194]. Third, activation of TRPV1 can induce filopodia elongation due to Ca^{2+}-mediated vesicular fusion. However, filopodial elongation may also occur in absence of TRPV1 activation or even in the absence of functional TRPV1 suggesting that TRP channels are capable to regulate but not essential for filopodial functions [196]. Besides TRPV1, expression of TRPV2, a close homolog of TRPV1, in HEK cells also results in filopodia formation [197].

TRPV1 activation results in elongation of dendritic spines of cortical neurons [196]. Within these structures, TRP channels are mostly involved in the regulation of exocytosis and endocytosis as TRP channels regulate the release as well as uptake of neurotransmitters and neuropeptides. For example, activation of TRPV1 results in release of substance-P and CGRP [87]. At the growth cones, TRP channels regulate the morphology and motility of the growth cones [198].

Actin-dependent fast and differential uptake of FM4-64 dye in TRPV1 expressing cells reflects active endocytotic uptake, which is much enhanced by the presence of TRPV1. The fast labelling of FM4-64 to limited cellular regions, like some areas of the cell body, filopodial regions and growth cones, suggests that these specific structures have fast-recycling vesicles. [196]. Accumulating reports of TRPV5 indicate a more general role of TRPV channels in vesicle fusion also [199]. In fact a lot more

strongly suggests that the localization of these TRP channels in synaptic sites is highly regulated and this regulation can be tissue dependent. Presence of these TRPC channels in the synaptosome was mostly due to the interaction with PDZ domain-containing protein INAD (protein responsible for the inactivation-no-after-potential Drosophila mutant). However, from these biochemical studies it is not clear if all these TRPC channels are present exclusively in the synaptic vesicles or PSD region or in both regions.

1.5.2. Role of TRP channels in regulating vesicular dynamics

TRP channels can regulate the cytoskeletal and vesicular dynamics to a large extent. The coordinated regulation of the sub-cellular compartments forms the basis of neuronal functions like filopodial dynamics, synaptogenesis, neurite extension and cellular migration. For example, several TRP channels are involved in regulation of filopodial structure and dynamics. This is made possible by the fact that within filopodia, TRP channels regulate several structural, cytoskeletal, vesicular and regulatory proteins as well as intracellular Ca^{2+}, all of which are involved in filopodial dynamics. TRPV1 expression induces filopodia both in neuronal and non-neuronal cells [196]. Similarly, TRPV4 is also present in the filopodial tips and activation of TRPV4 results in filopodial elongation as well as building of several filopodial structures [194]. The extensive filopodia formation due to expression of TRP channels and regulation of these filopodial structures can be summarized as following points. First, ectopic expression of TRP channels most likely enhances the endogenous expression of motor proteins that are involved in the filopodia formation and/or regulation of filopodial dynamics through regulation of actin dynamics. At-least over-expression of TRPV1

it has been demonstrated that Capsaicin can induce substance-P and neuropeptide-Y release from isolated synaposomes, indicating that functional TRPV1 is present in these synaptosomes and it can regulate synaptic release [192,193]. Indeed, physical presence of TRPV1 has been detected in the synaptosomal fractions [194]. By using immunoblot analysis Goswami et al. have demonstrated the presence of multiple forms of TRPV1 in the synaptosomal fractions prepared from rat spinal cord [194]. Presence of these multiple higher molecular weight bands specific for TRPV1 strongly indicates the multitude of complexity of the TRPV1 complexes at the synaptic structures. As expected, a band at 97 kDa that matches well with the monomeric form of TRPV1 was observed in synaptosome fraction as well as in post-synaptic density fraction. However, in the post-synaptic density (PSD) fraction another band, at ~200 kDa matches well with the dimeric form of TRPV1. Presence of this dimeric-sized band exclusively at the PSD fraction strongly suggests that within PSD compartment, a part of the TRPV1 is most likely present in a complex which is very tight in nature and even resistant to SDS-PAGE separation. In addition, apart from the monomeric band at 97 KDa, a smeary appearance of TRPV1 is visible which strongly correlates with the glycosylated form of TRPV1. However, this glycosylated form of TRPV1 is not visible in PSD fraction, indicating that within PSD fraction, mostly de-glycosylated form of TRPV1 is present. In a similar manner, presence of TRPV4 in the dendritic spines has also been reported [195]. In addition, a previous study has demonstrated that presence of several TRPC channels, namely TRPC1, TRPC3, TRPC5, TRPC6 and TRPC7 (but not TRPC4) in the synaptosomal fractions isolated from rat brain [190]. The same study revealed that TRPC3, TRPC6, and TRPC7 are present in the synaptosomes isolated from rat cerebella. This differential distribution of TRPCs in different synaptosome preparation

responsiveness in bronchial asthma [183], obesity-related metabolic syndrome [184], in pulmonary hypertension in chronic obstructive pulmonary disease [185].

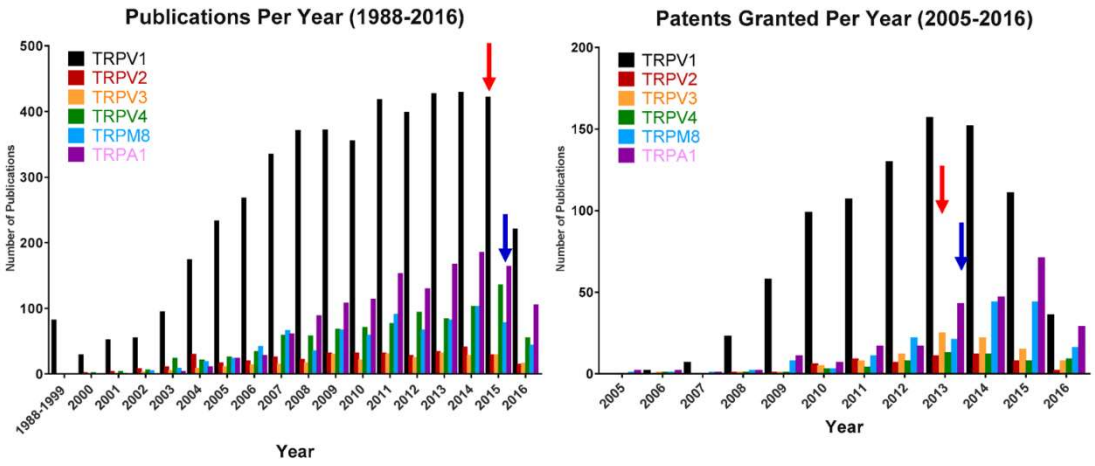

Fig. 8. Thermosensitive TRP channels are target of intensive research as reflected by Number of Publications and Patents granted per year. Total publications per year abd Total Patents granted per year (TRPV1 -black bars; TRPM8 -blue bars) relative to other thermo-TRPs (TRPV2, TRPV3, TRPV4, and TRPA1). Data as of 25/05/2016 obtained from Pubmed. Adapted from [186].

1.5. Role of TRP channels in Neuronal cells

1.5.1. TRP channels at Neuronal Synapse

TRPV1 channel activation has been shown to be necessary and sufficient to trigger long-term synaptic depression (LTD) on hippocampal interneurons [187]. TRPM1 has been shown to mediate synaptic transmission in rod bipolar cells [188]. In the adult rat spinal cord, TRPA1 is localized at presynaptic terminals on substantia gelatinosa neurons and the primary afferent terminals innervating onto spinal inhibitory interneurons, where, upon activation TRPA1 enhances glutamate release [189]. Similarly, TRPC members [190] and TRPA1 channels have been detected in the isolated synaptosomes [191].

The presence of several TRP channels in the synaptosomal fractions (bio-chemical preparation of synaptic units) and dendritic spines matches well with the involvement of TRP channels in various synaptic release and transmissions. Previously

osteosarcoma and malignancy, indicates that TRPM8 plays a crucial role in bone homeostasis [176]. TRPM8 also affects the endocrine system. For example, TRPM8 deficient mice show high rates of insulin clearance [177]. TRPM8 has been shown to be relevant for the excretory system as well. TRPM8 inhibition increases micturition intervals, micturition volume, and bladder capacity and decreases bladder overactivity [178]. TRPM8 polymorphisms are associated with increased risks for Irritable Bowel Syndrome [179].

1.4.2. TRPM8 in pathophysiology

TRPM8 is a cold and pain receptor present in small-diameter unmyelinated C-fibers and in lightly myelinated Aδ fibers [167] and is a vital target in development of pain killers [169]. Multiple genome-wide association studies have implicated the TRPM8 in migraine [180], which has been correlated with its expression in the brain meninges. Given the fact that cold may trigger migraine in certain individuals while menthol can alleviate headache, both agonists and antagonists of TRPM8 are being extensively pursued in the field of migraine research [180]. TRPM8 has emerged as a diagnostic/prognostic marker for prostate cancer as its expression levels are high in the early stages of prostate cancer and low in late stages [181]. Since TRPM8 activation has been found to kill the cancer cells, clinical trials are now undergoing to test the efficacy of TRPM8 agonists in treating various stages of prostate cancer [181]. Besides, prostate cancer, TRPM8 has also been found to be upregulated in osteosarcoma, neuroblastoma, urothelial carcinoma of urinary bladder, and breast carcinoma, as compared to the corresponding normal tissues in human [182]. TRPM8 gene polymorphisms have been found to be associated with cold-induced airway hyper-

hyperalgesia [167]. Besides, TRPV1 is also associated with both acute and chronic pain occurring in case of a wide range of pathophysiological states related to inflammation, such as arthritis, pancreatitis, irritable bowel syndrome, colitis, cancer pain, and migraine headache [168] and have been a major target for therapeutics towards pain relief [169]. Single Neucleotide Polymorphism variants of TRPV1 gene have been found to be associated with migraine [170]. TRPV1 gene variants with higher activity lead to more severe asthma [171] while loss-of-function TRPV1 variant is associated with lower risk of active childhood asthma [172].

1.4. TRPM8

1.4.1. TRPM8 in physiological processes

The expression and functional relevance of TRPM8 has been well described in the sensory and afferent neurons innervating the esophagus, stomach, intestine and colon [157]. Peppermint oil provides relief from abdominal pain and inflammation [173]. TRPM8-deficient mice are highly susceptible to DSS-induced colitis, whereas in wild-type mice with Trinitrobenzene Sulphonic acid (TNBS) and/or DSS-induced colitis, icilin treatment significantly reduces the severity of colitis [174]. TRPM8 is primarily expressed in human bronchial epithelial cells and nerves innervating the lungs [159] and TRPM8 modulators are emerging as drug targets in treatment of several airway related disorders like cough, asthma and Chronic Obstructive Pulmonary Disease [160]. TRPM8 has so far not been reported in any skeletal tissues, except for mRNA level expression in human and murine osteoblastic cell lines [175]. However the findings of a recent study demonstrating significantly higher levels of TRPM8 mRNA and protein in osteosarcoma and further increase in TRPM8 levels in higher stages of

pulmonary system including the human primary bronchial epithelial cells [159] and its antagonists have been a major target in treatment of several airway related disorders like cough, asthma and Chronic Obstructive Pulmonary Disease (COPD) [160]. The importance of TRPV1 in skeletal system is evident from the fact that TRPV1 antagonist Capsazepine inhibits differentiation of both osteoclast and osteoblast *in vitro* and also inhibits ovariectomy-induced osteoclastic bone resorption *in vivo* [161]. TRPV1 deletion impairs bone fracture healing and inhibits *in vivo* osteoclast and osteoblast differentiation [162]. Besides, TRPV1 has been shown to be involved in bone cancer associated pain and TRPV1 antagonists provide pain relief in such cases [163]. TRPV1 signaling also modulates energy and glucose homeostasis. On the other hand dietary Capsaicin intake has been shown to increase insulin secretion, decrease glucose levels, decreases white adipose tissue (fat) accumulation and decreases food intake by increasing satiety [164]. TRPV1 is present in the tubules of the medulla and renal cortex [1165]. TRPV1 activation increases the glomerular filtration rate, enhances renal sodium, water excretion and improves chronic and acute renal failure [166].

1.3.2. TRPV1 in pathophysiology

Several reviews have extensively discussed the role of TRPV1 in pathophysiology. TRPV1 has been largely implicated in pain related disorders [167], gastro-intestinal tract disords [157], airway disorders [160] and several aother diseses [1].

TRPV1 is widely expressed within the components of the peripheral and central nervous systems that are involved in pain sensation. It constitutes an essential component of mechanisms involved in injury-induced pain hypersensitivity and thermal

turret that is reported to make TRPV1 as temperature insensitive channel [150]. However, deletion of 15 residues from the turret region is also reported to retain thermosensitivity of TRPV1 [151]. All these experimental data indicates the complexity of the turret loop region in the context of thermosensitivity. The identity of the thermo-sensor domain in TRP channels still lacks consensus. Even after solving the cryo-EM structure of TRPV1 [44,45], TRPV2 [56,57], and TRPA1 [54], TRPM8 [43], the mechanism of thermal gating of TRP channels remain as enigma.

1.3. TRPV1

1.3.1. TRPV1 in physiological processes

Although TRPV1 was initially discovered as a pain receptor, its presence in several other non-neuronal tissues enables it to play a vital role in regulating other physiological functions as well. TRPV1 has been shown to be an important player in the circulatory system, and is relevant for regulation of hypertension and tumor angiogenesis [152,153]. TRPV1 activation by dietary intake of capsaicin has been shown to increase vasorelaxation, decrease blood pressure and prevent hypertension in genetically pre-disposed hypertensive rats [154,155]. TRPV1 has been also shown to mediate cellular uptake of anandamide and to promote endothelial cell proliferation and tube-formation [156].

Due to its sensitivity to acidic pH, TRPV1 is likely to be relevant in the digestive system. In fact, expression and functional relevance of TRPV1 has been well described in the sensory and afferent neurons innervating the esophagus, stomach, intestine and colon [157]. In fact TRPV1 play protective role in Dextran sulfate sodium (DSS) induced colitis [158]. TRPV1 has been described to be present in the broncho-

terminal 88 amino acids of C-terminus retain TRPV1 functionality and this form of TRPV1 remain fully sensitive to temperature and other stimuli [146]. The difference in these two observations can be linked with the presence/absence of extracellular calcium while performing these experiments [141]. Phosphorylation of Serine502 by PKC sensitize/inactivates TRPV1. Intact C-terminus appears to block accessibility of PKC to this site. When C-terminus is absent or truncated, PKC can act on Serine502 and phosphorylate it to make TRPV1 insensitive. However, when extracellular calcium is low/absent, PKC is unable to phosphorylate S502, leaving TRPV1 free to be activated by temperature (even if C-terminus is deleted or truncated). Hence, in the presence of extracellular calcium, PKC can phosphorylate S502 only when C-terminus is absent/truncated, thus making TRPV1 less sensitive to temperature. This suggests that extracellular Ca^{2+} has more significant effect on thermo sensitivity of TRPV1 [141].

Another attempt to identify the temperature sensor domain of TRP channels was made by Brauchi et al. where they demonstrated that the thermal gating properties of the heat sensor TRPV1 and cold sensor TRPM8 can be interchanged by swapping their C-terminus [147]. It is likely that the C-terminus of thermosensitive channel responds to temperature and interacts with the other regions of TRP channels to regulate pore gating [147]. However, Grandl et al. have shown that only 3 residues of the extracellular loop between "Pore loop" and Trans Membrane domain 6 are critical for thermosensitivity. Among several mutations tested, only TRPV1 mutations at Asn628, Asn652 and Tyr653 and corresponding positions in TRPV3 at Ile644, Asn647 and Tyr661 resulted in "loss-of-sensitivity" of the thermo-TRP channels to temperature. However these mutants are still sensitive to agonists and other physiological stimuli [148, 149]. Replacing 13 residues of TRPV1 pore turret loop forms an artificial pore

opening-closing [143]. The positive and negative values of entropy indicate that TRPV1 is more ordered in its closed state while TRPM8 is more ordered in its open state.

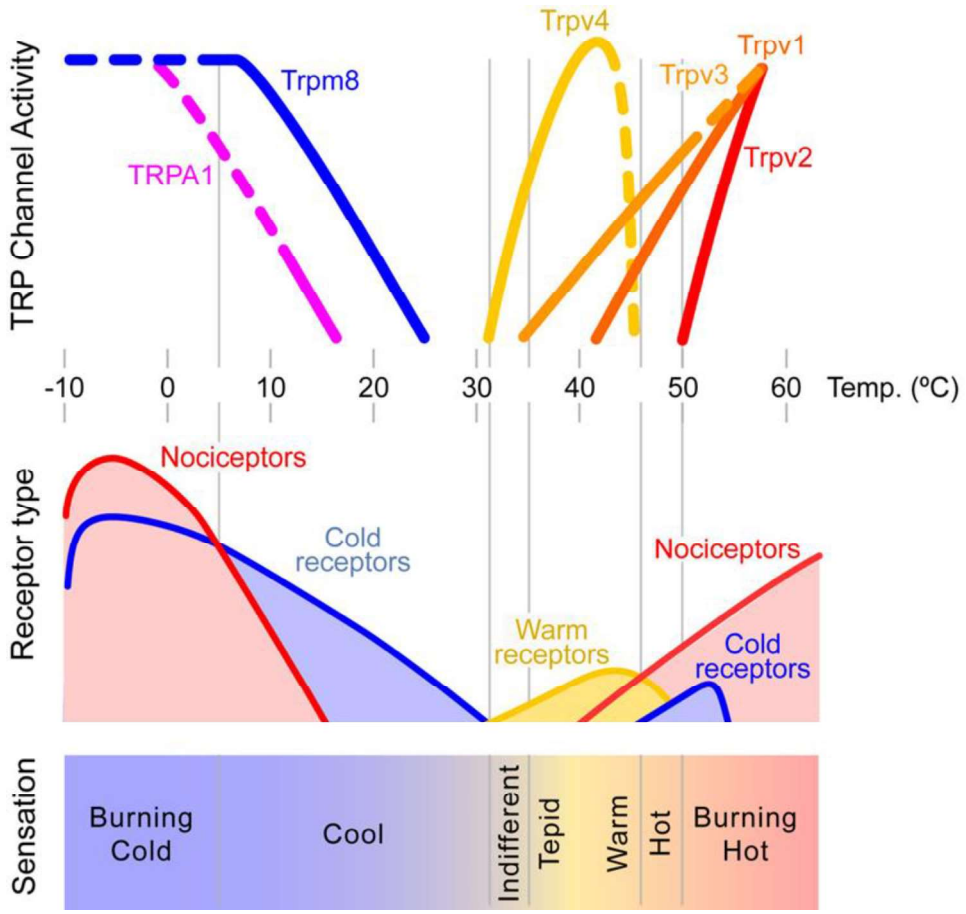

Figure 7. Activation of thermosensitive TRP channels corresponds to temperature sensations. Upper portion: Schematic representation of the thermal activation profile of various TRP channels when expressed in recombinant systems. Middle and Lower part: Schematic representation reflecting activity of sensory receptors in humans in response to sensations evoked by application different temperatures to the skin. Adapted from [144].

Several groups have attempted to identify the region responsible for thermo-gating in TRP channels, but there is no consensus yet as these reports have been contradictory of each other. For example, in 2003 it is reported that deleting entire C-terminus results in an insensitive TRPV1 [145], while deleting only the end portion of C-terminus reduces sensitivity of TRPV1 temperature, low pH and capsaicin (Q_{10} value drops from 25.6 to 4.7). A year later, in 2004 another group reported that deleting the

respectively while hibernating at sub-zero temperatures [138,139]. The molecular mechanism behind this extreme adaptability is yet unknown. In mammals, local exposure to cold initiates TRPA1 induced super oxide release leading to rapid vasoconstriction which is necessary to protect from heat loss. This is followed by TRPA1-dependent release of dilator peptides such as Substance P, CGRP and Nitric Oxide which induce vasodilation necessary to protect from local cold-induced tissue-damage [140]. Cold-treatment of two reptiles: Japanese grass lizard (*Takydromus tachydromoides*) and Japanese four-lined rat snake (*Elaphe quadrivirgata*) resulted in reduced TRPV4 expression in the muscles and tongue [134].

1.2.2. TRP channels in thermosensation and thermoregulation

Among the TRP channels, few TRP channels namely TRPV1, TRPV2, TRPV3, TRPV4, TRPM2, TRPM4, TRPM5, TRPM8 and TRPA1 have been reported to be highly thermosensitive in nature [141] (**Fig. 7**). These thermosensitive TRP channels show much higher temperature sensitivity (Q_{10} > 10) than other non-TRP Thermosensitive channels (Q_{10} ~ 3) like the shaker Potassium channel and Connexin 38 [141]. The switching of thermosensitive TRP channels from closed to open state needs temperature induced large enthalpic change (non-covalent bonds breakage to cause structural changes). In order to reverse back to closed state a large entropic change is required. This is supported by the fact that the heat-sensitive TRPV1 channel has enthalpy value of nearly 150 kcal/mol, and needs entropy of around 140 kcal/mol at 25ºC in order to undergo open-close transitions [146]. Similarly, TRPM8 needs high enthalpy of around -150 kcal/mol and high entropy of around –113 kcal/mol at 25ºC for

gets activated at 46-48°C, much higher than that of mammals [137]. Similarly, chicken TRPM8 also gets activated 5°C below that of mammalian TRPM8 [123]. Birds have suitably modified their thermoreceptors to cope up with the environmental variations they encounter and their somatosensory neurons are equipped for an efficient crosstalk between the environment and their behavioral requirements.

Mammals have a narrow range of body temperature (36-38°C). Yet few mammals have developed the ability to adjust their body temperatures to suit extreme environmental temperature changes. The desert-inhabiting round-tailed ground squirrel (*Spremophilus tereticaudus*) can raise their body temperature to 43°C which enables them to tolerate temperatures as high as 46°C. Even the Chipmunks (*Eutamias minimus*) can tolerate temperatures upto 43°C for prolonged periods. However the molecular mechanism behind this adaptability is not yet known. The Vampire bats have two variants of TRPV1: TRPV1-L is the full length TRPV1 that is present on dorsal root ganglia entrusted with responsibility to transmit sensory signals from peripheral parts to the brain; TRPV1-S is the C-terminus truncated isoform that is present in trigeminal neurons innervating the head and highly specialized anatomical structure of "leaf-pits" that enable them to detect their hot prey. Interestingly, TRPV1-L is activated at 42°C, whereas TRPV1-S is activated at 39°C, thus necessitating a thorough insulation of trigeminal neurons from "leaf-pits" from rest of the body so that it doesn't get activated by normal body temperature of the bats. Several mammals also hibernate to avoid extreme temperatures. For example lemur, rats, mice, hamsters, squirrels, woodchucks, chipmunks have been known to undergo hibernation. Interestingly, squirrels like 13-lined ground squirrel (*Spermophilus tridecemlineatus*) and the arctic ground squirrel (*Spermophilus undulatus*) can lower their body temperatures to 2–4°C and -2.9°C

[129]. In addition to painful temperature avoidance, the amphibians also undergo hibernation to survive in extreme cold environment and aestivation to survive in extreme hot environment [130-132].

Similar to amphibians, reptiles also have TRPA1 orthologue that is activated at 28-30°C [129]. In addition, the TRPA1 also acts as infrared sensor in pit-bearing snakes, enabling them to locate their prey [133]. During hibernation TRPV4 expression was found to be reduced by much larger extent in the muscles, tongue, brain, heart and lung. However, TRPV4 mRNA levels remained intact in the skin after entering hibernation or cold-treatment, suggesting that TRPV4 may serve as environmental thermosensor in the reptilian skin throughout its life cycle, even during hibernation [134]. Interestingly, TRPV4 has been also found to be responsible for temperature induced sex determination in reptiles [135]. In the American alligator (*Alligator mississippiensis*), fertilized eggs when incubated during the critical temperature sensitive period (cTSP) at 33°C produce male offspring, while upon incubating at <30 °C results in female offspring. The alligator TRPV4 ortholog (AmTRPV4) has been found to be highly expressed during cTSP and gets activated at temperatures near 37.3 °C (just 4°C above cTSP) [135]. Pharmacological inhibition of AmTRPV4 reduces the expression of genes responsible for male differentiation (AMH, SOX9) even when incubated at male producing temperatures, while pharmacological activation of AmTRPV4 increases production of SOX9 even at female producing temperatures [135].

Birds have core body temperatures of 40-44°C (much higher than that of mammals: 36-38°C) [136]. Birds have an efficient mechanism to maintain their high body temperature in all seasons, during long flights and also in different temperature zones, be it tropical or arctic. Biophysical analysis of chicken TRPV1 reveals that it

mammalian TRPV1, which gets activated above 42°C, Zebrafish TRPV1 has thermal activation threshold of 32°C, which matches the thermal tolerance limit for most fishes [124]. The molecular basis behind such differences in thermal thresholds can be manifold, yet shortening of the C-terminus of TRPV1 (due to truncation at TRPV1 gene of Zebrafish and several other fishes) has been linked with this different thermal threshold [124]. In agreement with that, molecular cloning of similar truncation in mammalian TRPV1 also reduced the thermal threshold of TRPV1 from 42°C to nearly 30°C, thereby confirming the observation observed in fishes [125,126].

Amphibians are diverged from fishes about 400MYA and acquired the capability of inhabiting much wider thermal tolerance zone. Among the various metabolic and biochemical adaptations that enabled amphibians to survive in diverse environmental niches, one important criteria was the lowering of thermal thresholds of their somatosensory neurons to 10°C [123]. Consistent with this observation, the thermal activation of TRPM8 orthologues in Western clawed frog (*Xenopus tropicalis*) and South African clawed frog (*Xenopus laevis*) was found to be shifted to 14°C [123]. Interestingly, in western clawed frog, TRPV3 gets activated at 16°C, most likely due to its highly diverged N- and C-terminus [127]. The lower activation threshold of amphibian TRPM8 and TRPV3 probably enabled them to tolerate prolonged periods of hypothermia during the winters. The frogs display nocifensive behavior above 38°C indicating that their heat sensitive machinery is similar to that of mammals. This is substantiated by the fact that frog TRPV1 gets activated at 40°C, similar to that of mammalian TRPV1 (activation threshold at 42°C) [128]. However, along with TRPV1, frog TRPA1 could also contribute to heat sensitivity. Frog TRPA1 gets activated at 38°C, much higher in contrast with the role of mammalian TRPA1 as a cold detector

is critical for the normal physiology. Even single cells response to sudden and short temperature changes, and such responses can affect the whole organism. For multicellular organisms, the response to temperature changes is implicated at multiple levels, thus promoting evolutionary adaptation among the species. In case of vertebrates, the primary afferents of the somatosensory neurons are the first to respond to temperature changes. A subtype of these neurons detect non-harmful temperatures which is critical for behavioral responses that enable animals to find suitable and habitable niche, maintain own body temperature, find food, etc. Another subtype of neurons detect noxious and damaging temperatures, critical for avoidance behavior and survivability of the animal. The TRP channels constitute an important part of the molecular machineries that are linked to the thermosensitivity in vertebrates. In spite of the high rates of conservation of TRP channels among vertebrates, each vertebrate order appears to employ distinct molecular strategy to adjust to their physiology and behavior to the environmental temperature.

Fishes are poikilothermic and found in every thermal domain of the globe including both hot equatorial, moderate tropical, cool temperate and the cold arctic regions. Most of the fishes can tolerate a wide range of temperatures ranging from 2°C to 32°C [119,120]. In spite of being distantly related, these fishes have evolved mechanisms to adapt to cooler water temperatures. A common feature in several (but not all) fishes is the loss of TRPM8 gene, which is responsible to detect cooler temperatures from 10°C to 26°C in mammals [121-123]. It is likely that the loss of cold-sensitive TRPM8 genes in several fishes enabled then to tolerate and adapt to cooler water temperatures. However, fishes are not much tolerant to warmer temperatures beyond 32°C likely due to the presence of functional TRPV channels in fishes. Unlike

Several natural products like Menthol, isopulegol, geraniol, linalool, eucalyptol and hydroxy-citronellal activate TRPM8 [103]. TRPM8 is inhibited by Arachidonic acid, docosahexaenoic acid and eicosapentaenoic [104]. Lysophosphatidylcholine, lysophosphatidylinositol and lysophosphatidylserine and sphingosylphosphorylcholine are agonists of TRPM8 [105]. Pregnenolone and other naturally occurring steroids are reported to activate TRPM3, as do sphingolipids such as sphingosine [106,107]. Spermine, an activator of TRPV1, inhibits TRPM4 and TRPM5 [108]. Carvacrol, the TRPV3 activator, inhibits TRPM7 [109].

TRPA1 is activated by noxious cold temperatures (<17°C) [110], isothiocyanates like Allyl isothiocyanate present in mustard oil [111], Thiosulfinates like Allicin (enriched in Garlic) [112], Cinnamaldehyde from *Cinnamomum cassia* and *Cinnamomum zeylanicum* [113]. Besides, Acrolein and Crotonaldehyde, which are constituents of cigarette smoke [114] also stimulate TRPA1 present in airways. Endogenous molecules like α,β-unsaturated aldehyde 4-hydroxynonenal, as well as ethanol metabolite acetaldehyde [115], Prostaglandin D2 metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) [116] activate TRPA1. ∆9-THC and cannabinol also activate TRPA1 [117].

1.2. TRP channels and thermosensation

1.2.1. Evolutionary adaptation to thermosensation: Role of TRP channels

One of the basic properties of living beings is their sensory mechanisms, i.e. their ability to sense and respond to different physical stimuli including changes in temperature. Temperature can have vital to detrimental effect on structure-function of biological macromolecules ranging from nucleic acids to proteins and lipids and thereby on the functioning of cells, tissues and entire organisms [118]. Temperature sensitivity

TRPV2 is a thermosensitive TRP channel with an activation threshold of >52°C. It also gets activated by 2-aminoethoxydiphenyl borate (2-APB) [89]. TRPV2 is expressed in muscle cells where it can be activated by osmotic pressure and membrane stretch [90]. The psychotropic cannabinoid namely Δ9-tetrahydrocannabinol (THC) from the marijuana [89], as well as the non-psychotropic constituents of *Cannabis sativa*: namely cannabidiol and cannabinol [91] also activate TRPV2. Probenecid appears to be the most selective TRPV2 agonist [92] known till now. Endogenous lysophospholipids, in particular lysophosphatidylcholine and lysophosphatidylinositol have recently been described as endogenous modulators of TRPV2 [93].

TRPV3 has an activation threshold between 33°C and 39°C [94]. It is also activated by several plant products like Camphor: a naturally occurring monoterpene isolated from *Cinnamomum camphora* [95]; Carvacrol: the major component in *Origanum vulgare*; Thymol: an important constituent of *Thymus vulgaris*; and the vanilloid Eugenol: the principal active component of the clove plant *Syzygium aromaticum* [96]. Farnesyl pyrophosphate (FPP), an intermediate metabolite in the mevalonate pathway, was recently reported to activate TRPV3 [97].

TRPV4 was originally discovered to be an osmolarity-sensitive channel [98,99], gets activated by warm temperatures (> 25°C) as well as mechanical stimuli [100]. The binding site of phorbol ester 4α-phorbol 12,13-didecanoate (4αPDD) at the third transmembrane domain of TRPV4, is also the same binding site as capsaicin in TRPV1 [101]. Endogenous compounds 5,6-Epoxyeicosatrienoic acid, Arachidonic acid and its precursors Anandamide and 2-Arachidonoylglycerol, act as an endogenous TRPV4 activators [102].

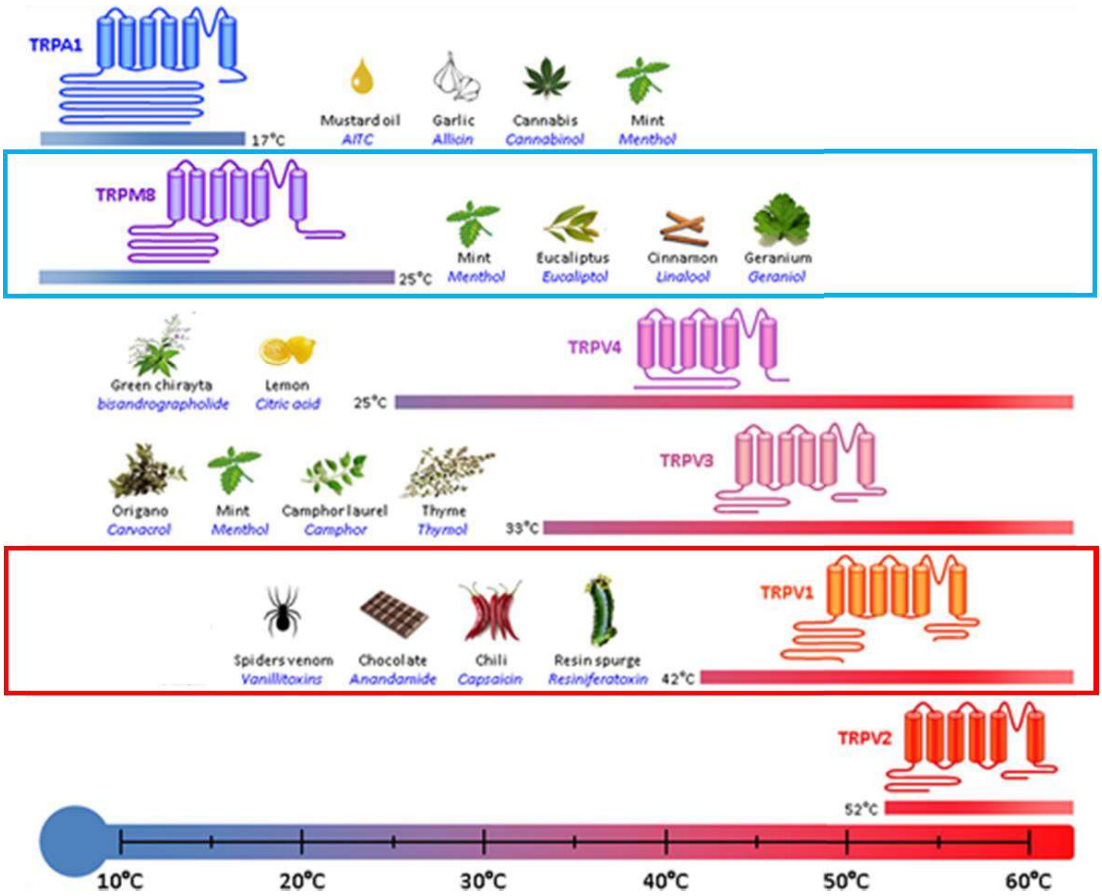

Fig. 6. Polymodal nature of Thermosensitive TRP channels. Thermosensitive TRP channels are activated by several natural compounds, few of which are depicted in the figure. Adapted from [85].

TRPV1 is activated by Capsaicin (the main component in chili, EC50 200nM in DRG neurons), acidic pH (activation threshold < pH 5.5) and heat (activation threshold > 43°C) [86]. In addition, the TRPV1 also gets activated by Vanilloids like Resiniferatoxin, Eugenol, Gingerol and Curcumin. Compounds which lack functional vanillyl moiety, especially polyamines, unsaturated dialdehydes, triprenyl phenols, canabionids as well as certain animal toxin peptides also modulate TRPV1 [87]. Several endogenous molecules like anandamide, NADA (N-arachidonoyl-dopamine) and its hydroxylated metabolites also modulate TRPV1 [88].

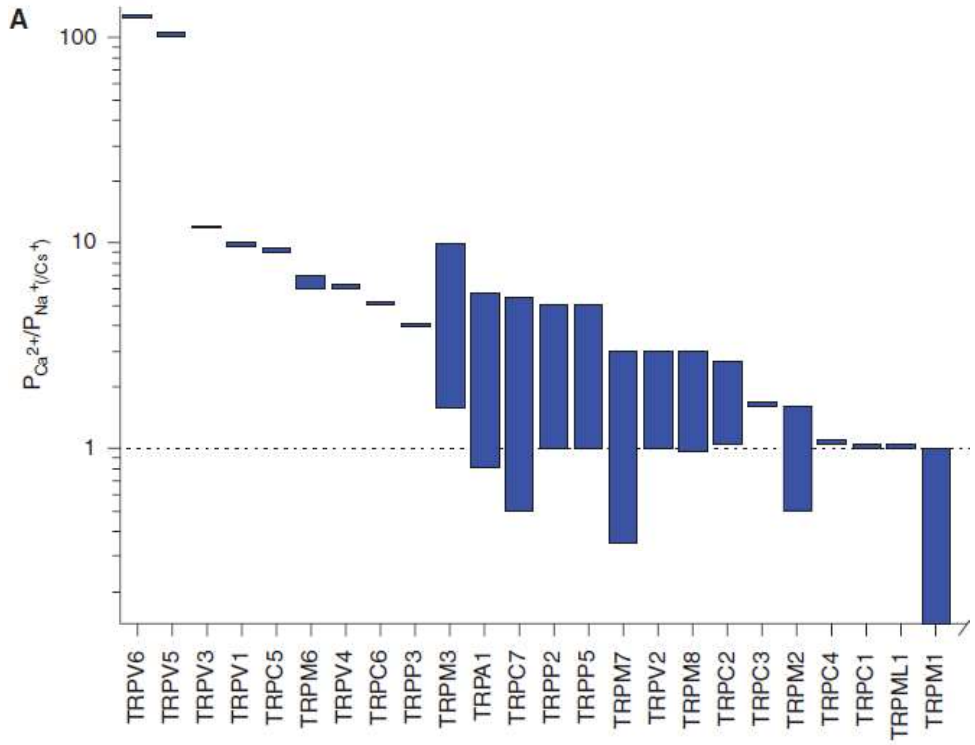

Fig. 5. Ca^{2+} vs Na^+ selectivity of TRP channels. Among TRP channels, TRPV5 and TRPV6 are highly Ca^{2+} selective. TRPV1, TRPV3, TRPV4, TRPC5, TRPC6 and TRPP3 are mildly Ca^{2+} selective. Rest TRP channels including TRPM8 are almost non selective for Ca^{2+}. Adapted from [83].

1.1.5. Regulation of TRP channels: Endogenous and Exogenous modulators

TRP channels are polymodal in nature, meaning they can be modulated by a plethora of endogenous and exogenous factors (**Fig. 6**). A common mechanism of TRP channel activation is by the G-protein coupled receptors and/or receptor tyrosine kinases mediated hydrolysis of phosphatidylinositol [4,5] bisphosphate (PIP2) to produce diacylglycerol (DAG), or inositol [1, 4, 5] trisphosphate (IP3) [84]. However, few TRP channels have got very specific modulators. These include physical stimuli such as temperature, pressure, light as well as chemical stimuli that include several natural products and synthetic ligands.

hypothalamus of brain. TRPM6 and TRPM7 channels are the major Mg^{2+} reabsorption channels in kidney and intestine [73]. In addition, these two channels also regulate intracellular levels of trace elements like Zn^{2+}, Cu^{2+}, Mn^{2+} and Co^{2+} [74]. TRPM8 acts as the cold-sensor (gets activated below 25°C), menthol receptor, testosterone receptor, regulator of sperm motility and acrosome reaction and it has also been implicated in prostate cancer. Mutations in TRPM1 are associated with congenital stationary night blindness [75]. Mutation in TRPM4 has been reported to cause a cardiac bundle branch disorder Progressive Familial Heart Block type 1 (PFHB1) [76]. Impaired intestinal Mg^{2+} absorption and renal Mg^{2+} leak is common due to a mutation in TRPM6 which is associated with hypomagnesemia with secondary hypocalcemia (HSH/HOMG) [77].

The sole member of TRPA subfamily, i.e. TRPA1 acts as a thermosensor in few species, and has been implicated in itch sensation, chemo sensation, nociception and olfactory responses. TRPA1 has been implicated in Familial Episodic Pain Syndrome (FEPS) [78].

The TRPML1, TRPML2 and TRPML3 mostly reside in intracellular vesicles where they are involved in regulating Endosomal, Lysosomal functions along with Autophagy [79]. Mucolipidosis type IV (MLIV) is an autosomal-recessive neurodegenerative lysosomal storage disorder caused by mutations in TRPML1 [80].

The TRPP members are mostly involved in renal functions and serve as flow-sensor in endothelium. Mutation in TRPP2 leads to Polycystic Kidney Disease (PKD) associated with enlarged kidneys and renal failure [81]. Recent studies have suggested that Ca^{2+} signalling mediated by TRPP2, could regulate establishment of left-right asymmetry during early vertebrate embryogenesis [82].

molecules. Efforts by various groups has led to increasing knowledge about the localization and functional relevance of specific TRP channels in various cell types.

Within the TRPC subfamily, TRPC1, TRPC3, TRPC5 and TRPC6 are ubiquitously present in brain and neurons and regulate brain development and functions related to neuronal plasticity [63]. TRPC2 helps in pheromone detection, determining sexual behavior, while TRPC4 and TRPC7 are involved in vascular and respiratory control, respectively [63]. Mutations in TRPC6 have been linked to the human proteinuric kidney disease, termed as Focal and Segmental Glomerulosclerosis [64].

Within the TRPV subfamily, TRPV1, TRPV2, TRPV3 and TRPV4 have been studied mainly in sensory neurons and are responsible for nociception, thermosensation and neurogenesis. TRPV3 has been implicated to be important for maintaining skin integrity, wound healing and hair growth. TRPV5 and TRPV6 are highly Ca^{2+}-selective (**Fig. 5**) and play critical role in Ca^{2+} reabsorption in intestines, kidney and in Vitamin D3 mediated keratinocyte development in the skin [65]. Mutations in TRPV3 causes Olmsted syndrome [66]. TRPV4 also acts as an osmosensor as well as a mechanosensor and TRPV4 has been implicated in bone homeostasis, voiding control, vasculature maintenance. Mutations in TRPV4 have been reported to result in different inherited disorders of bone growth, Brachyolmia and Skeletal Dysplasia [67,68] and neurodegenerative diseases like Scapuloperoneal Spinal Muscular Atrophy (SPSMA) and Charcot–Marie–Tooth disease type 2C (CMT2C) [69-72].

Among the TRPM members, TRPM1 acts as a light sensor in retina and tumor repressor in melanoma cells. TRPM2 is critical for insulin release by pancreas. TRPM3 acts as the steroid hormone (pregnanolone) sensor. TRPM4 regulates histamine release by mast cells, catecholamine release by chromaffin cells and vasopressin release at

of TRPV6 at 3.25Å resolution [62] all of which have shown that the TRPs assemble as tetramers surrounding variably charged gates of different size (**Fig. 4**).

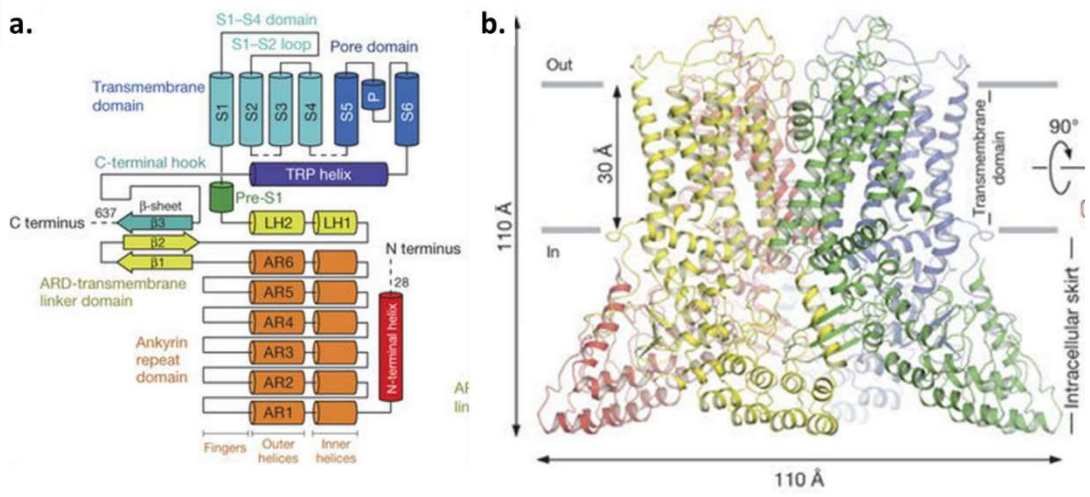

Fig. 4. Structural features of TRPV5. Linear diagram depicting major structural domains of TRPV6 (**a.**). 3.25 Å resolution Crystal Structure derived ribbon diagram of rat TRPV6 (**b.**). Adapted from [62].

1.1.4. Functions of TRP channels

Every cell type within the tissues is likely to express one or more type of TRP channels. Most TRP channels are present in the plasma membrane, where they regulate intracellular levels of Ca^{2+}, Mg^{2+} and trace metal ions either directly or indirectly via transcellular machinery. This in turn affects several physiological processes, including sensory functions (like nociception and temperature sensation, taste transduction, pheromone signaling, etc.), homeostatic functions (like osmoregulation, thermoregulation, hormonal secretion, minerals reabsorption, etc.), motor functions like release of neurotransmitters, vasomotor control and muscle contraction. Often the specific functions carried out by TRP channels at specific locations is made possible by the interaction between TRP channels and associated signaling and scaffolding

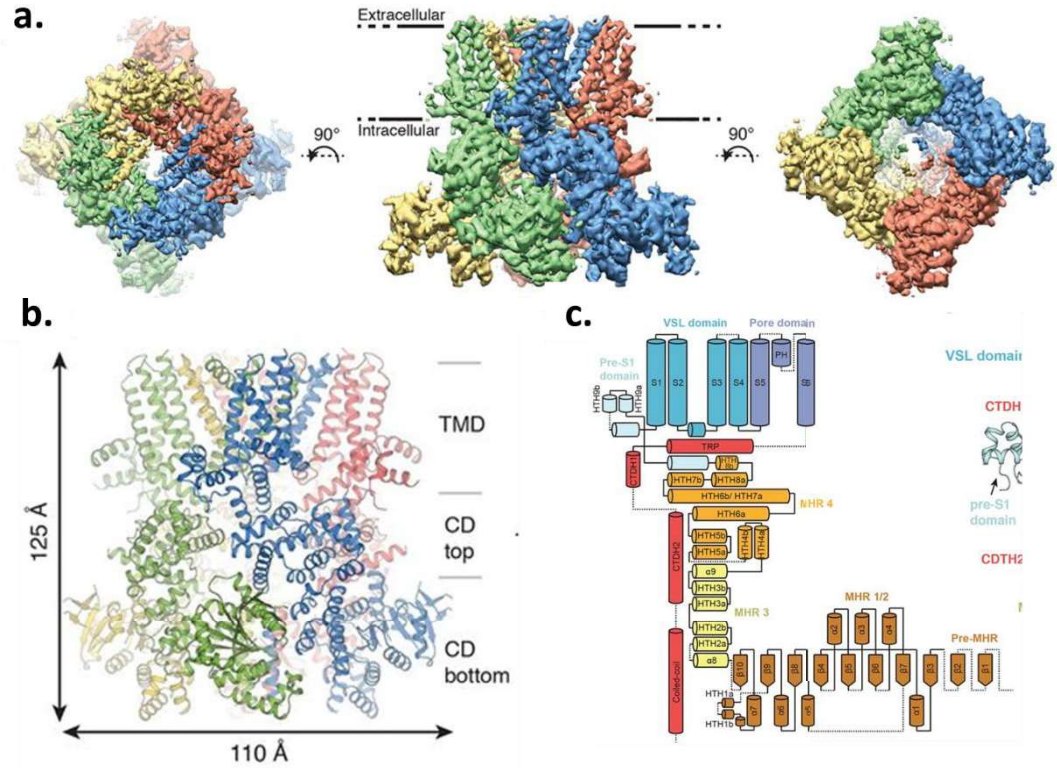

Fig. 3. Structural features of TRPM8. (**a**) Cryo-EM reconstruction and (**b**) model of TRPM8 viewed from the extracellular side (left), from the membrane plane (middle), and from the cytosolic side (right). (**c**) Topology diagram delineating the protein domains with secondary structure elements. Adapted from [43].

A major leap in solving TRP channel structure was achieved by the success of David Julius's and Yifan Cheng's group at University of California, San Francisco in solving the structure of rat TRPV1 at 3.4 Å resolution [44, 45]. Their work confirmed that similar to the voltage-gated channels, TRPV1 tetramer has four-fold symmetry around a central ion-permeable pathway that is formed by the transmembrane segments 5-6 (S5-S6) and the pore loop, and is flanked by S1-S4 voltage-sensor-like domains. TRPV1 has a short selectivity filter in its wide extracellular 'mouth'. The 'TRP domain' consists of a short α-helix and interacts with the S4-S5 linker, which plays a major role in allosteric modulation of TRPV1 [44, 45, 55]. The insight of TRP channel structure-function relationship was followed by subsequent characterization of human TRPA1 cryo-EM structure at ~4 Å resolution [54], TRPV2 cryo-EM structures at ~4 Å resolution [56, 57], TRPP2 cryo-EM structures at 4.2 Å resolution [58-60], cryo-EM structure of *Drosophila* TRPN at 3.6 Å resolution [61] and the crystal structure

channels are “bullet shaped”, where the dense “bullet-head” region include transmembrane segments, and a larger domain proposed to be the cytoplasmic domains. The same group reported a different structure for TRPC3 via cryo-EM studies [38]. This indicated that TRP channels in general adopt a “hanging basket” model in tertrameric form. However high resolution crystal structures for any single full-length TRP channel is still missing. Although high resolution structures of some fragments of TRP channels have been reported, like ankyrin repeats of TRPV subfamily members [46-50], C-terminal coiled-coil domain of TRPM7 [51], C-terminal coiled-coil domain TRPP2 [52] and the α-kinase domain of TRPM7 [53], yet a holistic view of channel structure representing full-length sequence is still missing. Each of these studies have either deleted few regions of the protein, or mutated several residues or have been able to poorly resolve some portions of the TRP channels under study.

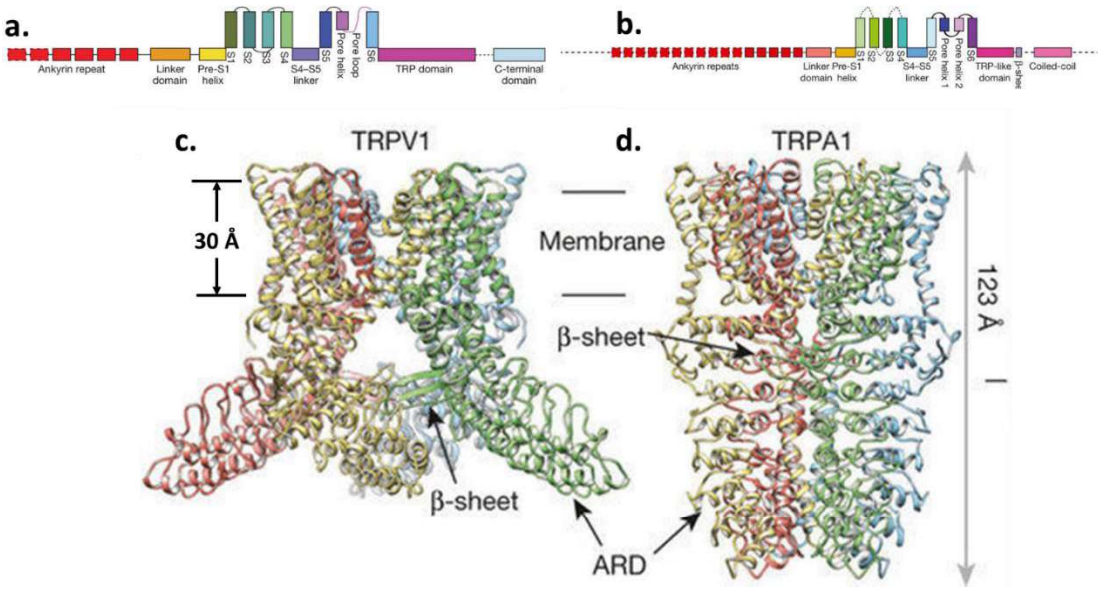

Fig. 2. Structural features of TRPV1 and TRPA1. Linear diagram depicting major structural domains of TRPV1 (**a.**) and TRPA1 (**b.**). 3.4 Å resolution cryo-EM derived ribbon diagram of rat TRPV1 apo-state atomic model (**c.**) compared with that of human TRPA1 (**d.**). Adapted from [44, 54].

repeat domain (ARD). There are 3 to 4 ARDs in TRPCs, 6 in TRPVs, 14 to 17 in TRPAs and around 29 in TRPNs. These ARD appear to be associated with tetramerization of the TRP channels and also involved in molecular interactions with other proteins and regulatory factors [25,26]. Several TRP channels contain motif sequences specialized for specific functions, such as EF-hand motif for Ca^{2+}-sensing, sequences for interaction with cholesterol, sites for phosphorylation, binding sites for tubulin, binding sites for PIP_2 and Calmodulin [27-30].

It is believed that most TRP channels function as homotetramers. However, the formation of heteromultimeric channels either between members of the same subfamily or between different subfamilies has been described in few cases such as incase of TRPCs [31] and TRPV channels [32]. Heterotetrameric channels can have entirely different properties and functions. However, it is still debatable whether these heterotetrameric channels are formed *in vivo* or not [33, 34].

So far, several TRP channel structures derived electron microscopy studies have been reported by [35]. These include those obtained via cryoEM: TRPV1 [36], TRPV4 [37], and TRPC3 [38], and those imaged via negative stain: TRPC3 [39], and TRPM2 [40], TRPM4 [41,42] and TRPM8 [43]. Cryo-EM images followed by single particle reconstruction confirmed that the TRP channel structure is fourfold symmetric and consists of two well-defined domains: the more compact transmembrane domain and the larger bulkier mass C- and N- termini at the cytoplasmic side. The TRPV4 cryo-.EM structure shows a great deal of similarity with TRPV1 structure [37]. The TRPV1 structure differs distinctly from the TRPM8 structure, which forms the basis of difference in their activation and function [44-46] (**Fig. 2, 3**). However structures for TRPC3 [39] and TRPM2 [40] determined via negative stain method indicated that these

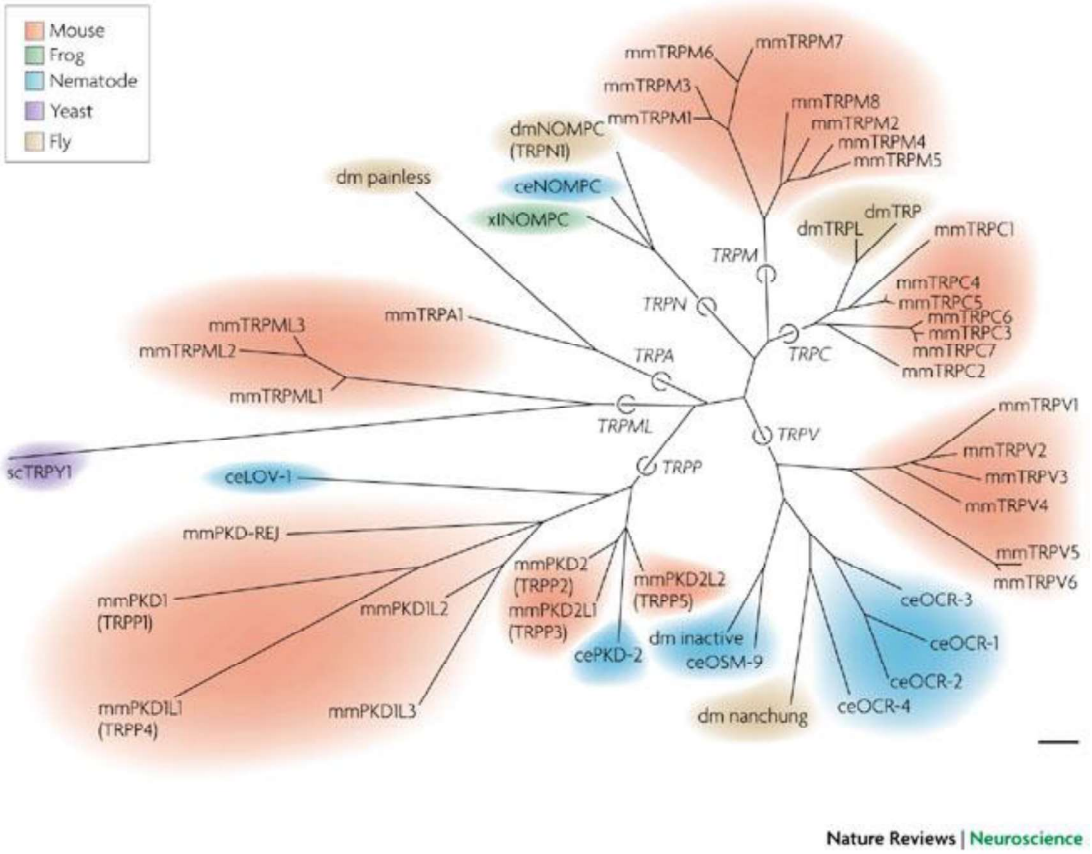

Fig. 1. The TRP channel tree. A phylogenic tree generated using ClustalX by aligning the transmembrane domains of all 33 transient receptor potential (TRP) channels from mouse and some from other species are shown. The seven main branches are denoted by circles at the branch roots. The letters and numbers following TRP denote TRP subfamily and member names, respectively. Different species are indicated by colors and by prefixes. [ce, *Caenorhabditis elegans*; dm, *Drosophila melanogaster*; mm, *Mus musculus*; sc, *Saccharomyces cerevisiae*; xl, *Xenopus laevis*.] Scale bar represents 0.2 nucleotide substitutions per site. Adapted from [23].

1.1.3. Structure of TRP channels

The TRP channels are typically characterized by the presence of six transmembrane helices, intracellular N-terminus and C-terminus regions. The pore loop is present between 5th and 6th transmembrane regions. Comparison between TRP channel pore loop and that of voltage-gated channels has revealed that the residues contributing to voltage sensitivity are absent within the TRP superfamily [24]. Several members of the TRP channels have a conserved sequence of 25 amino acids, the "TRP-box", immediately located after the 6th transmembrane helix [4]. The N- terminal cytoplasmic domain of TRP channels have variable number of repeats in the ankyrin

with the melanomic cell lines [13]. TRPMLs derive their name from TRPML1, mutation in which leads to a neurodegenerative lysosomal storage disorder Mucolipidosis type IV [14]. TRPPs derive their name from 'polycystins', named after TRPP2 (PKD2) mutation, which leads to autosomal polycystic kidney disease in humans [15]. The TRPA subfamily is characterized by the presence of about multiple ankyrin repeats. The TRPN subfamily members derive their names from the 'NO-mechano-potential C' (NOMP-C) channel of *Caenorhabditis elegans*. TRPN channels are not found in mammals, but they are expressed in invertebrates such as in flies and in worms. The only vertebrate where a member of TRPN family has been reported is Zebrafish [16]. The eighth subfamily TRPY (also known as 'yeast' TRP) is distantly related to rest of the seven classical TRP channel subfamilies. TRPY subfamily comprises of only one channel, namely TrpY1 that acts as a mechano-sensor of vacuolar osmotic pressure in yeast [17].

In spite of extensive genetic analysis, none of the terrestrial plants have been noted to have TRP-encoding genes [18]. However, a group of aquatic green alga *Chlamydomonas reinhardtii* appears to have genes that codes for a TRP-like channel responsible for Ca^{2+} signaling [19]. Several TRP channels have also been predicted to be present in parasites [20] but only a few have actually been experimentally reported. Two TRPML-like genes, namely lmmlA and lmmlB, have been described in *Leishmania* major [21]. A TRPA-like channel has been reported to regulate locomotor activity and respond to TRP channel drugs in *Schistosoma mansoni* [22]. All these evidence suggest that TRP channels are present in Metazoans, with variations in their structure and function.

Analysis of the deduced amino acid sequence in that report suggested that *trp* gene product is actually a membrane protein consisting of up to 8 trans-membrane segments, and the gene product shares many features with several receptor/transport proteins, yet has unique properties that distinguish it from the voltage-gated and ligand-gated ion channels reported at that time [6]. The use of patch clamping technique by Hardie established firmly that *trp* channel actually acts as a Ca^{2+} permeable channel [8]. The observation that the original Cosens-Manning trp mutant (designated trp^{CM}) shows faster electroretinogram decay to baseline and slower dark recovery kinetics when raised at 24°C (room temperature) relative to 19°C indicated that trp^{CM} is a developmental temperature-sensitive mutant [9]. This also suggested that apart from light, the *trp* channel could also be affected by temperature. A major mile-stone discovery happened in this research field when David Julius and co-workers cloned the Capsaicin Receptor from rat DRG neuron cDNA library [10]. They discovered that TRPV1 act as the "Capsaicin receptor" and it acts as a heat activated ion channel in the pain pathway [10]. This finding led to significant interest and funding in TRP channel research. Subsequently a large number of TRP channels have been discovered.

1.1.2. Diversity in TRP channels

As of now, more than 200 TRP channels across species and 28 Mammalian TRP channels have been reported [11] (**Fig. 1**). TRP channels have been classified into 7 subfamilies based on amino acid sequence and structural homology [12]. The TRPC subfamily ('canonical') consists of the closest homologs of Drosophila *trp* channels. The TRPVs ('vanilloid') have been named after the founding member Vanilloid Receptor 1 (TRPV1). The TRPM subfamily comprises homologs of TRPM1 which was initially named as "melastatin", due to the inverse correlation of its expression level

1. Introduction

Transient Receptor Potential (TRP) channels are the most diversified family of ion channels, polymodal in action, non-selective in cationic conductance and are involved in a wide range of sensory and cellular functions [1]. Intensive studies over the last 3 decades have established TRP channels as a major cellular sensor of environmental and physio-chemical signals and as important regulator of several cellular events. TRP channels have been implicated in several physiological roles and mutations/malfunctions of TRP channels have been implicated in diverse pathological conditions in humans. Due to their diversity in modes of activation-inhibition and diversity in effect on physiological conditions, much more remains to be studied to explore their role in neuronal and non-neuronal systems.

1.1. General Overview on TRP Channels

1.1.1. Discovery of TRP Channels

The first report of TRP channel was the observation by Cosens and Manning in 1969 that a group of spontaneously formed *Drosophila melanogaster* mutant flies behave as though blind under bright illumination, an effect that could be reversed by withdrawing bright light minimum for a minute [2]. Due to the transient response to sustained intense lights, this mutant was termed as 'transient receptor potential' or "trp" mutant by Minke et al. in 1975 [3]. Later on in 2002, an international committee of researchers collectively adapted "TRP" as the name of the entire TRP superfamily of ion channels [4].

In 1985, the DNA encoding *trp* gene was isolated and subsequently in 1989 the cloning, sequencing and molecular characterization of *trp* gene was reported [5-7].

Chapter 1

Introduction

and

Review of Literature

different functions in the sperm cells. The function/s of these channels also differs from species to species. Careful selection of TRPV1 and TRPM8 modulators and their dosage could be helpful in increasing fertilizing ability of vertebrate sperm.

penetration test using boar spermatozoa [259]. In contrast, in human sperm Capsazepine treatment isn't able to inhibit progesterone and ZP3-induced acrosomal reaction [271] indicating that that this function of TRPV1 is species specific. Interestingly, TRPM8 activation significantly reduced the number of sperm undergoing the progesterone-induced acrosomal reaction following capacitation in murine sperm [273]. Menthol, the TRPM8 activator has been shown not to affect motility of human sperm and to induce acrosomal reaction [271]. Menthol has also been shown to induce acrosomal reaction in murine sperm [272]. TRPV1 has been shown to be important for thermotaxis in human sperm [265]. SimilarlyTRPM8 has been shown to be important for thermotaxis of murine sperm [272]

3.4.4. TRPV1 and TRPM8 are differentially expressed during spermatogenesis

Besides playing significant role in mature sperm, TRPV1 and TRPM8 could also be relevant for spermatogenesis. In fact previous literature indicated the presence of TRPV1 and TRPM8 mRNA transcripts in rat spermatogenic cells [328]. However there has been no report on the protein level expression of TRPV1 and TRPM8 in different stages of spermatogenesis. Both TRPV1 and TRPM8 were abundantly present in different cells of mature testis, immature testis and various regions of epididymis. However, expression levels of both these channels are different in different stages of sperm development. This data indicates that TRPV1 and TRPM8 could also be relevant for spermatogenesis and therefore justifies the requirement of specific temperatures for spermatogenesis.

Taken together, all these results show that both TRPV1 and TRPM8 are endogenously expressed in vertebrate sperm and that they differentially regulate

3.4.3. TRPM8 but not TRPV1 is differentially expressed in capacitated and acrosome reacted mice sperm

There exists great deal of heterogeneity in sperm cell shape and size among the vertebrates. This also indicates that the expression pattern of TRPV1 and TRPM8 can also vary from species to species. The mice has a hook-like head with a thin acrosome. Interestingly while TRPV1 is present throughout the mice sperm and enriched at the mice sperm tail, TRPM8 is highly enriched at the acrosome of uncapacitated mice sperm. It is important to note that TRPM8 is not present in the acrosome of any other vertebrate (other than mice) sperm tested in this thesis work. This points out that TRPM8 expression pattern is "species specific". Interestingly, in capacitated sperm, TRPM8 localization changes to the post-acrosomal region and tail while in acrosome-reacted mice sperm, TRPM8 forms a major cluster at the post acrosomal region and is totally absent in the acrosomal region. However TRPV1 continues to localize throughout the mice sperm in all of these conditions. This indicates that specifically TRPM8 have some role in sperm capacitation and acrosomal reaction, at least in mice.

The importance of TRPV1 and TRPM8 in such sperm functions are also evident from previous reports by other groups. Anandamide, a major endocannabinoid, has been shown to mediate capacitation of bull sperm via TRPV1 and CB1 receptors [260]. TRPV1 levels have been shown to be undetectable in sperm from infertile human subjects [402]. TRPV1 activity has been shown to stabilize the plasma membrane of boar sperm and inhibition of TRPV1 for a long period induces acrosomal reaction in boar sperm [262,396]. In fact, the selective TRPV1 antagonist Capsazepine (CPZ) inhibited progesterone induced sperm/oocyte fusion, as evaluated by the hamster egg

in motility can be effectively blocked by TRPV1 specific inhibitor 5'-IRTX and general TRP channel inhibitor Ruthenium Red, indicating that TRPV1 along with other TRP channels regulate fish sperm motility. The increase in fish sperm motility can enhance the fertilization rates of fish sperm in the fish hatcheries, hence be of commercial importance for the fisheries industry. It was observed that this increase in sperm motility through TRPV1 activation also occurs in higher vertebrates like Duck sperm and Bull sperm. This indicates that the mechanism of motility control by TRPV1 is most likely conserved among vertebrates. The role of TRPM8 in sperm motility is interesting. TRPM8 activation appears to slow down bull sperm motility, but TRPM8 inhibition doesn't appear to affect bull sperm motility. This probably also indicates that the heat sensitive TRPV1 and cold-sensitive TRPM8 channels differentially regulate sperm motility.

Besides motility, capacitation and acrosomal reaction are also important factors regulating sperm fertilizing ability. The sperm needs to undergo capacitation only upon reaching the vicinity of the oocyte and immediately before fusing with the oocyte the acrosomal reaction must occur. Both these events are largely dependent on increase in intracellular Ca^{2+} levels and TRPV1 or TRPM8 activation can lead to increase in intracellular Ca^{2+} levels in sperm cells thereby causing premature capacitation and acrosomal reaction. Therefore the possibility of premature capacitation and acrosomal reaction induction upon TRPV1 and TRPM8 modulation was checked in bull sperm. It was noted that neither activation nor inhibition of TRPV1 or TRPM8 induce premature capacitation and acrosomal reaction. This suggests that TRPV1 or TRPM8 can be safely used for increasing sperm motility, without adversely affecting capacitation or acrosomal reaction.

almost no specific co-localization was observed, indicating that though TRPM8 localizes in close proximity of mitochondria at sperm neck, TRPM8 is not present within mitochondria.

TRPV1 expression was quite low in the mitochondrial region of bull sperm, hence the possibility of TRPV1 being present in sperm mitochondria wasn't explored further. However, in other vertebrates like fish and duck sperm, TRPV1 was found to co-localize with mitotracker red. In fact strong co-localization of TRPV1 was found with mitochondrial marker proteins Cytochrome C and HSP60 in fish sperm. This indicated that TRPV1 is present at the mitochondria and might regulate mitochondrial functions. These observations are strengthened by the fact that both TRPV1 and TRPM8 have been reported to be either present in mitochondria or regulate mitochondrial functions [398-401].

3.4.2. TRPV1 and TRPM8 are regulate sperm motility without affecting capacitation or acrosome reaction

Sperm motility is an essential parameter determining its fertilization ability, as the vertebrate sperm has to swim great distances to find the oocyte and fertilize it. The fact that TRPV1 and TRPM8 are chemosensitive, osmosensitive and thermosensitive, makes these two important candidates regulating sperm motility. Besides presence in and regulation of mitochondrial functions by these two channels and interaction with cytoskeleton further add to the fact that these two channels can modulate sperm motility. In fact it was observed in this study that TRPV1 activation can increase the percentage of motile fish sperm and helps in maintaining motility upto 90 minutes as compared to just 2 minutes motility in control conditions. This TRPV1 activation-mediated increase

mitochondria. Nevertheless, conserved expression of TRPM8 in the vertebrate sperm cells strongly suggests the evolutionary conserved role of TRPM8 and may also explain the thermosensitivity observed in these motile cells.

On other hand TRPV1 is present throughout the sperm of homeothermic animals (such as mammals and avian) and poikilothermic animals (such as fish, amphibians and reptiles). TRPV1 is enriched in the neck and tail region of the sperm of almost all species tested in this study. This indicates that TRPV1 plays an important role in sperm motility. Particularly, the mitochondrial region of sperm from most of the vertebrates have enhanced and specific localization of TRPV1 and TRPM8 indicating their possible role in regulating energy homeostasis of sperm cells, which is vital to enable their motility. Interestingly, non-motile human sperm have reduced percentage of cells expressing TRPV1 and TRPM8. Even the abundance of these two channels is decreased in non-motile sperm. In the non-motile human sperm both TRPV1 and TRPM8 are mislocalized and highly clustered at neck region of the sperm. This also indicates that expression and localization levels and location of these two channels are essential for sperm motility.

This observation is further strengthened by the fact that TRPM8 is present at detectable levels in only those bull sperm cells which have high mitochondrial potentiality. Those cells whose mitochondria potentiality is low, the expression profile of TRPM8 is either low or absent there. It indicates that there is a “one-to-one” correlation between mitochondria and TRPM8. In order to investigate the possibility of TRPM8 being present in the mitochondria, super-resolution imaging of mitotracker-red labelled bull sperm stained with TRPM8 was performed. Interestingly, TRPM8 clusters were found to cluster around the mitochondrial coil all along the neck region. However,

3.4.1. TRPV1 and TRPM8 are endogenously expressed in sperm

In this study, TRPV1 and TRPM8 were found to be endogenously expressed in vertebrate sperm (Piscean, amphibian, reptilian, avian and mammalian) [327,397]. However their localization pattern differs from species to species indicating that they may play different roles in the sperm of different species and such features may also be used as specific biomarkers.

This is supported by the fact that in fish sperm and in bull sperm, TRPV1 activation leads to enhanced motility, while TRPV1 inhibition leads to decreased motility. TRPV1 and TRPM8 activation as well as inhibition regulate sperm motility without affecting capacitation or acrosomal reaction of sperm. This indicates that modulators of TRPV1 and TRPM8 can be potential drugs for contraception as well as for motility-related infertility cases.

Detection of TRPM8 in the sperm cells of early vertebrates is intriguing. The sperm cell specific expression of TRPM8 suggests that TRPM8 may have played an important role in the adaptation (in response to temperature) of warm-blooded (homeothermic animals) and cold-blooded (poikilothermic) animals in different ecological niche, especially in animals (such as in fish and amphibians) where fertilization is exogenous in nature. Though we have detected TRPM8 expression in sperm cells from all the vertebrates that we have tested so far, an interesting pattern of TRPM8 localization is worth mentioning. In our analysis we noted that the localization of TRPM8 is mainly restricted in the sperm tail region of homeothermic animals (such as mammals and avian) with warm blood and having internal fertilization. In contrast, poikilothermic animals with cold blood (such as fish, amphibians and reptiles) the localization of TRPM8 in sperm is mainly restricted in the neck region which contains

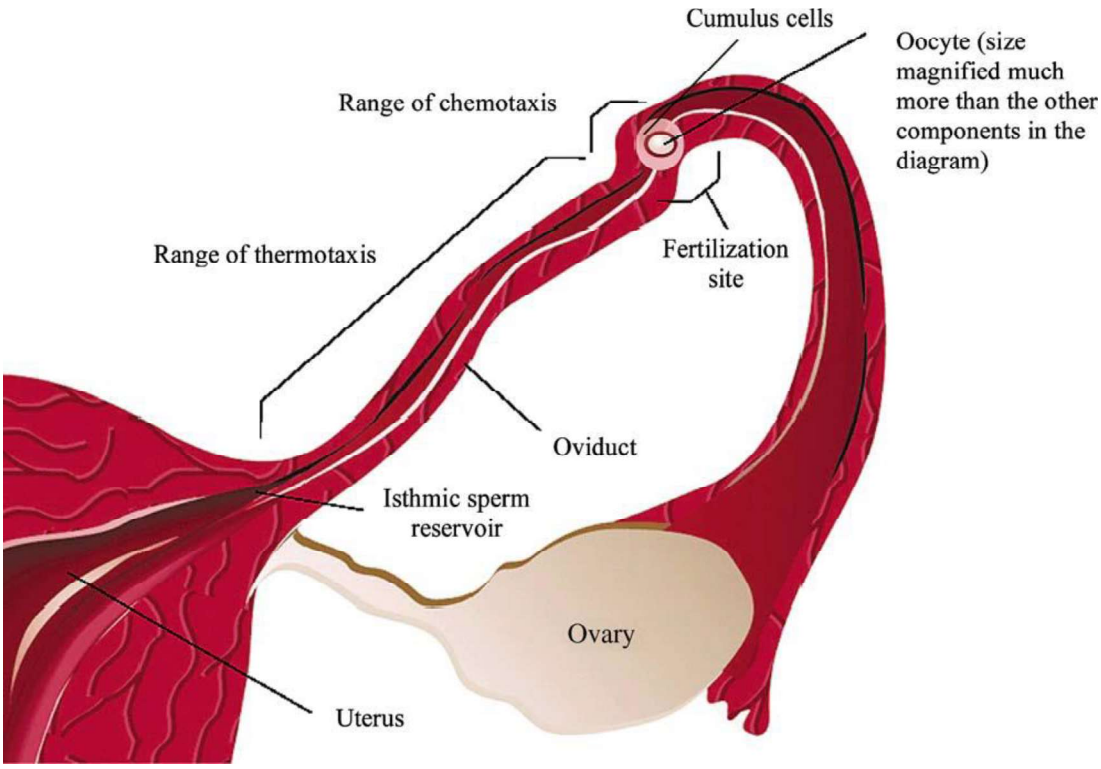

Figure 102. Mechanisms involved in guidance of sperm cells within the mammalian female genital tract. This schematic picture indicates the importance of thermotaxis over chemotaxis. Range of chemotaxis is quite small as the hormones secreted by oocyte dilute out after diffusing to long distances. Thermotaxis rage is quite long and appears to be a major factor guiding sperm from isothermic sperm reservoir to the site of fertilization. This picture represents human female genital tract (not drawn to scale). Image adapted from [394].

In mammals, the signaling events regulating thermotaxis of sperm are not well studied. The role of thermosensitive TRP channels in several vertebrates has been reported recently [395,396,260, 261,271,327]. The TRPV channels are capable of detecting thermal, chemical, osmotic, voltage and pH conditions [326]. Sperm development and function are regulated by these signaling cues. TRPVs can be expected to be involved in several of these steps. Hence it is necessary to determine the expression pattern of TRPV1 in vertebrate sperm and to decipher its functional role in determining fertilizing ability of sperm.

the oocyte, and ultimately fuse with the plasmalemma of the oocyte to deliver its genetic material [324]. These events require sperm cells to constantly and efficiently detect and respond to appropriate chemical and physical cues (like pH gradients, temperature) and only after reaching vicinity of the oocyte, the sperm has to undergo capacitation and finally fuse with the oocyte. Timing of each of these responses is critical and premature or inappropriate activation of these events can lead to failure in fertilization. Since sperm cells are mostly transcriptionally and translationally inactive, all cellular activities within it are carried out by the pool of proteins that has to be inherited during differentiation of spermatozoa85-387] and these proteins are responsible to regulate sperm functions via secondary messengers like intracellular Ca^{2+}. In ejaculated sperm cells, intracellular Ca^{2+} influences motility, chemotaxis [388,389], capacitation, hyperactivation [390, 391], acrosome reaction [392] etc.

Along with efficient Ca^{2+}-signaling, sperm cells must also sense several physical and chemical cues like pH, osmolarity and viscosity of the medium in order to re-orient themselves towards the oocyte [393]. Besides, another important guidance mechanism is thermotaxis, which is conserved sensory mechanism prevalent in among vertebrates. Mammalian spermatozoa are guided by temperature gradient from the cooler reservoir site (oviductal isthmus) towards the warmer fertilization site [393]. Rabbit and human spermatozoa are capable of sensing minute temperature differences (0.5°C or lower) during thermotaxis [393].

3.4 TRPV1 and TRPM8 are endogenously expressed in Vertebrate sperm and regulate fertilization potential of sperm

After exploring the expression and function of TRPV1 and TRPM8 in adherent cells (neurons), semi-adherent cells (macrophages) and non-adherent cells (T cells), the next target was actively swimming cells i.e. the sperm cells. Similar to other cells explored in this study, the sperm cells are also highly thermosensitive in nature and are also regulated by other physio-chemical parameters like pH, hormones, fluid-pressure etc., all of which are sensed by different TRP channels (**Fig. 101**).

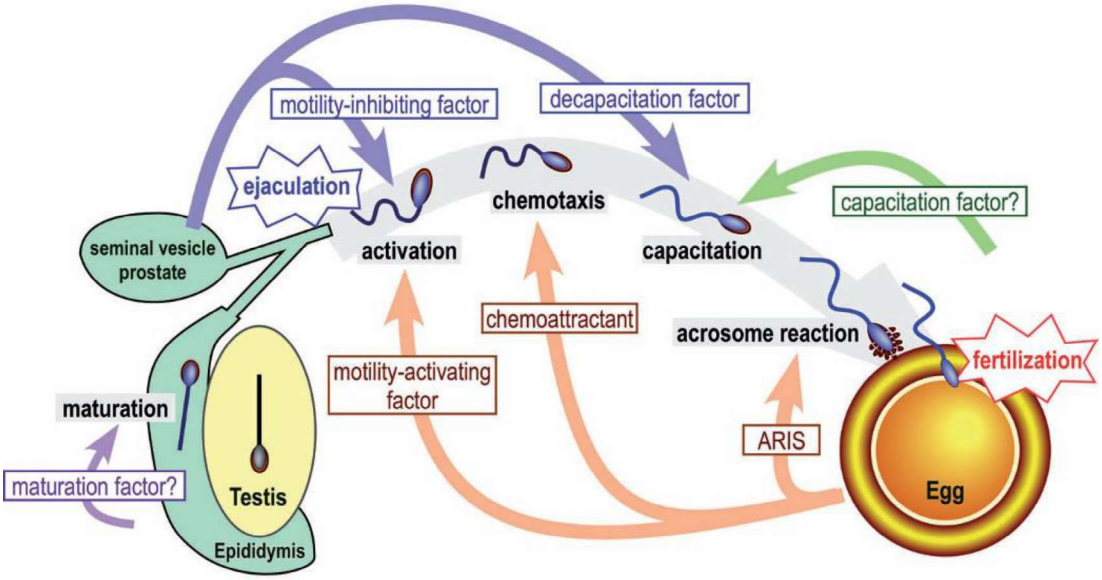

Fig. 101. Schematic drawing of the sperm cells journey and respective events during fertilization. Sperm cells mature in the testis, and remain inactive by motility inhibiting factors, till their release in female reproductive tract. Upon activation, sperm use chemotaxis as guiding signal to reach the vicinity of oocyte, where it undergoes capaciatation and acrosomal reaction. This is followed by fertilization with the oocyte. Each of these events are tightly regulated by Ion channels and chemicals released by the oocyte, which are sensed by these ion channels. Image adapted from [324].

During exogenous fertilization, sperm travel a long distance in aqueous media while during endogenous fertilization, sperm cells travel within the female reproductive tract to meet the oocyte. In higher mammals, sperm cells have to swim through the viscous mucus, find the oocyte, penetrate the cumulus and zona pellucida surrounding

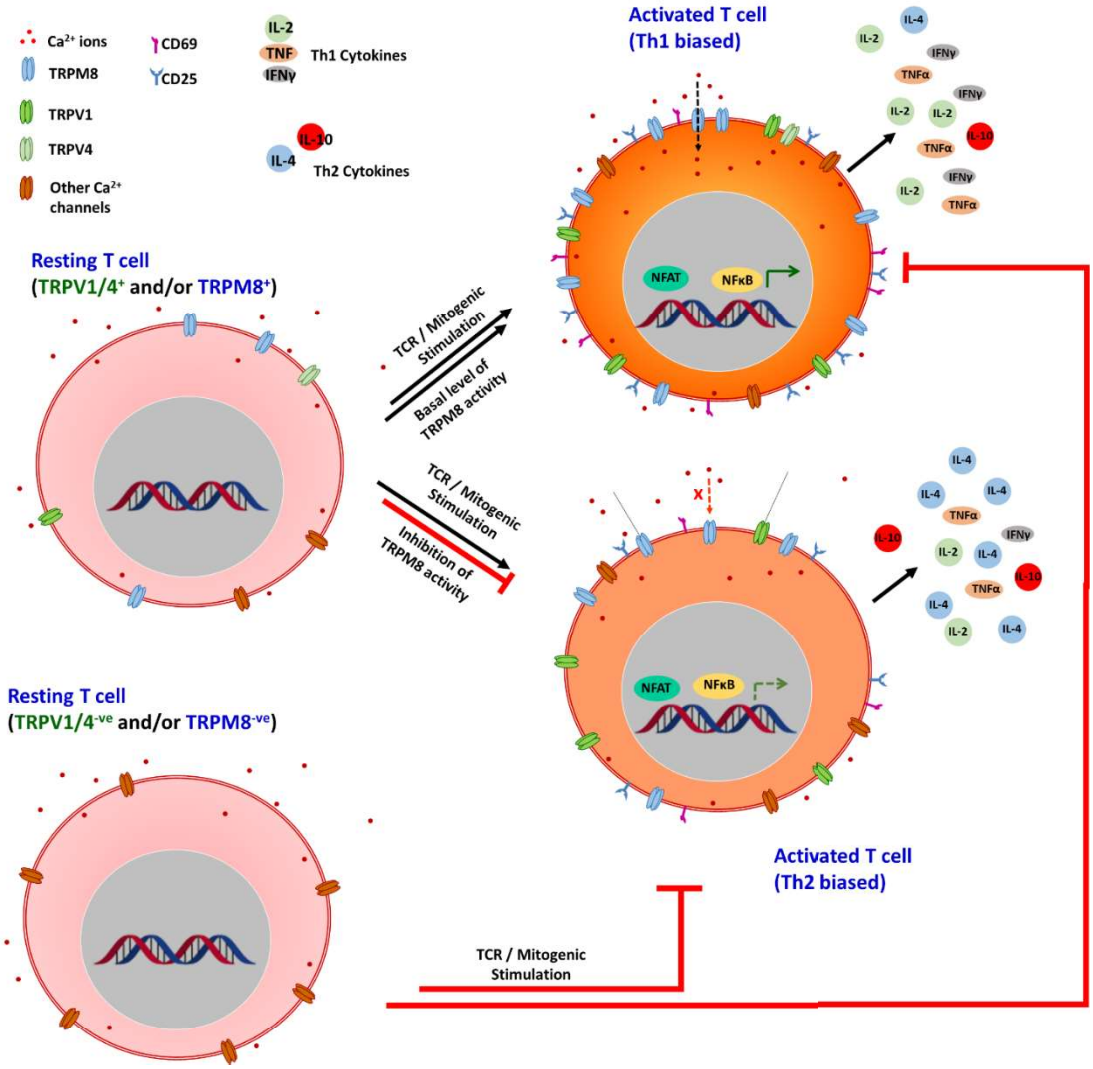

Figure 100. Proposed model depicting involvement of TRPM8 in T cell activation and effector responses. Functional TRPM8present in T cells appear to be involved in T cell activation and effector responses by modulating intracellular Ca^{2+}-levels and other signaling events resulting effector cytokine production and induction of T cell activation. Naïve T cells with low intracellular Ca^{2+} ions express TRPM8 channels mostly at the surface. However, activation of T cells triggers enhanced expression of TRPM8 channels at the surface and increases intracellular Ca^{2+} as well as expression of T cell activation markers (CD25, CD69), along with secretion of effector cytokines. In case of basal level activity of TRPM8 is on, it drives T cells to differentiate into Th1-biased T cells (producing larger amounts of IL-2, TNFα and IFNγ) whereas if TRPM8 activity is inhibited during TCR/Mitogenic stimulation, it induces T cells to differentiate into more of Th2-biased T cells (producing more IL-4). Naïve T cells that do not express TRPV1 or TRPM8 are most likely fail to get activated by ConA stimulation.

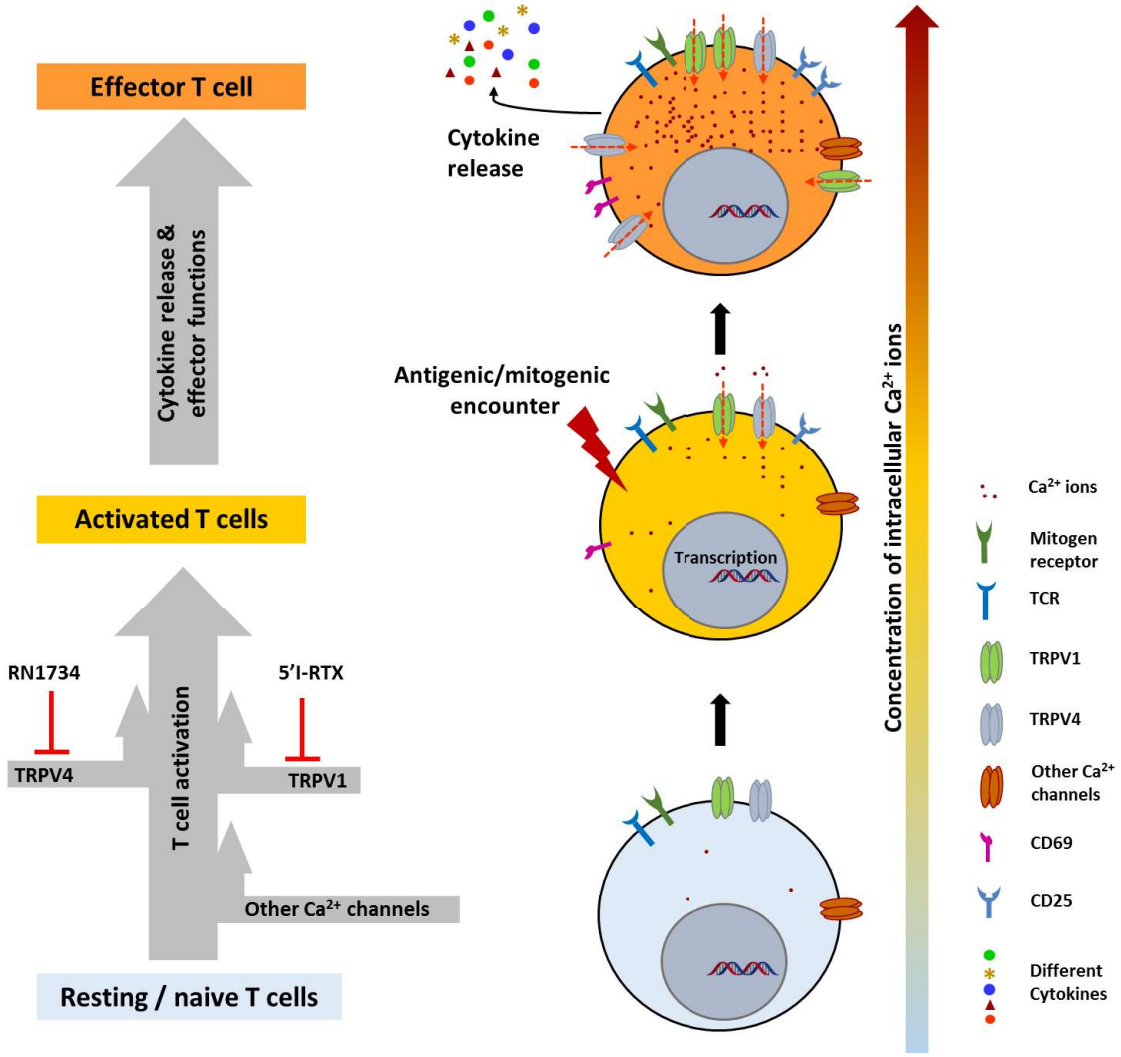

Fig. 99. Proposed model depicting involvement of TRPV1 channels in T cell activation and effector responses. TRPV1 channels present in T cells seem to be involved in diverse functions such as T cell activation, effector responses in association with cellular Ca^{2+} influx, effector cytokine production and induction of T cell activation markers (CD25, CD69). Involvement of TRPV1 channels in T cell activation follows sequential steps. Naive T cells with low intracellular Ca^{2+} ions express TRPV1 channels (as well as other TRPV channels such as TRPV4) at lower levels. However, activation of T cells coincides with enhanced expression of TRPV1 channels and further increment of intracellular Ca^{2+} as well as induction of T cell activation markers along with effector cytokine production. Such activation and effector function of T cells seem to be facilitated by TRPV1 (and also synergistically with TRPV4, as reported in [314]) as inhibition of those TRPs may restrict T cell responses. TRPV1 channel along with other TRPV channels are likely to contribute in these T cell functions in both Ca^{2+}-dependent and Ca^{2+}-independent manners.

During the process of activation of T cells, both TCR and ConA induce rapid T cell proliferation. TRPM8 inhibition is able to effectively reduce the TCR and ConA-mediated T cell proliferation. This could also be one of the mechanisms where basal levels of TRPM8 activity is essential for T cell activation. The inhibitory effects of AMTB on T cell effector functions allow us to investigate if TRPM8 inhibition affects T cell viability. Flow cytometric analysis using AnnexinV and 7AAD revealed that the only WS12 (5µM) or AMTB (10µM) used in this study, had any effect on T cell viability. Further, even in case of T cell activation-induced cell death, neither TRPM8 activation nor inhibition is detrimental to T cell viability.

This study revealed that the heat-sensitive TRPV1 and cold-sensitive TRPM8 channels are endogenously expressed in T cells and are also relevant for T cell activation as summarized (**Fig. 99 and Fig. 100**). We conclude that basal level TRPM8 activity is essential for proliferation and differentiation of $CD3^+$ T cells towards a protective Th1 response.

activator WS12 results higher T cell activation in case of TCR stimulated as well as in ConA stimulated conditions. This shows that TRPM8 activation can enhance T cell activation when used along with other antigenic or mitogenic stimulation. However TRPM8 activation or inhibition alone, has no effect on T cell activation. Although TRPM8 inhibition by AMTB is not able to block TCR-mediated activation, AMTB pre-treatment is able to block ConA mediated T cell activation significantly as evidenced from lower levels of $CD25^+$ or $CD69^+$ cells. This indicates that ConA and TCR stimulation involve different pathways in which TRPM8 has differential involvement.

In ConA-stimulated T cells, pre-treatment with TRPV1 inhibitor decreased release of pro-inflammatory cytokine IFNγ by T cells, but not of TNF or IL-2. These results indicate that TRPV1 regulates T cell activation towards a protective role. Similarly, ConA-mediated increase in these inflammatory cytokines (IL2, TNF, IFNγ) is effectively reduced upon TRPM8 inhibition. However, neither TRPM8 activation, nor inhibition affects TCR-mediated release of Th1-inducing inflammatory cytokines (such as IL2, TNF, IFNγ). In addition in TCR-stimulated cells, TRPM8 inhibition resulted in nearly 2-fold increase in the levels of Th2-inducing anti-inflammatory cytokine (IL4) and reduced levels of immune suppressor cytokine (IL10) in both TCR-stimulated and ConA- stimulated cells. Taken together, the cytokine profiles indicate that basal level of endogenous activity of TRPM8 is required for T cell activation and differentiation into Th1 effector cells. Inhibition of TRPM8 during T cell activation process can promote differentiation of T cells to Th2 type. The difference in cytokine profiles between TCR stimulation and ConA-mediated stimulation can be attributed to the difference in signaling pathways activated by these [384].

induce rapid rise in intracellular Ca^{2+}-levels in T cells. This probably suggest that endogenous TRPM8 activity is important for maintenance of intracellular Ca^{2+}-homeostasis as both inhibition of TRPM8 as well as activation alters Ca^{2+}-levels. Further experiments are needed to confirm the contribution of extracellular Ca^{2+}-entry and Ca^{2+}-release from intracellular stores. Localization of TRPV1 as well as TRPM8 within the cell can indicate its mode of action. For example, TRPV1 has been shown to be a functional ion channel present in the plasma membrane, Endoplasmic Reticulum as well as in the mitochondria [382]. Similarly, in LNCaP cell line, TRPM8 is almost exclusively present on the ER and is nearly absent at the plasma membrane, therefore TRPM8 activation by cold/Menthol/Icilin cause Ca^{2+} release from the ER [383]. In HEK293 cells, TRPM8 mainly localizes at the plasma membrane, and its activation doesn't release Ca^{2+} from ER [383]. However, in T cells, when WS12 is added along with AMTB, it fails to raise intracellular Ca^{2+}-levels, thereby confirming that the activator and inhibitor antagonize each other well.

Anti-CD3/anti-CD28 antibody cocktail is able to induce rapid Ca^{2+}-entry, an effect that can be blocked by pre-incubation of the T cells with TRPV1 inhibitor 5'-IRTX or AMTB. Although pre-incubation with 5'-RTX blocks the elevated Ca^{2+} levels caused by ConA, yet pre-incubation with AMTB is not able to do so. This suggests that TRPV1 and TRPM8 inhibition can inhibit TCR-mediated signaling but only TRPV1 inhibition can block mitogen-mediated signaling, at least in terms of intracellular Ca^{2+} levels.

Inhibition of TRPV1 prevented T cell activation by ConA or anti-CD3/anti-CD28 driven TCR stimulation. This supports the hypothesis that TRPV1 needs to be in the ON-state during T cell activation process. Further, pre-treatment with TRPM8

cells lacking both TRPV1 and TRPM8 are most likely fail to get activated by ConA. Therefore it is possible that the mechanism of ConA-mediated Ca^{2+} influx acts in a "TRPV1-dependent", but "TRPM8-independent" manner.

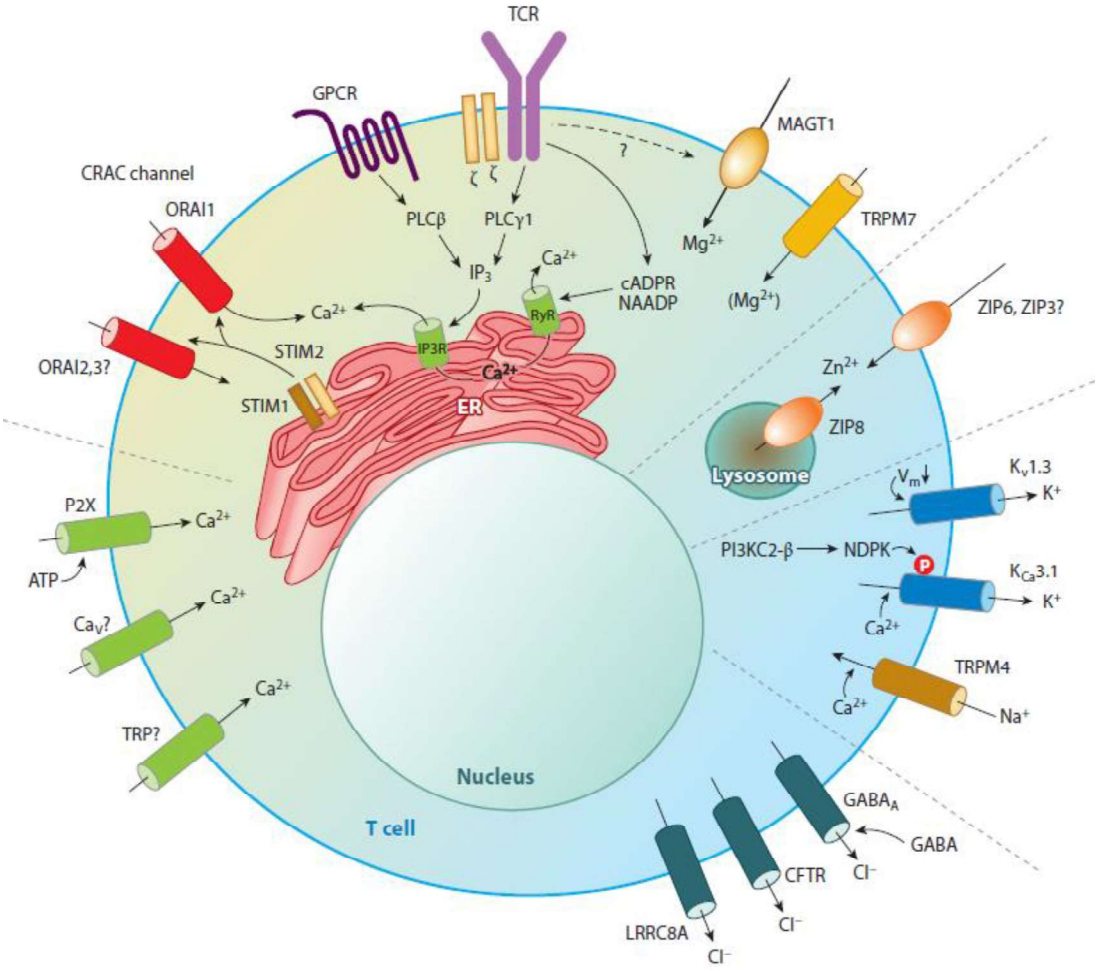

Fig. 98. Ion channels present in T cells. T cells contain several ion channels including TRP channels. These ion channels regulate calcium dynamics of T cells and hence affect T cell activation initiated upon antigen-TCR complex formation. Adapted from [344].

3.3.2. TRPV1 and TRPM8 differentially regulate T cell functions

We have verified the functionality of TRPV1 and TRPM8 in T cells by different means. Ca^{2+}-imaging experiments performed in presence of 2mM extracellular Ca^{2+} shows that only TRPV1 activators RTX and NADA increase intracellular Ca^{2+}-levels in T cells while both TRPM8 activator WS12 (5µM) and inhibitor AMTB (10µM)

3.3 TRPV1 and TRPM8 are endogenously expressed in T cells and regulates T cell activation

3.3.1. TRPV1 and TRPM8 are endogenously expressed in murine and human T cells

T cells form a vital part of adaptive immunity as they get stimulated by antigen presenting cells (APC) and carry out either effector functions or immune-regulatory functions. Many of these functions are tightly regulated by ion channels present in T cells (**Fig. 98**) including TRP channels. Since these functions are also dependent on Ca^{2+} signaling, it is logical to explore the physical expression patterns and functional relevance of TRPV1 and TRPM8 in T cells. TRPV1 has been reported by us and others to be relevant in T cell activation [314, 158]. Although TRPM8 is important for immune response in colitis model [219], the functionality of TRPM8 in regulating T cell activation and differentiation has not been assessed till date. In this study, we demonstrated that both TRPV1 and TRPM8 are endogenously expressed in nearly 100% of primary human and mouse $CD3^+$ T cells as well as in Jurkat cells (human leukemic T cell line). In case of resting T cells, TRPV1 localizes mainly in the intracellular pool, while TRPM8 is primarily present at the membrane. Upon T cell activation, accumulation of TRPV1 and TRPM8 at the surface increases, indicating that both these channels could play important role in T cell effector functions. Interestingly, a quarter of resting T cells is TRPV1 negative (only $TRPM8^+$), but upon activation, most of the T cells express TRPV1. Both TRPV1 and TRPM8 are co-expressed in resting as well as activated T cells and the proportion of $TRPV1^+$ $TRPM8^+$ T cells increases upon activation. This suggests both TRPV1 and TRPM8 likely play important role and possibly synergistically in T cell activation and effector functions. Notably

by a recent report which showed defective phagocytosis in TRPM8-deficient peritoneal macrophages and increased phagocytosis upon TRPM8 activation in wild-type macrophages [219]. This again indicate that both these channels can be modulating cytoskeletal dynamics in macrophages that is necessary to form phagocytic cups necessary for bacterial engulfment. Such regulation of cytoskeletal dynamics has been shown in neuronal cells previously in the context of TRPV1 [206] and in the context of TRPM8 (in this study). However whether this regulatory functions in a similar manner in macrophages remains to be explored in details. This idea is supported by the fact that a related thermosensitive channel TRPV2 has already been shown to induce rapid disassembly and re-polymerization of sub-membranous actin in macrophages upon bacterial encounter, leading to formation of phagocytic cups [220].

Endogenous TRPV1 activity has been shown to be necessary for adhesion of monocytes (THP-1 cells), and for endothelial cells [380]. In contrast, TRPV1 activation by Capsaicin has been shown to down-regulate expression of adhesion molecules ICAM-1 and VCAM-1 on endothelial cells and to reduce LPS-induced monocyte adhesion on endothelial cells [381]. The role of TRPM8 in cell adhesion has not been shown yet. In this study TRPV1 inhibition and TRPM8 inhibition significantly reduced macrophage adhesion indicating that basal level of TRPV1 and TRPM8 activity helps to form focal adhesion points during macrophage adhesion.

TRPV1 activation leads to elongated macrophages, while TRPV1 inhibition leads to more circular and enlarged macrophages. TRPV1 activation also induces higher and inhomogeneous distribution of intracellular Ca^{2+} in the leading edges. On the other hand, TRPM8 activation leads to shrinking of macrophages, resulting in decrease in cell spreading while TRPM8 inhibition leads to more circular and enlarged macrophages. These phenotypic changes indicate that TRPV1 and TRPM8 modulation may alter rate of vesicular fission-fusion at the macrophage membrane and thereby affecting the shape of the macrophages. While LPS-induced elongation was effectively blocked by both TRPV1 activation as well as by inhibition, neither TRPM8 activation nor inhibition had any significant effect. In LPS-treated macrophages, TRPV1 inhibition increases surface area of cells while TRPM8 inhibition drastically reduces the surface area of cells. This observation indicates that TRPV1 as well as TRPM8 regulate cytoskeletal dynamics and membrane dynamics (vesicular fission-fusion) which in turn regulate cell shape and size.

TRPV1 activation as well as TRPM8 activation promote bacterial phagocytosis, while TRPM8 inhibition was able to reduce bacterial phagocytosis. This is supported

cells [367] and astrocytes [375]. In all of these reports, inhibition or deletion of TRPV1 leads to decreased cell migration.

TRPM8 in the context of cell migration has been studied mostly in cancer cells. The fact that TRPM8 expression is highly up-regulated in several cancers like that of the prostate, and gets dramatically reduced in cells undergoing metastasis [182] indicates that TRPM8 has a role in preventing migration of cancer cells. Experimentally it has been shown that TRPM8 activation blocks the migration of prostate cancer cells [369, 376-378]. Interestingly, TRPM8 has been shown to inhibit endothelial cell migration in an ion conductance independent manner [379] by binding to a small GTPase Rap1 and inactivating conformational change of Integrin, required for endothelial cell migration.

In this study, TRPV1 activation is found to decrease the rate of macrophage migration, while TRPV1 inhibition leads to enhanced macrophage migration. However, TRPM8 activation increases macrophage migration while TRPM8 inhibition reduces macrophage migration. The migration data obtained for both TRPV1 and TRPM8 is in sharp contrast with the published literature on cells other than macrophages. This indicates that the effect observed by us is specific only for macrophages. Migration of cells is dependent upon increased rate of microtubule polymerization at the leading edge, and increased depolymerization at the trailing edge. Hence it is likely that TRPV1 and TRPM8 affect macrophage migration by altering rates of cytoskeletal dynamics. In fact, previously it has been shown that TRPV1 activation leads to rapid microtubule disassembly [206]. However, the effect of TRPM8 on microtubule dynamics is not yet known. Further work needs to be done to explore the modulation of Ca^{2+} and cytoskeletal dynamics by TRPV1 and TRPM8 during macrophage migration.

in most of the cellular aspects, these channels show significant differences. The major TRPV1 pool is localized intracellularly in resting and LPS-activated conditions, while the majority of TRPM8 pool is localized at the membrane. This indicates that a small percentage of TRPV1 at the macrophage membrane is sufficient for immunological function in macrophages, while larger population of TRPM8 has to be present at the surface for effective immunological response. The surface accumulation of both TRPV1 and TRPM8 increase upon LPS-mediated macrophage activation which again indicates that surface expression of these two channels is essential for immune response.

3.2.2. TRPV1 and TRPM8 differentially regulate macrophage structure-function

Macrophages need to migrate to the site of infection, injury or inflammation in order to carry out their immunological function. Ca^{2+} regulates downstream signaling events that results in cytoskeletal remodeling, followed by retraction of the trailing edge of migrating cells and expression-localization of adhesion molecules [366]. By influencing intracellular Ca^{2+} levels, TRPV1 activation regulates cytoskeletal rearrangement including disassembly of microtubules and reorganization of F-actin that drive cell migration [198, 367]. TRPV1 activation by Capsaicin increases migration of hepatoblastoma cells, corneal epithelial cells and smooth muscle cells [367-369]. Other members of the TRP family, like the TRPC and TRPM channels, have also been shown to influence cell motility and migration [370-372]. Specifically, TRPV1 and TRPM8 have been shown to regulate migration in different types of cells. Activation of mitochondrial TRPV1 has been shown to increase microglial cell migration [373]. TRPV1 activation also promotes directional cell migration in order to carry out wound closure as reported in case of keratinocytes [374], rat pulmonary arterial smooth muscle

depolymerization which is essential for phagocytic receptor clustering (**Fig. 97**). Besides, TRPV2-deficient macrophages respond inefficient motility in response to chemo-attractants [220]. This indicates that TRPV channels are capable of regulating cytoskeletal re-organization in a similar manner as shown in case of neuronal cells. The TRPV2-deficient mice also have higher pathogen load and higher mortality when challenged with pathogenic bacteria.

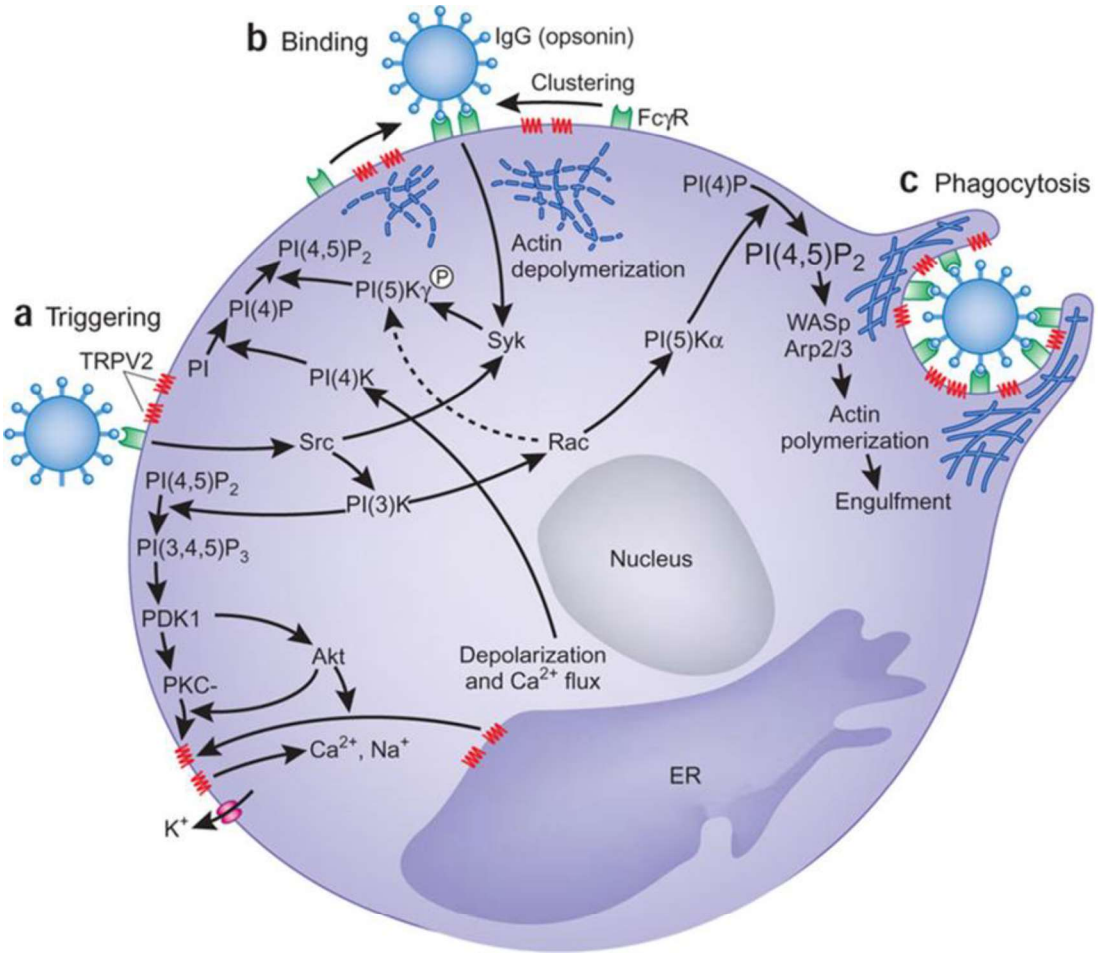

Fig. 97. Role of TRPV2 channels in phagocytosis. Model depicting the interaction between TRPV2 and FcγR receptors during triggering of phagocytosis. TRPV2 activation starts signaling cascade which engages FcγR receptors to attach with foreign particles for their internalization into the macrophage through phagosome formation. Image adapted from [365].

In this study, nearly 100% of the Raw264.7 macrophages and peritoneal macrophages were found to be positive for TRPV1 and TRPM8 expression. However,

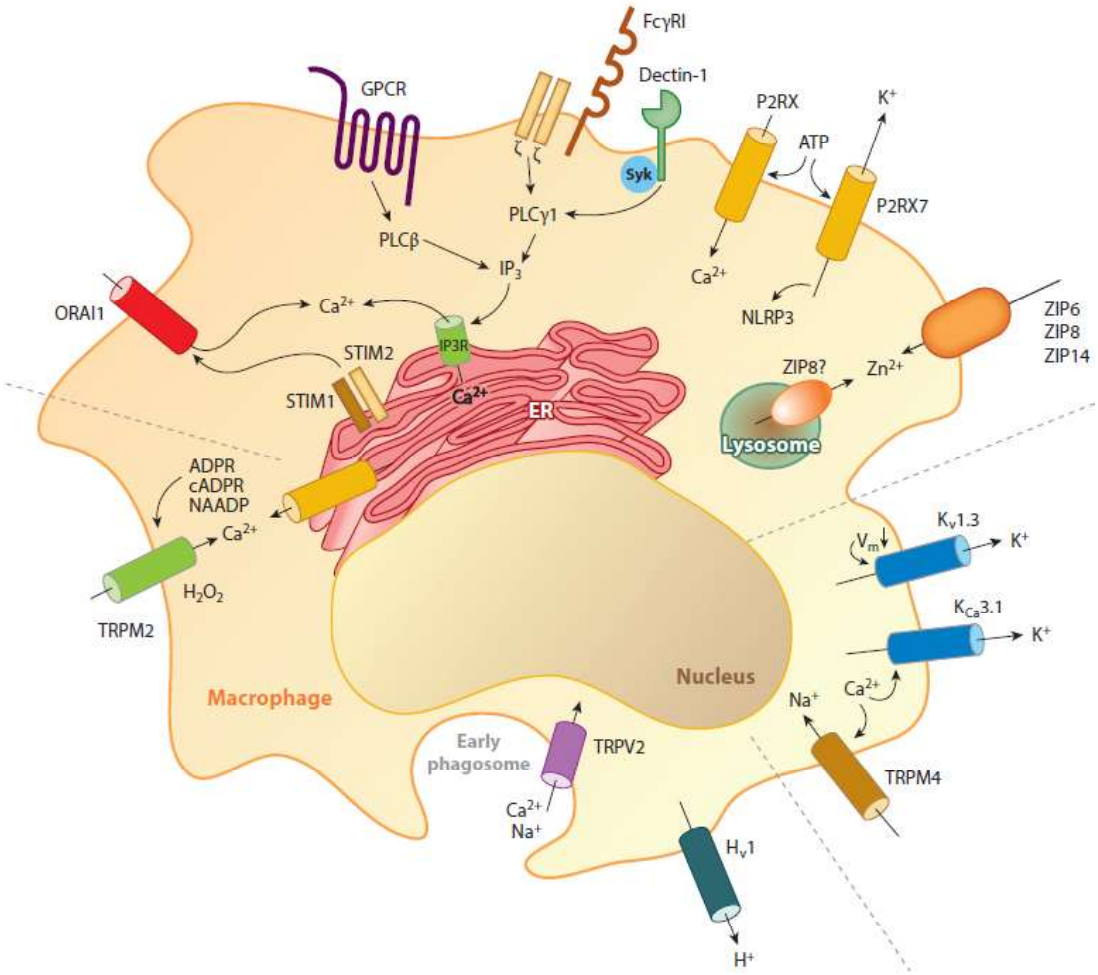

Fig. 96. Ion channels in Macrophages. Macrophages express several ion channels that regulate a wide range of macrophage functions from Ca^{2+} dynamics to phagosome formation. Adapted from [344].

3.2.1. TRPV1 and TRPM8 are endogenously expressed in macrophages

TRPV1 and TRPM8 channels have been relatively under-explored in non-neuronal cells like immune cells. Although few TRP channels, namely, TRPV2, TRPV4, and TRPM8 have been reported to regulate phagocytosis in macrophages [219, 220, 225], the cellular role of TRPV1 and TRPM8 is poorly understood. For example TRPV2 has been shown to be important for particle binding and phagocytosis by macrophages [220]. TRPV2 channels get re-localized to the phagosomes in murine macrophages and depolarize the plasma membrane, resulting in partial actin

3.2 TRPV1 and TRPM8 are endogenously expressed in Macrophages and regulates cellular activities in macrophages

Innate immune responses are largely dependent on phagocytosis, antigen processing and antigen presentation by macrophages and dendritic cells. Proper antigen presentation by these cells to T cells can trigger an effective adaptive immune response which is essential for survival of the host. Several ion channels have been found to be functionally present in macrophages, including TRP channels (**Fig. 96**). These ion channels regulate Ca^{2+}-mediated signaling in macrophages, cytoskeletal remodeling, vesicular trafficking, membrane dynamics and antigen presentation events and are thus important for efficient macrophage functions.

dynamic microtubules (such as tyrosinated tubulin detected by YL1/2 antibody) used in this study. However this possibility needs experimental validation and it also remains to be explored if TRPM8 inhibition also depolymerizes stable microtubules characterized by specific markers (like detyrosinated and acetylated tubulin, γ-tubulin, etc.).

3.1.5. TRPM8 inhibition enhances neuritogenesis

Using F11 cells transfected with TRPV1, previously it was shown that TRPV1 overexpression leads to enhanced filopodia formation and elongation [28, 198]. These reports have established that TRPV1 is important for neurogenesis [364]. Since we have established that unlike TRPV1 which is undetectable endogenously in F11 cells, TRPM8 is abundantly expressed endogenously in F11 cell, allowing us to explore the direct effect of TRPM8 modulation on neuritogenesis without transfecting the cells. In this study we found that TRPM8 activation is unable to enhance the number of cells with neurites, nor is it able to alter the length of primary and secondary neurites. However long term TRPM8 inhibition (18 hours in this study) leads to substantially higher number of cells developing neurites and also the length of primary and secondary neurites increases significantly. Although the mechanism behind this observation is not clear, yet this suggests that inhibition of endogenous activity of TRPM8 reorganizes the cytoskeleton, thus initiating neurites and long term TRPM8 inhibition results in increased rate of vesicular fusion to membrane, contributing additional membrane components for neurite elongation.

transmembrane ion channels, are present on plasma membrane, and upon activation they trigger heavy calcium entry into cells, stimulating the cells to trigger receptor internalization. This acts as one of the mechanisms to desensitize the active ion channels present on the plasma membrane. As a result, the endocytosed vesicles bring in extracellular substance into the cell (in this case transferrin). The time frame of endocytosis indicates that TRPM8 triggered endocytosis is quite fast process. Interestingly, Goswami et al. have reported earlier that TRPV1 acts as a synaptic protein and TRPV1 activation in F11 cells leads to fast exocytosis [194, 196]. All these studies indicate that the heat-sensor TRPV1 and cold-sensor TRPM8 act in opposite manner in terms of vesicular recycling [194, 196].

3.1.4. TRPM8 inhibition destabilizes microtubules

Using F11 cells transfected with TRPV1, previously it has been shown that TRPV1 activation leads to rapid microtubule retraction, collapse of growth cone and neurites [28, 206]. However in this study it was observed that TRPM8 activation neither depolymerize microtubules nor actin filaments. Rather, TRPM8 inhibitor treatment depolymerizes microtubules without affecting filamentous actin. It is well established that Ca^{2+} depolymerizes microtubules *in vitro* as well as *in vivo* [360, 361] via two distinct processes, i.e. by dynamic destabilization and/or by signal cascade-mediated fragmentation of microtubules. TRPV1 activation mediated microtubule disassembly has been attributed to sudden increase in intracellular Ca^{2+}, [28] as well as by signaling cascade [206]. However, a similar logic may not be applicable for TRPM8 as TRPM8 inhibition doesn't increase intracellular calcium levels. It is possible that TRPM8 inhibition triggers and releases microtubule severing enzymes which then depolymerize

temperature sensing. Among all, the CRAC-motifs present in the TM4 - Loop4 - TM5 - Pore loop - TM6 –TRP box region are highly conserved throughout vertebrate evolution and seems to be the major stretch regulated by cholesterol. This analysis also matches well with the reports demonstrating the role of critical regions and different TRPM8 mutants that revel altered behaviors. For example, region consisting amino acid number 40-86 (which covers CRAC-2 and CRAC-like 3) is important for TRPM8 channel localization to plasma membrane and subsequent tetramerization [357]. Similarly, Voets et al. described that specific TRPM8 mutants, namely R842A, H845A, R851Q, R862A, K856A and K856R have defects in voltage-sensing ability and/or temperature sensitivity (threshold for cold activation) [358]. Notably these mutants are positioned at the putative CRAC-29 and CRAC-30 motifs. Similarly, Fujita et al. have demonstrated that three TRPM8 mutants, namely K995Q, R998Q, R1008Q which are located within CRAC-32 have defective PIP_2 binding and thus have altered threshold for cold-induced activation of TRPM8 [359]. These results strongly indicate that some of these CRAC- as well as CRAC-like motifs have functional importance and indicate a possible role of cholesterol in the regulation of TRPM8 function.

3.1.3. TRPM8 undergoes fast recycling in F11 cells and influences endocytosis

When cultured in cholesterol supplemented medium (F12 Ham's containing 10% FBS), the TRPM8 clusters show rapid recycling within and near the cluster. This is indicated by the fact that bleaching an small area (which barely includes 5-10% of the cluster) to the TRPM8 cluster bleaches the entire TRPM8 cluster. Besides TRPM8 activation increases endocytosis of fluorescently labelled transferrin in a dose-dependent manner but doesn't affect exocytosis of transferrin. TRP channels being

association between TRPM8 and lipid raft components is very strong. This data supplements the previous reports that show that TRPM8-lipid raft association to be essential to maintain TRPM8 activation thresholds at lower temperatures and thus have the physiological relevance [347, 348]. So far several reports demonstrate that cholesterol interactions as well as cholesterol-mediated regulation of different TRP channels are of importance (such as members belonging to TRPV and TRPC sub family) [350-354]. The involvement of cholesterol in the regulation of TRPM8 channel properties also fits well with the proposed multi-step model of activation [355, 73]. However, the molecular mechanism by which cholesterol regulates the TRPM8 channel function is not clear. Our analysis suggests a possibility that TRPM8 interacts with one (or more) unit of cholesterol and TRPM8-cholesterol complexes may have different thermosensitivity than cholesterol-free TRPM8. Notably, a similar mechanism of cholesterol-mediated regulation of the pore size has been documented for TRPV1-TRPV4 via Nicotinic Acid which is clinically prescribed for reducing blood cholesterol levels [356].

3.1.2. TRPM8 has several highly conserved cholesterol binding motifs throughout vertebrate evolution

The strong interaction between cholesterol and TRPM8 indicate that there could be some cholesterol binding motifs in TRPM8. It was noted that N-terminus and the transmembrane region and loops in the human TRPM8 sequence have several CRAC or CRAC-like motifs. Besides, most of these regions are highly conserved and some of these regions remain essentially unaltered. This observation strongly suggests that these regions are important for functions such as pore formation, channel gating and

clusters and either remains stationary or exhibits different types of differentiation patterns at the plasma membrane [347]. Interestingly, even in the absence of activation stimuli like cold or menthol, most TRPM8 containing "cellular particles" keep moving all over the plasma membrane and periodically stay in about 1600 nm sized confined microdomains for 2-8 sec [347]. Removal of cholesterol with methyl-beta-cyclodextrin (MβCD) has been shown to stabilize TRPM8 motion in the PM and results in larger TRPM8 current amplitude suggesting that TRPM8 needs membrane cholesterol for proper lateral diffusion [347]. This is in agreement with a previous report that suggests that menthol and cold-mediated responses of TRPM8 are increased upon preventing association between TRPM8 and lipid raft, and that TRPM8 activates at higher temperatures than 25°C when outside lipid rafts [348]. Though most of these reports are based on TRPM8 overexpression based systems, but provide important information regarding the role of TRPM8 association with lipid rafts at molecular level.

Using F11 cells (cloned initially by the fusion of embryonic DRG neurons with mouse neuroblastoma cells, [349] it was observed that TRPM8 is endogenously expressed both at the surface and in intracellular pools (**Fig. 2.1.1.1**). Further it was also noted that endogenous TRPM8 exists as distinct punctate structures in the cell body as well as in the neurites (**Fig. 2.1.1.2**). The verification of endogenous expression of TRPM8 enabled us to probe for its functional role in F11 cells without having to rely on over-expression based systems. However, when TRPM8-GFP was transfected into F11 cells, it was noticed that these TRPM8 clusters are tightly associated with cholesterol and lipid raft structures (detected by Fillipin and Caveolin, Flotillin). The fact that even after cholesterol depletion by Pravastatin or by treatment MβCD, TRPM8 clusters continued to retain cholesterol. Caveolin and Flotillin staining show that the

thermosensitive cells and regulate functions that involve vesicular recycling, cytoskeletal reorganization and Ca^{2+}-homeostasis. This in turn regulates plethora of different cell lineage-specific functions such as neuritogenesis, immune modulation and sperm motility. Interestingly in most cases TRPV1 and TRPM8 have differential or precise opposite roles in regulating all these cellular functions.

Although TRPV1 and TRPM8 have been functionally characterized in terms of pain sensation and thermosensation in DRG neurons, in the DRG neuron derived F11 cell line endogenous levels of TRPV1 could not be detected at mRNA level [345] or at protein level [346]. F11 cells even don't respond to TRPV1 activator Capsaicin [345]. Most of the studies on TRPV1 using F11 cells has been in TRPV1-overexpression based systems. Since this study mostly deals with the endogenous role of TRPV1 and TRPM8 in different cellular systems, it wasn't preferred to do overexpression based studies using TRPV1. Interestingly, I found that TRPM8 is endogenously present in F11 neurons, therefore in this work only TRPM8 has been studied for its role in neuronal system.

3.1 TRPM8 regulates neuronal structure and function by influencing cytoskeletal and vesicular dynamics

3.1.1. TRPM8 is associated with cholesterol-enriched structures in DRG-neuronal cell line F11

Initially TRPM8 was described to be functionally expressed in primary DRG neurons where it acts as a sensor for low temperature and pain [276]. Using TRPM8-EGFP transfected HEK and F11 cell lines, it has been shown that TRPM8-EGFP forms

The four model systems used in this work are highly thermosensitive, yet have their own unique cellular features, environmental conditions and physiological challenges. Therefore the specific functions carried out by these systems have been used as read-out systems in this work. The neuronal cells are adherent cells with chemotactic and theromotactic ability, can extend their neurites to great lengths and nonlinear pathways for their sensory/motor functions. The macrophage cells are highly migratory, and can migrate to sites of infection/inflammation, adhere there, invade tissues and mediate phagocytosis and pathogen clearance. T cells are floating cells and attach only to antigen presenting cells, other T cells and B cells to form immunological synapse and relay information. Sperm cells can swim and sense thermal as well as chemical gradient in the female reproductive tract. Sperm cells also travel great lengths to finally fuse with the oocyte. In addition, vertebrate sperm carry out external or internal fertilization, thereby sperm of each species has its own unique range of requirements and challenges. The expression and functional relevance of TRPV1 and TRPM8 was largely unexplored in each of these four cell types, hence this study was aimed to characterize these aspects through expression analysis and functional analysis specific to these cell types and also relevant functions. This work establishes the involvement of these hot- and cold-sensitive ion channels as key regulators for complex cell signaling events that have immense importance in biological systems.

In this work, relevance of TRPV1 and TRPM8 have also been explored in peripheral neurons, different immune cells like macrophages and T cells and also in mature sperm cells. In all these cellular systems, endogenous expression, localization, molecular organization and diverse cellular functions have been investigated. This work suggests that functional TRPV1 and TRPM8 channels are present in different

All organisms have in-built molecular mechanisms to detect temperature changes in the environment and ability to respond in a manner that facilitates adaptation or avoidance of adverse temperature environment not only at the individual level, but also at the tissue and cellular levels [330, 126]. This thermosensory mechanism is vital for every living organism as both environmental as well as internal body temperatures have profound effect on the physiological and behavioral processes of the organisms [331,332]. In vertebrates, the environmental temperature is sensed by somatosensory neuronal endings present in the skin, which express a group of ion channels that are specialized to detect specific temperatures [333-336].

Transient receptor Transient Receptor Potential (TRP) channels represent a group of non-selective cation channels that can be modulated by several endogenous and exogenous factors [337]. TRPV1 acts as a heat sensitive channel, while TRPM8 acts as a cold sensitive channel, at least in higher order mammals. Both TRPV1 and TRPM8 have been primarily characterized in sensory neurons [10,338,276] and have been under intensive study in pain research [167]. Due to their role in thermosensation, the expression of these two channels were initially thought to be restricted to neuronal systems only. However recently TRPV1 and TRPM8 have also been reported to be present in several non-neuronal cells like keratinocytes [339], muscle cells [17] [340], endothelial cells [341], sperm cells [342, 343], immune cells [344, 231], etc. TRP channels regulate different cellular functions. Since the available information is fragmented and do not clearly describe the endogenous role of these channels, therefore expression profile combined with functional studies using neuronal and non-neuronal systems is very much necessary.

Chapter 3

Discussion

TRPV1 and TRPM8 can be potential drugs for contraception as well as for motility-related infertility cases.

Taken together this study shows that both TRPV1 and TRPM8 are important regulators of not only neuronal cells, but also of non-neuronal cells like macrophages, T cells and sperm. The findings of this study need to be explored in-depth to understand the biochemical machinery at play and the cellular signaling involved in these processes. Careful use of natural or synthetic modulators of TRPV1 or TRPM8 can be helpful in enhancing neuritogenesis in patients with neuronal degeneration. Such understandings can help in activating immune system against harmful pathogens and in treating infertility cases related to abnormal sperm motility.

these channels are essential for T cell effector functions. Inhibition of TRPV1 prevents T cell activation by ConA, which supports our hypothesis that TRPV1 needs to be in the "on-state" during T cell activation process. This was further confirmed by reduced surface expression of T cell activation markers CD25 and CD69, and decreased release of IFNγ by T cells pre-treated with TRPV1 inhibitor, even after incubation with ConA. These results indicate that TRPV1 regulates T cell activation and TRPV1 expression is upregulated in activated T cells, mostly to carry out immunological effector functions by activated T cells.

In this study, TRPV1 and TRPM8 were found to be endogenously expressed in vertebrate sperm (piscean, amphibian, reptilian, avian and mammalian). However their localization pattern differs from species to species indicating that they may play different roles in the sperm of different species. Such differences in localization and expression might be essential as both cell morphometry as well the extracellular environments of sperm cells differ from species to species. The mitochondrial region of sperm from most of the vertebrates have enhanced expression of TRPV1 and TRPM8 indicating their possible role in regulating energy homeostasis of sperm cells, which is vital to enable their motility. Non-motile human sperm have reduced percentage of cells expressing TRPV1 and TRPM8. Even the abundance of these two channels is decreased in non-motile sperm. This indicates that these two channels are essential for sperm motility. This is supported by the fact that in fish sperm and bull sperm, TRPV1 activation leads to enhanced motility, while TRPV1 inhibition leads to decrease in motility. TRPV1 and TRPM8 activation and inhibition regulate sperm motility without affecting capacitation or acrosomal reaction of sperm. This indicates that modulators of

mediated macrophage activation which again indicates that surface expression of these two channels is essential for immune response. TRPM8 inhibition significantly reduces macrophage adhesion indicating that TRPM8 activation helps to form focal adhesion points during macrophage adhesion. TRPV1 activation leads to elongated macrophages, while TRPV1 inhibition leads to more circular and enlarged macrophages. On the other hand, TRPM8 activation leads to shrinking of macrophages, resulting in decrease in cell spreading while TRPM8 inhibition leads to more circular and enlarged macrophages. These phenotypic changes indicate that TRPV1 and TRPM8 modulation can alter rate of vesicular fission-fusion at the macrophage membrane and alter cytoskeletal dynamics thereby affecting the shape of the macrophages.

TRPV1 activation leads to decrease in rates of macrophage migration, while TRPV1 inhibition leads to enhanced macrophage migration. However, TRPM8 activation increases macrophage migration while TRPM8 inhibition reduces macrophage migration. Migration of cells is also dependent upon increased rate of microtubule polymerization at the leading edge, and increased depolymerization at the trailing edge. Hence it is likely that TRPV1 and TRPM8 affect macrophage migration by altering rates of cytoskeletal dynamics. However, the effect of TRPM8 on microtubule dynamics is not yet known. TRPV1 activation promotes bacterial phagocytosis, while TRPM8 inhibition reduces bacterial phagocytosis, which again indicate that both these channels can be modulating cytoskeletal dynamics in macrophages.

In case of resting T cells, TRPV1 is localizes mainly in the intracellular pool, while TRPM8 is primarily present at the membrane. Upon T cell activation, accumulation of TRPV1 and TRPM8 at the cell surface increases, indicating that both

TRPV1 and TRPM8 channels have been reported to regulate sensory functions in primary sensory neurons and most of the research with TRPV1 and TRPM8 channels have been focused on their role as pain-receptors. However, over the last decade there is increasing evidence that TRP channels are also present in several non-neuronal cells. However the exact cellular function and regulation of TRPV1 and TRPM8 channels in these neuronal and non-neuronal cells are still poorly understood.

In this study TRPM8 was found to be endogenously expressed in F11 neurons. TRPM8 activation increases the rate of Transferrin uptake while TRPM8 inhibition results in elongated neurites, thereby suggesting that TRPM8 activation results in rapid rate of endocytosis (vesicle pinching off from membrane) while long term TRPM8 inhibition results in increased rate of vesicular exocytosis (vesicle fusing to membrane and contributing additional membrane fusion for neurite extension) leading to elongation of neurites. TRPM8 was found to co-localize with lipid raft components. This strong association was not disrupted even after depleting cellular cholesterol, indicating that TRPM8 clusters are strongly associated with lipid rafts.

TRPV1 and TRPM8 channels have been relatively under-explored in non-neuronal cells like immune cells. In this study, nearly 100% of the Raw264.7 macrophages and peritoneal macrophages were found to be positive for TRPV1 and TRPM8 expression. However, the major TRPV1 pool was intracellularly localized in resting and LPS-activated conditions, while the majority of TRPM8 was localized at the membrane. This indicates that a small percentage of TRPV1 at the macrophage membrane is sufficient for immunological function/s of macrophage cells, while larger population of TRPM8 has to be present at the surface for effective immunological response/s. The surface accumulation of both TRPV1 and TRPM8 increase upon LPS-

Chapter 4

Conclusions and Future Perspectives

to check the reliability and significance of the data points. P-values <0.05 were considered as statistically significant. Significance codes used: '***' for P-values ranging within 0 to 0.001; '**' for P-values ranging within 0.001 to 0.01; '*' for P-values between 0.01 to 0.05 and 'ns' for P-values above 0.05.

progressivity, average path velocity (VAP) μm/s, straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN= VSL/VCL×100%), straightness (STR =VAP/VCL×100%), amplitude of lateral head displacement (ALH), % Rapid, Medium, Slow and Static sperm.

5.5.10. Motility assay for fish sperm

Freshly collected neat milt from Rohu fishes were collected at CIFA, Bhubaneswar transported to lab within 30–40 minutes of collection. The milt was maintained at 15–20°C during the transportation. Subsequently the milt was stored in 4–8°C freezer for up to 96 hours, during which all the motility experiments were performed. 4 μL water spot was made on microscopic slide (Globe Scientific, 1304) and thereafter a small quantity (0.1–0.2 μl) of Rohu milt was added on the spot. A coverslip (Fisher Scientific) was gently placed on the sample spot immediately and the sperm movement was captured by using Olympus microscope (BX51). The movement of Rohu spermatozoa was recorded at 400-500 frames/minute for 1 minute. The original movies were processed via Movie-maker software. For TRPV1 modulation, specific activator (NADA, Sigma Aldrich) or inhibitors like (5'-IRTX, Sigma Aldrich), Ruthenium Red (Sigma Aldrich) were used at indicated concentrations (diluted in water). For this assay, normal tap water was used as double-distilled water turn out to be lethal for the fish sperm.

5.6. Statistical tests

The data were imported in ''R'' software for statistical analysis or were analysed by Prism software. The Student's T-test or ANOVA test was done for the sets of data

pooled together into a 1.5ml tube and 100µl of semen was added into 3 tubes: (1) without any pharmacological agent, (2) with TRPM8 activator WS12 (10µM), (3) with TRPM8 inhibitor AMTB (10µM). The tubes were incubated for 1 hour in a water bath maintained at 37°C. After incubation for 1 hour, 10 µl of semen was spotted on a glass slide maintained at 37°C, covered with a fresh coverslip and placed under 100X objective of Olympus (BX51) microscope. Motility of bull sperm in each of the tubes was recorded as a movie file of 1 minute each. The movies were compiled using movie maker software.

5.5.9. Evaluation of Bull sperm motility by CASA

Motility analysis of bull sperm cell was performed by Computer Assisted Semen Analysis (CASA) to get quantitative values of multiple motility parameters (Experiments done at FSB Cuttack). For each condition, more than 300 sperms were tracked and analysed with the Hamilton Thorne IVOS computer-assisted semen analysis (CASA) system CASA IVOS 12.3 (Hamilton Thorne Biosciences, Beverly, MA). Cells were adjusted to a concentration of $1x10^6$ cells/ml prior to loading into Leja4 chamber of 20µm depth. Camera was set at 1.77 magnifications. Video-recording of 5–7 phase contrast microscopy fields of sperm samples were made over 1–2 minutes. Temperature was kept constant at 37°C. Recording was done at a frame rate of 60 frame/s (60 Hz) and 30 frames per field. At least 4 recordings per frames were made for each sample at each time point to achieve the statistically significant values. For some experiments, a time kinetics were done by taking readings at 0, 30, 60, 120, 150, and at 180 minutes respectively. For each set, minimum of 300 sperms were taken into account. The following sperm motility parameters were measured: % motility, %

(4µg/ml) or soluble α-CD3 (10µg/ml) for 10 minutes. Fluo4-AM signal was detected using Zeiss LSM780 microscope and with same settings. The images were analyzed by using LSM-software and intensities specific for Ca^{2+}-loaded Fluo-4 are represented in artificial rainbow color with a pseudo scale (Red indicating highest level of Ca^{2+} while blue indicate lowest levels of Ca^{2+}).

5.5.6. ELISA

Culture supernatant from the respective experiments were collected and stored in -20°C and the ELISA was performed for cytokine markers namely IFN-γ, IL-2, IL-4, IL-10 and TNF using BD biosciences Sandwich ELISA kits as per the manufacturer's instructions. The readings were acquired in a microplate reader (BIO-RAD iMARK) at 450nm.

5.5.7. Fluorescence Recovery After Photobleaching (FRAP)

F11 cells were cultured on a glass coverslips and TRPM8-GFP was expressed by transient transfection. Around 36 hours post-transfection, the cells were used for FRAP experiments using 63X oil immersion objective of Zeiss LSM 780 Confocal Microscope. 100% Laser power was used with 50 iterations to bleach the samples. Post-bleaching at least 100 frames were recorded at 0.5 sec time interval.

5.5.8. Video recording of Bull sperm motility

Sperm motility videos were recorded using frozen straws from Jersey bull sperm (commercially sold by the Frozen Semen Bank, Cuttack). After thawing the straws for 2 minutes at 37°C in water bath, the semen from two straws (~200µl per straw) was

antibody (1:500, Invitrogen). For visualization of the cholesterol directly, fixed cells were quenched of residual paraformaldehyde by incubating with 1% Glycine solution for 30 minutes and then stained with Filipin dye (125µg/ml, Sigma–Aldrich) for 1 hour.

5.5.5. Ca^{2+}-imaging

Ca^{2+} imaging of F11 neurons and primary peritoneal macrophages was performed similar to that described for T cells with minor modifications. The culture media of F11 cells and macrophages were replaced with HBSS buffer containing 2mM Ca^{2+} 1 hour prior to live cell imaging, and 30 minutes thereafter, cells were loaded with Ca^{2+}-sensitive dye (Fluo-4 AM, 2 µM for 30 minutes).

The coverslips were then added to the live cell chamber for Ca^{2+} imaging and images were acquired in every 5 sec intervals. The cells were stimulated with specific agonists alone or in combination of agonists and antagonists as described. Fluo-4 AM signal was acquired using a Zeiss microscope (LSM 780) or Olympus fluorescence microscope (IX83) and with the same settings. The images were analyzed using LSM software or Image J and intensity of Fluo-4 signal was quantified and also represented with artificial rainbow color with a pseudo scale (red indicating the highest level of Ca^{2+} and blue indicating the lowest levels of Ca^{2+}).

For Ca^{2+}-imaging of T cells, primary murine splenic T cells in their resting state were loaded with the Ca^{2+}-sensitive dye (Fluo4-AM, 2µM for 30 minutes). The cell suspension was added to the live cell chamber for Ca^{2+}-imaging and images were acquired in every 5 seconds. The cells were stimulated with specific agonists alone or in combination of agonists and antagonists as described. In some cases, the cells were pre-incubated with TRP channel inhibitors for 2 hours and then stimulated with ConA

drying for 24 hours at room temperature, images were acquired using 63X Oil immersion objective of LSM 780 Confocal microscope (Carl Zeiss, Germany). The images were processed using LSM image browser software.

5.5.3. Flow cytometry

For investigating TRPV channels expression, cells were stained with individual TRPV channel-specific antibodies mentioned above and subsequently flow cytometric analysis was performed. For evaluating immune markers expression profiles, mouse T cells were incubated in with anti- CD25 PE, CD69 PE and CD3PE Cy5 mAb dissolved in Assay buffer (1X PBS, 1%BSA and 0.05% Sodium Azide) for 30 minutes on ice and then washed further. Purity of human T cells was also evaluated by anti-human CD3 PE mAb. Stained cells were washed two times with the same assay buffer before line-gated acquisition of around 10000 cells were performed. Stained cells were acquired using FACS Calibur flow cytometer (BD Biosciences). Data was analyzed using Cell Quest Pro software (BD Biosciences). Percentage of cells expressing the markers are presented as Dot Plots while the Mean Fluorescence Intensity (MFI) values are reflected in histograms that indicate expression levels of the markers per cell.

5.5.4. Lipid Raft markers staining

F11 cells were seeded in 24 well plates and TRPM8-GFP was transfected using Lipofectamine 2000 Plus (Invitrogen) as per manufacturer's instructions. 36 hours after transfection, the cells were fixed with 4% PFA and immunostained with mouse monoclonal anti-Caveolin-1 (1:250, Sigma–Aldrich) or anti-Flotilin1 (1:250, Abcam) antibody and subsequently with anti-mouse Alexa-Fluor-594-conjugated secondary

or Olympus Fluorescence Microscope IX83 with 40Xor 60X objective and analyzed with the Zeiss LSM image examiner, Olympus Cell Sense software, Image J software.

5.5.2. Immunohistochemistry of tissues

After surgical removal, the tissues were fixed with 4% Paraformaldehyde for 12-24 hours and then transferred to 25% Sucrose for storage at 4°C till cryo-sectioning. After snap freezing in dry ice, the tissues were mounted on to the object plate holder of cryostat by embedding solution (Leica Biosystems). The object plate holder was attached to the object head maintained at -19°C, while the chamber was maintained at -20°C. Using CM3050 S cryostat (Leica Biosystems) sections of 25µm thickness were cut. The sections were attached to slides pre-coated with 0.1% poly-L-Lysine (Sigma-Aldrich). The slides were stored in -20°C freezer till processing. For immunohistochemistry (IHC), the slides were washed thrice with PBS at to room temperature (25°C) at 5 min interval each. The sections were permeabilized with 0.5% Titron X 100 (Sigma-Aldrich) for 30 minutes, blocked with 5% BSA in PBS for 45 minutes and then incubated with primary antibodies against TRPV1 or TRPM8 (Alomone Labs) at 1:300 dilution in 2% BSA overnight within a moist chamber at 4°C. The slides were then washed thrice with 0.1% PBS-T (PBS with 0.1% Tween20) for 5 minutes each and then incubated with anti-rabbit secondary antibody labelled with AlexaFluor 488 (Molecular Probes) at 1:1000 dilution in 2% BSA for 2 hours at room temperature. The sections were then washed thrice with 0.1% PBS-T and counterstained with DAPI (5µg/ml, Invitrogen) for 15 minutes at room temperature. After washing thrice with 0.1% PBS-T, the slices were layered with anti-bleaching reagent Fluoromount-G (Southern Biotech) and covered by coverslip (Fisher Scientific). Post-

with 2% PFA containing 0.2% Sodium Azide. The cells were then stained overnight with anti-βIII-Tubulin (1:500, Sigma Aldrich) and then with anti-mouse Alexa Fluor 594 (1:500, Invitrogen) and Phalloidin conjugated to Alexa Fluor 488 (1:500, Invitrogen), counter stained with DAPI. Images were acquired using Olympus Fluorescence Microscope (IX83) using 40X or 60X objectives and analysed via Olympus Cell Sense Software.

5.5. Method related Immunocytochemistry and microscopy

5.5.1. Immunofluorescence analysis and microscopy

For immuno-fluorescence analysis of Neuronal or Immune cells, these adherent cells were grown on glass coverslips and fixed with paraformaldehyde (PFA) (final concentration 2%) before subjecting to immunostaining. For immunofluorescence analysis of non-adherent cells like T cells and Sperm, immediately after harvesting, these cells were diluted in PBS and fixed with 2% PFA. The cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and then blocked with 5% BSA for 1 hour. The primary antibodies against TRP channels were used at 1: 200 dilution, and those against cellular markers were used at 1:500 dilution. In some experiments, blocking peptides were used to confirm the specificity of the immuno-reactivity. The ratio (w/w) of blocking peptides with specific antibody was 1:1. All primary antibodies were incubated for overnight at 4°C in PBS-T buffer (PBS buffer supplemented with 0.1% Tween-20) containing 2.5% BSA. AlexaFluor-488 tagged anti-rabbit antibody (Molecular probes) at 1:1000 dilution was used as secondary antibody. All images were taken on a confocal laser-scanning microscope (LSM-780, Zeiss) with a 63X-objective

experiments, cholesterol chelator βMCD (5mM, Sigma–Aldrich) was added to the medium to reduce membrane cholesterol.

5.4.12. Transferrin Uptake assay of Neurons

Around 10^5 F11 cells were seeded in 6 well plate (Corning). After 12 hours, the FBS containing media was replaced with media without FBS. The cells were serum starved for 1 hour. Thereafter, human Transferrin conjugated with Alexa Fluor 594 (1µg/ml, Invitrogen) was added along with different drugs (WS12 10µM, AMTB 10µM, AMTB + WS12 each at 10µM) or Ionomycin 1 µM) was added to the cells for 2, 5, 15 and 30 minutes respectively. Immediately after completion of incubation period, the cells were metabolically fixed by adding Sodium Azide (15mg/ml, Sigma Aldrich) and incubated for 5 minutes. Thereafter, cells were harvested by Trypsinization, neutralized with FBS and fixed with 2% PFA. After washing twice with PBS, cells were resuspended in FACS Buffer and 10,000 cells were acquired by FACS Calibur Flow Cytometer. Data was analyzed by Cell Quest Pro software and Mean Fluorescence Intensity values were plotted to show the extent of transferrin uptake into F11 cells which is indicative of rate of endocytosis.

5.4.13. Neuritogenesis Assay

10,000 F11 cells were seeded onto 18mm coverslips (Fisher Scientific) in 12 well plates (Corning) and allowed to grow for 12 hours. TRPM8 channel specific drugs (WS12 at5µM, 10µM; AMTB at 5µM, 10µM, AMTB + WS12 5µM, 10µM each) and Ionomycin (1 µM) were added to the cells for 3 hours, 6 hours, 18 hours and 24 hours respectively. Immediately after completion of incubation period, the cells were fixed

cells and 10% FBS containing DMEM was replaced with 2% FBS containing DMEM (to stop proliferation of cells) containing TRP channel modulatory drugs. Cells were imaged at 0 hours and at 12 hours (5 images per coverslip with 4X objective) to quantify the area covered by cells upon migration.

5.4.10. Phagocytosis assay of Macrophages

Murine Peritoneal macrophages were grown for 2 days after isolation. Cells were harvested using chilled PBS and 50,000 cells were seeded on 12mm coverslips in 24 well plates. DMEM containing antibiotics was replaced with DMEM without antibiotics. DH5α *E. coli* cells expressing pcJLA-RFP were harvested at mid-log phase and $5X10^5$ bacteria was added to each coverslip (1:10 MOI). Cells were incubated with bacteria for 20 min in CO_2 incubator. Cells were then fixed with 4% PFA containing 0.1% Sodium Azide. Cells were washed thrice with PBS and stained with Phalloidin-AF488 and DAPI. Imaging was done using an Olympus Fluorescence Microscope (IX83) and images were manually quantified to determine the number of phagocytosed bacteria per cell in each of the treatment conditions.

5.4.11. Cholesterol reduction/depletion in Neurons

Neuronal F11 cells were grown in Ham's F12 media supplemented with 10% FBS (HiMedia) prior to cholesterol depletion. For cholesterol reduction/depletion, cells were cultured in serum-free media for 24 hours and cholesterol biosynthesis inhibitor Pravastatin (1µM, Sigma–Aldrich) was added to the medium 12 hours before fixing the cells by 4% PFA. In certain cases 15 minutes before fixing or performing FRAP

experiments with human sperm were done as per the approval from Institutional Human Ethics Committee (NISER/IEC/2015-11 and KHPL-04/2013).

5.4.7. Mitochondrial Potentiality of Bull Sperm using MitoTracker red labelling

Bull sperm were incubated with or without TRP channel modulatory drugs for specified time points. About 10 minutes before fixing the sperm cells 5µM of MitotrackerRed (Invitrogen) was added to each sample. After 10 minutes of incubation with dye, the sperm were fixed with 2% PFA. The fluorescence intensity of MitotrackerRed is dependent on the oxidation of the dye and thus proportional to the mitochondrial membrane potential.

5.4.8. Cell Adhesion assay of Macrophages

Peritoneal macrophages were harvested using chilled PBS post-1-day culture and 5,000 cells in 100µl of DMEM media were aliquoted into tubes. TRP channel modulatory drugs were added to the tubes and incubated for 15 minutes. The 100µl of treated cells were then spotted at the center of glass coverslips and incubated either for 5 minutes or for 15 minutes. 100 µl of 4%PFA was added onto the cell after the incubation period was over. Cells were then stained with DAPI and counted using automated cell analyzer LionHeartFX (BioTek) which scans the entire coverslip and counts the cells based on DAPI signal.

5.4.9. Cell Migration assay of Macrophages

RAW264.7 cells were seeded into 35mm culture dishes ($1x10^6$ cells per dish) so that the dish was 100% confluent in 12 hours. A straight scratch was made using a 200 µl Micropipette Tip. Cells were washed thrice gently with PBS to remove floating

then immediately fixed in 4% PFA. Sperm from another avian species, white pekin Duck (*Anas platyrhynchos*) was collected by cloacal massage by trained professionals (at Central Avian Research Institute, Bhubaneswar). Reptilian sperm was collected from sexually mature male house lizards (*Hemidactylus leschenaultii*) collected from institutional campus (n = 3) and sacrificed by cervical dislocation. Testes was dissected out and immediately fixed in 4% PFA. Amphibian sperm was collected from common toad (*Duttaphrynus melanostictus*). Sexually mature male toads (n = 3) were collected from institutional campus and sacrificed by cervical dislocation. Testes were dissected and fixed immediately in 4% PFA. In case of chicken, lizard and common toad, the testis were smeared and centrifuged at 1000 RPM for 30 seconds. The supernatants containing sperm cells were used for further analysis. Sperm pellet was obtained by centrifugation at 6000 RPM for 5 minutes. Mature sperm from Rohu fish (*Labeo rohita)* were collected by professionals of Central Institute of Freshwater Aquaculture (CIFA, Bhubaneswar) from male broods of Rohu after inducing them with "Ovaprim" (0.2–0.3 ml/kg body weight) during peak breeding season (in the time of early August). In all cases, extreme care was taken to minimize the sufferings and the number of animals used. All experiments were done according to the approval from institutional animal ethics committee of NISER (NISER-IAEC/SBS-AH/07/13/10).

Human spermatozoa were collected from healthy proven fertile donors after 3 days of sexual abstinence (with informed consent). After liquefaction, semen analysis was done to check the sperm parameters and the swim-up (highly motile) and swim-down (nearly immotile) sperm were separated as described before with some minor modifications. Cells of both fractions were either treated with drugs or left untreated as controls and were then either fixed with 4% PFA or made into gel samples. All

5.4.5. Pharmacological modulation of T cells

Jurkat cells, purified human T cells and purified murine T cells were activated using ConA (4µg/ml) for 36 hours. Since most of the primary T cells were found to be activated during 36 hours-48 hours of ConA or anti-CD3/CD28 driven stimulation, all the T cell-based experiments were performed around 36 hours of plating the cells. Primary murine T cells were stimulated with plate-bound α-CD3 (2µg/ml) and soluble α-CD28 (2µg/ml) for 48 hours or with ConA (5µg/ml) for 36 hours before harvesting cultured-media soup or cells. Similarly, in certain experimental conditions, cells were treated with the following TRP channel modulators: RTX (100nM), 5'I-RTX (1µM to 10µM), WS12 (10µM), AMTB (10µM) for 36 hours. After 36 hours of treatment, cell culture media was collected for ELISA and the cells were harvested by centrifugation at 500 RPM for 2 minutes for further experiments. Trypan blue exclusion assay confirmed that >95% cells were alive after incubation with above mentioned concentration of TRP channel modulatory drugs for the duration (36-48 hours) mentioned.

5.4.6. Collection and isolation of sperm cells

Freshly ejaculated sperm from bovine (*Bos indicus*) were collected from healthy bulls after at least 48 hours of sexual abstinence by means of artificial vagina by trained professionals (at the Frozen Semen Bank, Cuttack). For collection of avian sperm, chicken (*Gallus gallus domesticus*) testis were collected (n = 4) from slaughter houses and were bought to the laboratory within 15 minutes. After removing the tunica albuginea (outer covering membrane), the testis was chopped into pieces, smeared and

5.4.3. Isolation of human T cells

Human peripheral blood mononuclear cells (hPBMC) were isolated by using HiSep (HiSep LSM LS001, Himedia) as per the manufacturer's instructions. Dynabeads Flow Comp TM Human CD3 T cell purification kit was used to purify T cells from hPBMC as per the manufacturer's instruction (Invitrogen). In brief, human blood collected from healthy donors in heparinized vials, was diluted with ice-cold PBS in a 15 ml centrifuge tube and overlaid on 2.5ml of HiSep LSM (Himedia). The suspension was centrifuged in a swinging bucket rotor for 30 minutes at 400g. Subsequently, the lymphocyte layer was collected by sterile Pasteur pipette, washed twice with isotonic phosphate buffered saline and cultured on Iscove's Modified Dulbecco's Medium (IMDM, PAN Biotech) supplemented with 10% FBS.

5.4.4. Isolation of murine Peritoneal Macrophages

Murine Peritoneal macrophages were isolated from the peritoneum of 6–8-week-old BALB/c mice. After cervical dislocation, about 8-10ml of chilled PBS containing 1% FBS was introduced to intact peritoneal cavity of mice using 10ml Syringe with 21 gauge size bore. After tapping the peritoneal area to dislodge the macrophages, the peritoneal lavage was collected and cells were pelleted by centrifugation at 1200rpm for 5 minutes. The cells were dissolved in 10% FBS containing DMEM and plated in 100mm cell culture dishes. 12 hours after plating, the non-adherent cells were removed by washing gently with room temperature PBS twice and cultured for 24 hours before harvesting and seeding the macrophages for further experiments.

TBS-T: 20 mM Tris, 150 mM NaCl. 0.1% (w/v) Tween-20.

Stripping buffer: 1% SDS, 20 mM Tris/HCl (pH 6.8), 1% (v/v) β-Mercaptoethanol.

5.4. Method related to cell biology

5.4.1. Cell culture

All the cells were grown in complete media containing 10% FBS, 2mM L-glutamine, 100µg/ml streptomycin, 100U/ml penicillin, 1µg/ml Amphotericin B. Cells were grown in a humidity controlled incubator maintained with 5% CO_2 and at 37°C. F11 cells were cultured in Ham's F12 medium. RAW264.7 cells and murine peritoneal Macrophages were maintained in DMEM media. Jurkat cells, murine T cells and human T cells were grown in IMDM media.

5.4.2. Isolation of murine T cells

Murine splenocytes from 6 - 8 week old male mice (BALB/c) were isolated as per the approval of the Institutional Animal Ethics Committee (IAEC protocol no. NISER/SBS/AH/IAEC-15). Single cell suspension of splenocytes was made by passing the suspended cells through cell strainer (pore size: 70 µm) and were cultured in 24 well polystyrene cell culture plate (3.5×10^6 cells/well) with Iscove's Modified Dulbecco's Medium (IMDM, PAN Biotech) supplemented with 10% FBS (Himedia, India). T cells were purified from the non-adherent splenocyte population by using BD IMag^{TM} Mouse T Lymphocyte Enrichment Set – DM kit as per manufacturer's instructions (Company name). The % purity of the purified T cells was verified by flow cytometry after staining with anti-CD3 antibody. More than 95% purity of $CD3^+$ T cell population was obtained in each case.

5.3.3. Western blot analysis

Western blot was performed by transferring the proteins to PVDF membrane by the semi-dry transfer method. In brief, after separating proteins by SDS-PAGE the gels were incubated briefly in a transfer buffer. Whatman paper and PVDF membranes were also soaked in transfer buffer. The gel was placed on the membrane sandwiched by two layers of Whatman papers. This combination was placed on a semi-dry transfer apparatus ensuring that the gel is connected to cathode while membrane is connected to anode. The apparatus was connected to power supply and electrotransfer was done maintaining a constant current of 17V (for a single gel) for 1 hour. The transfer efficiency of proteins from the gel to membrane was confirmed by staining the membrane with Ponceau Red dye solution. Further, the membrane was blocked with 5% non-fat skimmed milk dissolved in TBS-T buffer. After blocking, the membrane was incubated with primary antibody dissolved in TBS-T buffer for 12 hours, followed by 3 times wash in TBS-T buffer and incubation with secondary antibody dissolved in TBS-T buffer for 1 hour. Finally, the membrane was again washed thrice with TBS-T buffer. Finally, the membrane was developed through chemiluminescence method (Super Signal™ West Femto Maximum Sensitivity Substrate, Thermo Scientific) and detected by Chemidoc XRS apparatus (BioRad). In some experiments blots were stripped-off by incubating the blots in stripping buffer at 50°C for 30 minutes and re-probed again with a different primary antibody.

<u>Buffers and solutions used:</u>

Transfer buffer: 0.1% SDS, 20% (v/v) MeOH, 48 mM Tris/HCl, 39 mM Glycine.

Ponceau Red solution: 5% (w/v) Ponceau S dye, 5% (v/v) Acetic acid.

Buffers used:

Laemmli protein loading buffer (5X): 62.5 mM Tris HCl (pH 6.8), 5% β-mercaptoethanol (v/v), 50% Glycerol (v/v), 2% SDS (w/v), 0.1% (w/v) Bromo Phenol Blue. Volume was adjusted by adding water.

Resolving gel mixture: 10% Bis-Acrylamide (v/v), 375 mM Tris HCl (pH 8.8), 0.1% SDS (w/v), 0.1% Ammonium Persulfate, 0.005% TEMED in water.

Stacking gel mixture: 4% Bis-Acrylamide (v/v), 125 mM Tris HCl (pH 6.8), 0.1% SDS (w/v), 0.1% Ammonium Persulfate, 0.005% TEMED in water.

SDS-PAGE running buffer (1X): 196 mM Glycine, 0.1% SDS, 50 mM Tris-HCl (pH 8.3).

10% APS stock solution, 30% Bis-Acrylamide stock solution, 10% SDS solution, 100% TEMED solution and 1.5 M Tris-HCl (pH 8.8) solution (for separating gel)/or 0.5M M Tris-HCl (pH 6.8, for stacking gel) were used to prepare separating and stacking solutions. APS and TEMED were added just prior to layering the cassettes with stacking/resolving gel mixtures.

5.3.2. Coomassie staining of the protein bands in gel

For Coomassie staining, 0.1% Coomassie Blue dye dissolved in 50% methanol, 10% glacial acetic acid was used to stain the proteins present in the SDS-PAGE gel. Staining was usually performed overnight with agitation in staining solution. After staining, the gel was transferred to destaining solution I which contained 50% methanol + 10% acetic acid and incubated for 1-2 hours, then the gel was transferred to destaining solution I which contained 7% methanol + 10% acetic acid acetic. After destaining, blue protein bands were visible against a clear background. The gels were scanned with a scanner attached to a computer.

LB and plated on the LB-Agar plates containing the desired antibiotic. After 14 hours incubation at 37°C, single colonies were obtained and used for further experimental procedures.

5.3. Methods related to protein and Biochemistry

5.3.1. Separation of denatured proteins by SDS-PAGE

Protein samples were prepared for SDS-PAGE separation by completely denaturing the cell extracts by adding Laemmli protein loading buffer in 1:4 v/v (from a 5x stock) and heating the mixture at 95°C for 5 minutes. SDS-PAGE cassettes were assembled using a pair of clean glass plates (7 cm high and 10 cm wide) separated by a spacer (0.75 mm thickness). The cassettes were filled up to approximately 5 cm height with separating gel mixture and allowed to polymerize. A thin layer of water was slowly layered to the top of separating gel layer to make the top layer of separating gel smooth. After polymerization of the separating gel, the water was slowly removed and stacking gel mixture was layered on the top of the separating gel and thereafter a 15-well comb was inserted within. Once the stacking gel got polymerized, comb was removed slowly without disturbing the wells. The cassettes were inserted into the electrophoresis chamber vertically, which was then filled with the electrophoresis running buffer and the denatured protein samples were loaded into the wells using a Hamilton syringe. The apparatus was connected to a constant current source (10mAmp) for electrophoresis. Bromophenol blue, the tracking dye present in the Laemmli buffer, enables visualization of migrated proteins within the gel. When the dye front came close to the bottom end of the gel, electrophoresis was stopped. The separated proteins in the gel were visualized by Coomassie Blue staining. For Western Blot analysis, the gel was used to transfer the proteins to a PVDF membrane.

5.2. Methods related to molecular biology

5.2.1. Competent *E. coli* cell preparation

A single colony of *E. coli* was inoculated in 3ml of Luria-Bertani (LB) broth and grown overnight at 37ºC, 220rpm shaking. From the starter culture, 1ml of culture was added to 100 ml of LB medium and incubated at 37°C, 220rpm till OD_{600} 0.4-0.5 was reached. Culture was incubated for 10min on ice and centrifuged at 3000rpm for 5 minutes at 4°C. The bacterial pellet was re-suspended in 30 ml of ice-cold 100mM $CaCl_2$ solution and incubated for 30 minutes on ice. The suspension was again centrifuged for 5 minutes at 4°C. Finally, the pellet was re-suspended in ice-cold 100mM $CaCl_2$ solution supplemented with 10% glycerol, and then distributed into tubes, stored at -80°C till use.

LB media composition: 10g Tryptone, 5g Yeast extract, 10g NaCl dissolved in 1 liter of double distilled water and autoclaved at 121°C at 15 lbs for 20 minutes.

5.2.2. Transformation of *E. coli*

For transformation *E. coli* competent cells (DH5α strain) were taken out from -80°C freezer and thawed on ice for 10 minutes. Subsequently 100µL cells were taken in a 1.5mL microfuge tube and approximately 100ng of plasmid DNA was added to the competent cells. This mixture was then incubated for 10 minutes on ice, after which it was given a heat shock at 42ºC for 45 seconds in a water bath. Immediately after heat shock, the tube was placed on ice for 2 minutes. Subsequently, 800µL LB media was added to the mixture and the cells were allowed to grow in incubator-shaker for 1 hour at 37°C, with constant shaking at 220rpm. After 1 hour the transformed cells were centrifuged at 13,000 RPM for 30 seconds, resulting pellet was re-suspended in 100 µL

Amphibian sperm (*Duttaphrynus melanostictus*) [3]	NISER-Bhubaneswar, India
Duck sperm (*Anas platrhyncos*) [3]	CARI, Bhubaneswar, India
Chicken Sperm (*Galus galus domesticus*) [3]	NISER-Bhubaneswar, India
Mouse Sperm (*Mus musculus*)[4]	NISER-Bhubaneswar, India
Bovine sperm (*Bos gaourus*) [3]	Frozen Semen Bank, Cuttack, India
Human sperm (*Homo sapiens*)[5]	Kar clinic Pvt Ltd, Bhubaneswar, India

[1]IAEC number: NISER/SBS/AH/IAEC-25

[2]IAEC number: NISER/SBS/AH/IAEC-15

[3]IAEC number: NISER-IAEC/SBS-AH/07/13/10

[4]IAEC number: NISER/SBS/AH/IAEC-17

[5]IEC numbers: KHPL-04/2013, NISER/IEC/2015-11

5.1.7. Bacterial cell lines

Bacterial cell lines	Source
E coli DH5α strain	Dr. Luna Goswami, KIIT School of Biotechnology, Bhubaneswar, India

5.1.8. Software

Purpose	Software	Source
Image Analysis	Fiji (Image J)	NIH, USA
Image Analysis	Zen, LSM	Zeiss, Germany
Image Analysis	Cell Sense Software	Olympus, Japan
Flow Cytometry	Cell Quest Pro	BD, USA
Statistical Analysis	GraphPad Prism	GraphPad Software, Inc., USA
Graph Plotting	Excel	Microsoft Corp., USA
Western Blotting	Quantity One	BioRad, USA

anti-Rabbit Alexa Fluor 647	Chicken	Molecular Probes	1:500

Secondary antibodies used in western blotting

Description	Host	Source	Dilution
HRP labeled anti-mouse	Donkey	GE Healthcare	1:10,000
HRP labeled anti-rabbit	Donkey	GE Healthcare	1:10,000

Blocking peptides

Peptide Sequence	Blocking activity against	Source
C-terminus (824-838aa) of rat TRPV1 (EDAEVFKDSMVPGEK)	TRPV1	Alomone
3rd extracellular loop (605-619aa) of rat TRPV1 (NSLPMESTPHKSRGS)	TRPV1	Alomone
3rd extracellular loop "PORE" (917-929aa) of human TRPM8 (SDVDGTTYDFAHC)	TRPM8	Alomone

5.1.5. Constructs used

Vectors	Source
TRPM8-GFP	Dr. Sebastian Brauchi, Universidad Austral de Chile, Valdivia, Chile.
pcJLA-RFP	Dr. Mrutyunjay Suar, KIIT School of Biotechnology, Bhubaneswar, India

5.1.6. Cell lines and Primary cells

Cell lines and Primary cells	Source
F11 (neuronal cell line)	Prof. Ferdinand Hucho (Freie Universität, Berlin, Germany)
RAW264.7 murine macrophages	NCCS, Pune, India
Murine Peritoneal Macrophages[1] (from Balb/c mice)	NISER-Bhubaneswar, India
Jurkat T cells	NCCS, Pune, India
Murine splenic T cells[2] (from Balb/c mice)	NISER-Bhubaneswar, India
Fish sperm (*Labeo rohita*)[3]	CIFA, Bhubaneswar, India
House Lizard sperm (*Hemidactylus flaviviridus*) [3]	NISER-Bhubaneswar, India

5.1.3. Primary antibodies used

Antibodies	Host	Source	Application/s	Dilution
α-Tubulin	Mo	Invitrogen	WB, IF	1:500
β-III tubulin	Mo	Sigma Aldrich	WB, IF	1:500
Actin	Mo	Abcam	WB, IF	1:500
Caveolin1	Mo	Sigma Aldrich	IF	1:500
CD11b	Mo	DSHB	IF	1:500
Focal Adhesion Kinase	Mo	Abcam	IF	1:500
Flotillin1	Rb	Sigma Aldrich	IF	1:500
GAPDH	Rb	Sigma Aldrich	WB	1:30,000
Phospho Tyrosine	Rb	Sigma Aldrich	WB, IF	1:500
Myosin IIB	Mo	DSHB	IF	1:500
Vinculin	Mo	Sigma Aldrich	IF	1:500
TRPV1-Ct	Rb	Alomone	WB, IF	1:200
TRPV1-ext	Rb	Alomone	WB, IF	1:200
TRPV1-Ct	Rb	Sigma Aldrich	WB, IF	1:200
TRPM8-ext	Rb	Alomone	WB, IF	1:200
CD25PE,CD69PE, CD3PE-Cy5	Mo	BD Biosciences	Flow Cytometry	1:750
CD3-PE	Hu	BD Biosciences	Flow Cytometry	1:750
CD3 and CD28 (azide free)	Mo	BD Biosciences	In-vitro Stimulation	1:750
CD90.2	Mo	Tonbo Biosciences	Flow Cytometry	1:750

Mo: mouse monoclonal; **Rb:** rabbit polyclonal; **Hu:** Human, **WB:** Western Blot; Dilution with respect to Western Blot analysis.

5.1.4. Secondary antibodies and related reagents

Description	Host	Source	Dilution
anti-Mouse Alexa Fluor 488	Chicken	Molecular Probes	1:1000
anti-Rabbit Alexa Fluor 488	Chicken	Molecular Probes	1:1000
anti-Mouse Alexa Fluor 594	Chicken	Molecular Probes	1:500
anti-Rabbit Alexa Fluor 594	Chicken	Molecular Probes	1:500
anti-Mouse Alexa Fluor 647	Chicken	Molecular Probes	1:500

Whatman paper	Whatman
Yeast extract	Himedia
TRP channel Drugs:	
Resiniferatoxin (RTX)	Sigma Aldrich
5'-Iodo Resiniferatoxin (5'-IRTX)	Sigma Aldrich
WS12	Sigma Aldrich
AMTB	Sigma Aldrich
4α-Phorbol Didecanoate (4αPDD)	Sigma Aldrich
RN1734	Tocris
Ruthenium Red	Sigma Aldrich
NADA	Sigma Aldrich
Media and Serum:	
DMEM	PAN-Biotech
F12 Ham's media	HiMedia
IMDM	PAN-Biotech
Fetal Bovine Serum (FBS) -Australian Origin	HiMedia, PAN-Biotech

5.1.2. Kits and Markers

Plasmid DNA isolation (midi prep) kit	Qiagen
Lipofectamine 2000 cell transfection kit	Invitrogen
West Femto Maximum Sensitivity Chemiluminescence Kit	Thermo Scientific
Bradford protein estimation kit	Sigma Aldrich
Pre-stained protein marker	Fermentas
DB IMag™ Mouse T Lymphocyte Enrichment Set - DM	Invitrogen
HiSep LSM LS001	Himedia
Dynabeads Flow Comp TM Human CD3 T cell purification kit	Invitrogen
Mouse IL-10 OptEIA ELISA Set, 555252	BD Biosciences
Mouse TNF OptEIA ELISA Set II, 558534	BD Biosciences
Mouse IL-4 OptEIA ELISA Set, 555232	BD Biosciences
Mouse IFN-Gamma OptEIA ELISA Set, 555138	BD Biosciences
Mouse IL-2 OptEIA ELISA Set, 555148	BD Biosciences

JC-1	Invitrogen
Kanamycin	Sigma Aldrich
LB powder	HiMedia
LysoTracker Red AM	Invitrogen
Methanol	MP Biomedicals
MitoTracker Red AM	Invitrogen
$MgCl_2$	Sigma Aldrich
$NaBH_4$	MP biomedical
Paraformaldehyde	Sigma Aldrich
Peanut Agglutinin (PNA) AF488	Invitrogen
Peanut Agglutinin (PNA) AF647	Invitrogen
Penicillin-Streptomycin Solution	HiMedia
Phalloidin-AF488	Invitrogen
PMSF	Sigma Aldrich
PIPES	Sigma Aldrich
Probenecid	Invitrogen
Propidium Iodide	Sigma Aldrich
PVDF membrane	Millipore
Poly L-Lysine Solution 1%	Sigma Aldrich
Ponceau S	Sigma Aldrich
Potassium Hydroxide	Sigma Aldrich
Skimmed Milk powder	HiMedia
Sodium Azide	Sigma Aldrich
Sodium Chloride	Sigma Aldrich
Sodium Dodecyl Sulphate (SDS)	Sigma Aldrich
Sodium Hydroxide	Sigma Aldrich
Sucrose	Sigma Aldrich
TEMED	Sigma Aldrich
Transferrin-AF488 (human)	Invitrogen
Transferrin-AF594 (human)	Invitrogen
Tris base	Sigma Aldrich
Triton X100	Sigma Aldrich
Trypan Blue	HiMedia
Trypsin	HiMedia
Tryptone	HiMedia
Tween 20	Sigma Aldrich

5.1. Materials used

5.1.1. Chemicals

Chemical used	Source
Acetic Acid (Glacial)	Amresco
Acrylamide	Sigma Aldrich
Agar	Himedia
Ampicillin Sodium Salt	Sigma Aldrich
Ammonium per sulphate (APS)	Sigma Aldrich
Amphotericin B	MP Biomedicals
β-Mercaptoethanol	Sigma Aldrich
Bis-Acrylamide	Sigma Aldrich
Bromophenol Blue	Sigma Aldrich
Bovine Serum Albumin (BSA)	Sigma Aldrich
6-CFDA	Sigma Aldrich
Cover Slips	Fisher Scientific
Complete Protease Inhibitor Cocktail	Sigma Aldrich
Concanavalin-A (ConA)	HiMedia
Coomassie Brilliant Blue G250	MP Biomedicals
DAPI	Invitrogen
DMSO	Sigma Aldrich
DMSO –Anhydrous	Invitrogen
EDTA	Sigma Aldrich
EGTA	Sigma Aldrich
Ethanol	Merck
Filipin III	Sigma Aldrich
Fluo4-AM	Invitrogen
Fluoromount G	Southern Biotech
Gentamycin	HiMedia
Glycerol	Sigma Aldrich
Glycine	Sigma Aldrich
Hydrogen Chloride	Sigma Aldrich
HEPES	Sigma Aldrich
Ionomycin	Sigma Aldrich
IPTG	MP biomedical

Chapter 5

Materials and Methods

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Chapter 6

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