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Klinik für Augenheilkunde
Direktorin: Prof. Dr. Antonia Joussen

Habilitationsschrift

Functional expression of temperature-sensitive transient receptor potential channels (TRPs) in cultured human corneal and conjunctival cells: Relevance in the pathophysiology of ocular surface diseases

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Dr. phil. nat. Stefan Mergler

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Dekan: **Professor Dr. med. Axel Radlach Pries**

1. Gutachter/in: **Professor Dr. Juana Gallar Martinez (Alicante)**

2. Gutachter/in: **Professor Dr. Dr. Solon Thanos (Münster)**

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1. Introduction

1.1 Human cornea

The cornea can be described as a transparent window of the front part of the eye. The horizontal diameter of an adult cornea is 11.5 – 12 mm, whereas the vertical diameter is about 1 mm smaller (1). Unlike the human conjunctiva, the human cornea does not contain blood vessels but consists of 3 cellular layers (epithelium, stroma, endothelium) and 2 interfaces (Bowman's layer and Descemet's membrane) (2) (Fig. 1.1). The most significant property of the cornea with the anterior chamber and lens is its transparency. Thereby, each cell layer of the cornea plays a specific role as described in the following sections.

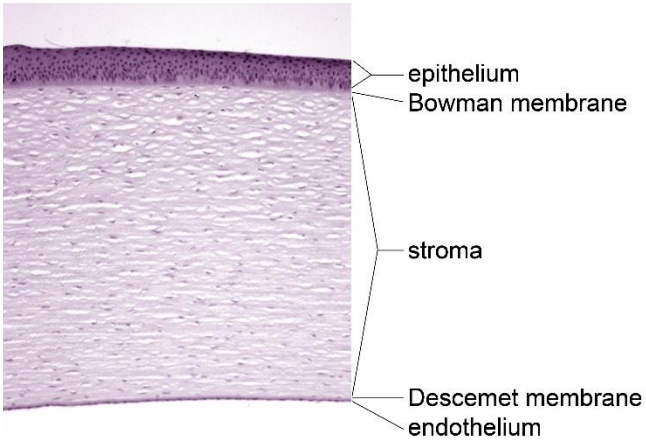

Fig. 1.1: Histological structure of the human cornea. Vertical section of human cornea. HE staining, magnification 100x. Photo kindly provided by M. Valtink, TU Dresden (3).

1.1.1 Corneal epithelium

The human corneal epithelium (HCE) is about 50 µm thick and is located at the outside of the cornea, facing the air. It consists of several cell layers such as the basal-, wing- and superficial cell layer. The latter one is anatomically and physiologically connected with the overlying tear film. The HCE is kept moist with tears and is continuous with the human conjunctival epithelium (section 1.2). Human corneal epithelial cells (HCEC) in the upper layers undergo continuous terminal differentiation and are shed into the tears. Notably, they contribute to the mucus component of the tear film by producing membrane bound specific mucins that can be shed into the aqueous component of the tear film. The replacement of HCEC in the upper layers is dependent on maintenance of the proliferating basal layer. This renewal process is dependent on the presence of a host of cytokines or growth factors that stimulate this process

through interactions with their cognate receptors (4). Another relevant characteristic of the epithelium is its barrier function. In general, this barrier protects the cornea and intraocular structures from infection by pathogens. The HCE is a stratified squamous epithelium from which the terminally differentiated, superficial cells are continuously shed. As cells are shed, the epithelium is maintained by mitosis, which occurs in the basal cell layer. The function of the HCE is maintained by migration of new basal cells into the cornea from the intervening transition area (limbus). The cells migrate centripetally and originate from committed stem cells in the limbal epithelium (5). Overall, the HCE is maintained by a balance among the processes of cell migration, mitosis, and shedding of superficial cells (4). From the electrophysiological side, it has a relatively high transepithelial potential which is consistent with a low ionic conductance of the apical epithelial cell membranes and a high resistance of the tight junctions of the paracellular pathway (6). Interestingly, synthetic peptides can modulate the corneal epithelial resistance to increase the permeation of therapeutic agents across this barrier (7). Regarding metabolic pathways, the corneal epithelium primarily uses glucose and glycogen for energy production which can be influenced by ultraviolet radiation (8). Glucose reaches the cells by diffusion from the aqueous humor and the corneal epithelial cells store high levels of glycogen. Concerning oxygen supply, the corneal epithelium receives its oxygen directly from the atmosphere under open-eye conditions (9).

1.1.2 Corneal stroma and nerve fibers

The human corneal stroma (substantia propria) is the middle connective tissue layer (Fig. 1.1) that is approximately 500 µm thick and constitutes more than 90% of the total corneal thickness (2). Anatomically, Bowman's layer (8 – 14 µm) is located between the corneal epithelium and the corneal stroma (Fig. 1.1.2) (2). It serves as a basement membrane of the HCE and is composed of collagen and laminin. Additionally, it protects the corneal stroma. The corneal stroma is fibrous, tough, unyielding, and correspondingly transparent in connection with its matrix of collagen fibrils. Biochemically, there is a difference between the anterior and the posterior corneal stroma regarding water, glucose and sulfate content.

Anatomically, corneal nerve fibers are embedded in the corneal stroma (stromal nerve bundles) (Fig. 1.1.2) (10). They are directly associated with the high sensitivity of the whole cornea. There is a high density of sensory nerve endings, which are unmyelinated and sensitive to touch, temperature and chemicals.

Fig. 1.1.2: Simplified figure of the corneal stroma containing nerve fibers. The corneoscleral limbus approximately begins from the indicated dashed line (10).

The electrophysiological properties of primary sensory neurons innervating the cornea are attributable to the functional characteristics of their peripheral nerve terminals (11). Finally, the Descemet's membrane (5 – 20 µm) is located between corneal stroma and corneal endothelium (Fig. 1.1.2) (2). This thin layer does not contain cells and is directly connected with the corneal endothelium as a basement membrane.

1.1.3 Corneal endothelium

The human corneal endothelium (HCE_n) is a monolayer of cells that forms a leaky barrier at the posterior cornea and faces the anterior chamber of the eye (Fig. 1.1). Human corneal endothelial cells (HCE_nC) are structured like a hexagonal mosaic on Descemet's membrane similar as in HCE_nC of pigs (Fig. 1.1.3).

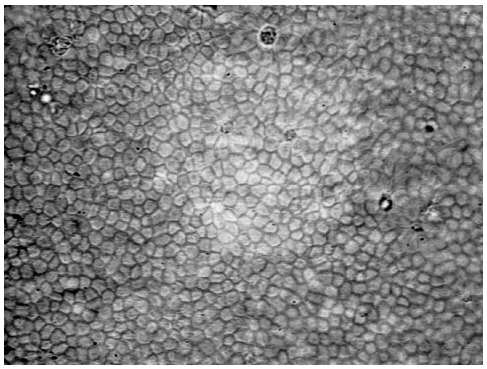

Fig. 1.1.3: Light micrograph of the corneal endothelium of a pig (S. Mergler) (12).

Unlike the corneal endothelium of other vertebrates, the HCE_n does not contain mitotically active cells (13). Functionally, the corneal endothelium plays an essential role to maintain the transparency of the cornea due to its two basic functions. Firstly, it forms a barrier, eliciting

net fluid transport from the stroma, whose physico-chemical properties sustain fluid imbibition into the anterior chamber. The corneal endothelium has a low electrical resistance (14) due to the relatively high permeability of intercellular junctions at the cells' apicolateral membranes. The low resistance endothelial barrier function is dependent on the presence of calcium in the extracellular milieu with junctional disruption occurring in a calcium-free solution or with calcium channel antagonists. Accordingly, this results in individualization and rounding of cells and rapid corneal swelling (15). Therefore, corneal endothelial function and integrity are dependent on mechanisms that regulate intracellular calcium levels as well as external calcium for appropriate cell-to-cell apposition. Secondly, the endothelium mediates fluid egress to offset the fluid being imbibed by the stroma. These offsetting functions of the HCE_n maintain corneal thinness and deturgescence as prerequisites for corneal transparency. This is described by the pump-leak hypothesis, which states that either osmotically or electro-osmotically coupled fluid flow out of the stroma into the anterior chamber is equal and opposite to fluid being imbibed into the stroma through paracellular pathways between neighboring endothelial cells (16). The osmotic gradient is needed to elicit net fluid efflux from the stroma across the endothelial layer into the anterior chamber. This is realized by ion transporter ATPases, co-transporters and ion channels in the endothelial layer with the active transport mechanism being temperature dependent. Temperature reversal studies showed that the cornea swells after cooling, and that corneal swelling is reversed upon rewarming to a physiologic temperature. Therefore, membrane barriers and pump functions must be impaired at lower temperatures (17). The Na^+-K^+-ATPase is an essential component of endothelial pump function and is localized at the lateral cell membranes of corneal endothelial cells. The importance of Na^+-K^+-ATPase activity for corneal transparency is indicated by the finding that exposure to ouabain, a specific inhibitor of this enzyme, caused corneal swelling. Additionally, it prevented reversion of corneal swelling when the endothelium was rewarmed after cooling. Specifically, ouabain inhibited endothelial active sodium flux (18;19). Taken together, the barrier and pump function are crucial for viability of the corneal endothelium and its role in corneal tissue function.

1.2 Human conjunctiva

Anatomically, the conjunctiva is a mucous membrane that covers the inner surface of the upper and lower lids and extends to the limbus on the surface of the globe. The conjunctiva, the limbus and the cornea comprise the tissues at the ocular surface. These three regions have

both structural and functional features in common. They are covered by a stratified, squamous, non-keratinizing epithelium at the surface of the eye. Functionally, all three regions of this human conjunctival epithelium (HC_jE) support the tear film and serve as a barrier to fluid loss and pathogen entrance. Specifically, the conjunctiva helps to lubricate the eye by contributing to the mucous component of the tear film. The HC_jE does not appear to vary dramatically in the three conjunctival regions, but the number of cell layers is varying. Unlike other ocular tissues, the conjunctival epithelium has goblet cells intercalated between the epithelial cells. Neural stimulation of the cornea induces conjunctival goblet cell mucous secretion (20). Functionally, the HC_jE contributes to immune surveillance and helps to prevent the entrance of microbes into the eye similar to the HCE. The *substantia propria* of the conjunctiva consists of highly vascularized loose connective tissue, which is rich in immune cells in comparison to the limbus and the cornea.

1.3 Pathophysiology and pathogenesis of dry eye syndrome

The relevance of the corneal- and conjunctival epithelium as a barrier between the tears and the stroma is demonstrated by the response of the cornea and conjunctiva to an epithelial abrasion. Moreover, removal of the corneal epithelium leads to imbibition of fluid from the tears into the stroma (swelling) and loss of transparency subsequent to increases in tissue thickness that exceed 20%. On the other hand, decreased tear production or increased tear film evaporation can also lead to pathophysiological changes. They may result in the so-called "Dry Eye Syndrome" (DES), which is also known as *keratoconjunctivitis sicca* (KCS). Due to the report of the Dry Eye Workshop (DEWS) it is defined as a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by an increased osmolarity of the tear film and inflammation of the ocular surface (21). The aetiology, pathophysiology and pathogenesis of DES is complex with many interacting factors involved (22;23). Specifically, DES is caused either by insufficient tear production (aqueous tear deficiency = ATD) or excessive tear evaporation (evaporative dry eye = EDE). The reason for the very common EDE has just been further explored in another workshop (Meibomian Gland Disease – MGD Workshop). Due to the definition of MGD, it is a chronic, diffuse abnormality of the meibomian glands, commonly characterized by terminal duct obstruction and/or qualitative/quantitative changes in the glandular secretion. This may result in alteration of the tear film, symptoms of eye irritation, clinically apparent inflammation, and ocular

surface disease (24-27). Although the etiology of MGD may differ from that of ATD (which is due to insufficient lacrimal gland production), the two conditions share many clinical features including symptoms of ocular surface irritation and visual fluctuation, altered tear film stability, and potential ocular surface compromise. When MGD is of sufficient degree, it may give rise to the second major subtype of dry eye syndrome, "evaporative" dry eye. The definition and classification of dry eye syndrome is given in the "Report of the Definition and Classification Subcommittee of the International Dry Eye Workshop" (2007) (21;26;27) (Fig. 1.3). The DES subtypes are not mutually exclusive as previously acknowledged (21). ATD and EDE results in tear *hyperosmolarity* that leads to discomfort and ocular damage (28). Normally, the osmolarity of the tear film is 302 ± 6.3 mOsM (29). In DES, however, the osmolarity increases up to 330 - 340 mOsM (30). Therefore, hyperosmolarity is a precipitating event leading to the pathological changes associated with dry eye. In addition, hyperosmolarity is associated with pro-inflammatory stress to the ocular surface (31). Furthermore, programmed cell death of ocular surface cells can arise in experimentally induced DES (32). Concerning ATD, the hyperosmolarity eliciting ATD is a function of declines in tear flow rate, and increases in tear film evaporative rates. With EDE, the osmolarity inducing this condition is also dependent on declines in tear flow rates (25). Reduction in tear film quality and volume leading to EDE can be a consequence of increased evaporation rate. These responses may be unsuitable due to of hormonal imbalance, altered blink rates, aberrant tear film lipid layer thickness and composition due to meibomian gland dysfunction (25). In a healthy state, the tear film protects corneal surface health and prevents pain by moisturizing and nourishing the corneal surface. However, with insufficient tear production or excessive evaporation, the tear film becomes *hypertonic*, developing a high concentration of salt (hyperosmotic environment), leading to corneal surface desiccation. In this context, regulation of lacrimal gland secretory processes has relevance in DES (33). An unhealthy balance within the tear film leads to the aforementioned symptoms of dry eye. Therefore, therapies targeted at recovering or stabilizing the lipid layer are relevant to treat DES. This can be carried out either as monotherapy or in conjunction with therapies designed to enhance aqueous production (34). In the future, accurate measurement of tear film osmolarity may be an important test in the diagnosis of DES. Recent studies described that single osmolarity measurements are more effective than other diagnosis tests for identifying DES (e.g. lactate determination, Schirmer test, staining of the ocular surface) (35). Accordingly, the aim of DES therapy should be to constitute a normal osmolarity of the tear film.

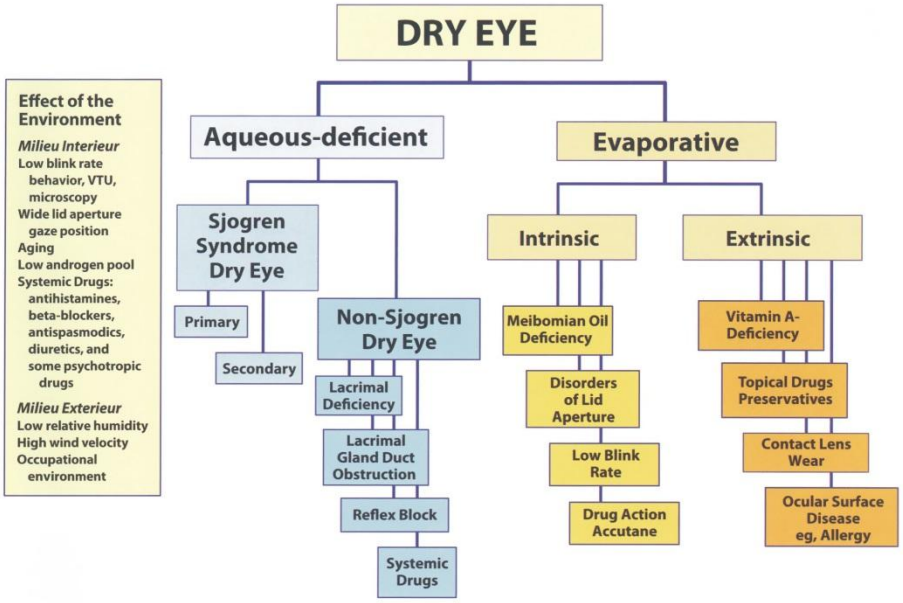

Fig. 1.3: Major etiological causes of dry eye. The left hand box lists environmental parameters which may lead to develop DES. There is an aqueous-deficient dry eye (blue box) as well as evaporative DES (light brown box) (21).

Taken together, one promising approach to treat DES is to develop a hypotonic buffer serving to offset dry eye-induced shrinkage of corneal surface cells. The success of this endeavor depends on gaining a better understanding of the mechanisms controlling ion transport mechanisms. These mechanisms mediate osmolyte fluxes across the anterior ocular surface since exposure to anisoosmolality can trigger mechanisms leading to typical properties of DES.

1.4 Pathophysiology of human corneal endothelium

Human corneal endothelial cells (HCE_nC) are a non-regenerating cells and the number of HCE_nC decreases with age from approx. 3,500 cells/mm² at birth to approx. 2,000 cells/m² at high age (36). As cells decrease in number, neighboring cells enlarge to cover cell-free areas, thereby becoming thinner and attenuated. Despite this constant loss of cells, normal thickness and transparency of the cornea are maintained because corneal decompensation occurs only when endothelial cell density falls below approx. 500 cells/mm² (37). Additional stress imposed on the endothelium markedly augments the normal aging process and may accelerate endothelial cell loss. Factors reducing endothelial cell density or inducing cell damage are inherited or acquired endothelial dystrophies or diseases like glaucoma, surgical procedures such as intraocular lens surgery or penetrating keratoplasty, or drug toxicity. The resulting corneal decompensation is associated with a marked reduction in Na^+-K^+ pump site density,

indicating exhaustion of the pump's physiologic reserve and its insufficiency to offset fluid imbibition by the stroma. As a consequence of endothelial failure, corneal thickness increases due to fluid retention in the stroma. Once corneal thickness has increased by 20%, the cornea becomes opaque. Currently, the only available treatment for corneal decompensation is transplantation of a donor cornea (keratoplasty). More than 100,000 corneal transplantations are performed each year (US and Europe), making it the most frequently performed transplantation in human medicine. The success of this procedure depends on a vital function of the donor corneal endothelium. As a quality criterion, endothelial cell density of the donor cornea is evaluated prior to transplantation and only corneas with a minimum of 2,000 cells/mm² are transplanted. This criterion severely limits the availability of donor corneas that are suitable for transplantation, and the need for donor corneas cannot be covered. Therefore, other procedures inhibiting a decline in endothelial cell density are under investigation such as transplanting corneal endothelial cells, genetic manipulation of corneal endothelial cells, and/or stimulating fluid secretion by the remaining endothelial cells (38-42). All these approaches are entirely at an experimental stage. Another problem that needs attention is to suppress a potential and usually slowly developing endothelial instability and dysfunction after keratoplasty, which results in late postoperative endothelial failure (43).

1.5 Plasma membrane receptors

Ocular cells such as HCE_nC, HCEC and human conjunctival epithelial cells (HC_jEC) express membrane receptors such as insulin-like growth factor-1 receptor (IGF1R), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), basic fibroblast growth factor (FGF-2) and its receptor-1 (flg- type), transforming growth factor beta (TGF-ß1) and vascular endothelial growth factor (VEGF) (44-49). The activation of growth factor receptors by specific ligands arranges basic cellular processes such as cell growth, migration, differentiation, proliferation and survival. Specific expression levels of such growth factor receptors may be a hallmark of the behavior of the aforementioned cell types. Accordingly, appropriate receptor modulators can be used for a modern targeted therapy of DES or improvement of HCE_nC survival. Regarding HCE_nC, the expression of such receptors also impacts on morphometric HCE_nC parameters and HCE_nC density in culture-preserved human corneas (50). Addition of certain growth factors to culture media in order to stimulate specific pathways via their receptors was already shown to stabilize endothelial cell density on cultured donor corneas under serum-free conditions (51). Therefore, it seems considerable

that growth factor receptors expressed in HCE_nC might be used as targets to increase donor cornea longevity during organ cultivation. Regarding HCEC and HC_jEC, current application of growth factors such as epidermal growth factor (EGF) presented clinical improvements on DES by stabilizing the tear film and maintaining the integrity of epithelium. Studies indicated that EGF has potential as a therapeutic agent in clinical treatment of dry eye disease (52;53).

1.6 Ion channels and their role in calcium regulation and apoptosis

Cell biological processes such as cell growth, differentiation, proliferation or secretion are regulated by ion channels. They regulate fluxes of essential signaling ions, thereby regulating cell volume and maintaining cellular membrane potential. In addition, they also play a role in apoptosis in various cell types (54-59) including corneal endothelium (12), since this process is - like almost all cellular processes - regulated by calcium. Changes of intracellular calcium concentration can either lead to an extracellular calcium influx through membrane channels or lead to an intracellular calcium release from stores (e.g. endoplasmic reticulum) by Ca^{2+}-releasing channels (e.g. IP_3, ryanodine). Notably, excessive apoptosis or calcium overload may be an important context in the pathogenesis of corneal dystrophy (60). Regarding keratoplasty, excessive apoptosis also holds responsible for the loss of up to 20% of donor corneas, causing irreversible declines in endothelial cell number, thereby adding to donor cornea shortage (61). In general, the apoptotic processes can be activated by increased intracellular calcium levels as well as by changes in (mostly increased) ion channel activities (12;57;62). It remains to be investigated if this also applies to HCEC and HC_jEC as well as HCE_nC. Gaining a better understanding of the mechanisms, a control of intracellular calcium homeostasis through Ca^{2+} permeable channels in these cells may help to identify trigger mechanisms that lead to cell demise. Regarding clinical aspects of DES, a petrolatum based calcium ointment indicated improved symptoms and ocular surface staining in DES patients. However, some of the effects may be due to lipids in the petrolatum vehicle (63;64).

1.7 Transient receptor potential channels (TRPs)

Various studies showed that dysfunction of voltage dependent ion channels and TRPs are linked with increases in cytosolic Ca^{2+} and apoptosis (65-71). TRPs are ligand-operated, non-selective cation channels that exhibit varying degrees of preferential Ca^{2+} permeability (72). In humans, TRPs play an important role in modulating taste sensation, eliciting responses to painful stimuli, temperature and pheromones. TRPs can be activated by different mechanisms

including physical activators, e.g. change in temperature or mechanical stimuli. In addition, TRPs can be activated via calcium store depletion (store-operated calcium channels; SOCs) and by activated G protein-coupled receptors (GPCRs) or receptor-linked tyrosine kinases (RTKs). TRPs are expressed in numerous tissues under physiological and pathophysiological conditions (73-75). To date, over 30 of these channels have been discovered in humans. Structurally, TRPs consist of 7 putative transmembrane domains with a pore loop between the fifth and sixth segment. Based on sequence and functional similarities, TRP channels are divided into seven main subfamilies: TRPC (canonical/classical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), TRPML (mucolipin) and TRPN (Drosophila NOMP protein) (74;76). TRPs form homo- and heteromeric complexes whose Ca^{2+} selectivity is variable. Except for TRPM4 and TRPM5, all TRPs are permeable to Ca^{2+} (77;78). Notably, several (hereditary) diseases are caused by defects in TRPs (TRP-channelopathies) [reviewed by Nilius et al. (78;79)].

1.7.1 TRPV1

The well-known capsaicin receptor (TRPV1) is a heat-sensitive receptor and belongs to the temperature-sensitive TRPs (thermo-TRPs) (80). TRPV1 is the most investigated TRP channel and belongs to the TRP vanilloid channel subfamily (TRPVs). TRPV1 can be selectively activated by a component in pepper extracts, capsaicin (CAP) and its ultrapotent analogue resiniferatoxin (RTX) as well as by physical heat (> 43 °C) (81;82) (Fig. 1.7.1).

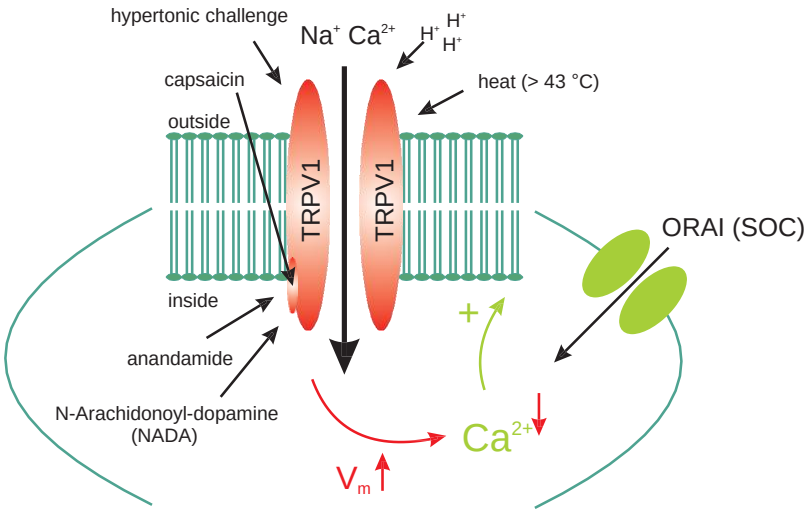

Fig. 1.7.1: Schematic representation of TRPV1. This TRP channel possesses endogenous activators such as endocannabinoids (anandamide, NADA). TRPV1 can also be activated by heat (> 43 °C) and is permeable for monovalent and divalent cations like other unspecific cation channels. Notably, membrane depolarization ($V_m\uparrow$) by TRPV1 channels results in a reduced Ca^{2+} entry ($Ca^{2+}\downarrow$) via ORAI (SOC = store-operated channel). This Ca^{2+} process accordingly modulates TRPV1 function to modify the intracellular Ca^{2+} content (3).

TRPV1 is a receptor for various inflammatory stimuli, hypertonicity, protons, and heat as well as lipid metabolites. Notably, TRPV1 is expressed in numerous human tissues especially in sensory neurons [review (82)], but also expressed in cornea and conjunctiva [review (3)]. Furthermore, TRPV1 is also (over)expressed in various tumor tissues [review (83)]. TRPV1 can also be activated at room temperature when proton concentration is increased (pH < 6) (84). Furthermore, various controlling mechanisms are involved in triggering specific responses through Ca^{2+} signaling (85). Capsazepine (CPZ) is the corresponding selective TRPV1 antagonist. Interestingly, TRPV1 activity is not only ligand-dependent, but also partially voltage-dependent (86). In this context, membrane depolarization by TRPV1 channels suppressed Ca^{2+} entry through another Ca^{2+} dependent channel such as ORAI1, which is a pore-forming transmembrane protein. Activation of this channel elicits SOC activation in non-excitable cells (Fig. 1.7.1). More specifically, ORAI1 is encoded by the *ORAI1* gene and causes activation of a very high Ca^{2+} permeable ion channel named as I_{CRAC} (calcium released activated Ca^{2+} current) when activated by Ca^{2+} store depletion (87). Activation of ORAI1 is supposed to depend on transfer to the plasma membrane of a so-called intracellular store membrane associated stromal interaction molecule 1 (STIM1). This protein is important concerning the Ca^{2+} filling state of intracellular stores. STIM1 moves to open SOC by interacting with ORAI1 upon intracellular Ca^{2+} store depletion. So far, the role of TRPV1 in connection with ORAI1 was not studied in ocular surface tissues and cells. Notably, intracellular Ca^{2+} accumulation plays a crucial role in TRPV1-stimulated apoptosis. Elevations in intracellular calcium concentration by capsaicin-induced TRPV1 activation stimulate apoptotic cell death via the activation of Ca^{2+}-dependent enzymes like phospholipases, proteases, and endonucleases. In contrast, reduction of extracellular calcium prevents TRPV1-induced apoptosis (88).

1.7.2 TRPV4

TRPV4 is also known as *osmosensor* and is a Ca^{2+}- and Mg^{2+}-selective channel which exhibits a functional role in cell-volume regulation (89). TRPV4 is also thermosensitive and responds to moderate heat (> 27 - 35 °C) as well as to phorbol esters (4α-PDD) and several endogenous substances including arachidonic acid (AA), the endocannabinoids anandamide and 2-AG, and cytochrome P-450 metabolites of AA, such as epoxyeicosatrienoic acids (74;90-93). TRPV4 can also be modulated by internal and external Ca^{2+} (94). Like TRPV1, TRPV4 is expressed in various human tissues and could firstly be demonstrated in HCEC (95), in which TRPV4 led a Ca^{2+} influx which occurs in response to swelling induced

regulatory volume decrease (RVD) behavior (95). At this point, the regulation of cell volume is essential for the function of corneal epithelium. The role of TRPV4 in the other ocular surface cells is not elucidated so far.

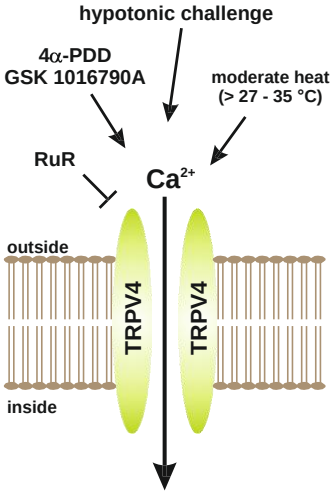

Fig. 1.7.2: Schematic representation of TRPV4: Moderate heating (> 27 - 35 °C) can activate this channel. Pharmacologically, specific agonists such as 4α-PDD and GSK1016790A (GlaxoSmithKline) can activate TRPV4. Ruthenium Red (RuR) is an inhibitor of TRPV4, but also blocks TRPV1-3 (drawn by S. Mergler).

1.7.3 TRPM8

TRPM8 is a cold receptor and can be activated by moderate cooling (< 28 °C) or pharmacologically by the cooling agent menthol, eucalyptol and icilin (80;96;97). This results in a transient rise in intracellular Ca^{2+} concentration (98-100). Furthermore, TRPM8 controls the cell cycle and regulates Ca^{2+} homeostasis, which is important for cell survival (101). For example, the viability of human melanoma is clearly suppressed in the presence of menthol in a concentration-dependent manner. This implies that Ca^{2+} permeability via TRPM8 channels may at least partly contribute to the regulation of cellular viability (101). BCTC is the corresponding selective TRPM8 antagonist (102). Physiologically, TRPM8 (as well as TRPA1 being also a cold receptor) participate in thermosensation and nociception (96;103). TRPM8 was initially identified as a prostate-specific gene (Trp-p8) (104). TRPM8 is (over)expressed in prostate cancer cells and is suggested to be a promising target for the treatment of prostate cancer (105). Like TRPV1, there is also a high (co) expression of TRPM8 in other tumor cells (99;106). TRPM8 is also co-expressed in sensory neurons including corneal afferent nerves (107;108). Activation of TRPM8 by cooling in cold-sensitive corneal afferents gives rise to a *wetness* perception, whereas a role of cold-insensitive afferents in basal tearing and other ocular dryness-related functions such as eye blink and the *dryness* sensation is still not yet clarified (108;109).

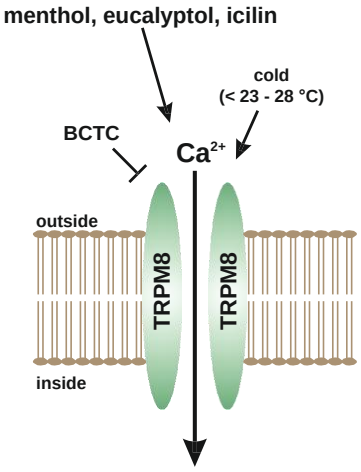

Fig. 1.7.3: Schematic representation of TRPM8: This channel can be activated by moderate cooling (< 28 °C) and is permeable for mono-valent and divalent cations like other unspecific cation channels (drawn by S. Mergler).

2 Investigation of human ocular surface cells

In this habilitation thesis, mainly permanent cell lines and primary cell cultures were investigated. Specifically, human corneal endothelial cells (HCEC-12), human corneal epithelial cells (HCEC-SV40) and human conjunctival epithelial cells (IOBA-NHC) were used as established cell models for corresponding ocular tissues. Furthermore, primary cell cultures of human corneal endothelial cells were additionally used.

2.1 Culture of human corneal and conjunctival cells

In general, cells were cultivated as previously described (110-112). In brief, cells were cultivated in Dulbecco's minimal essential medium (DMEM)/F12, 5 - 10% FCS, growth factors and antibiotics. The cultures were maintained in 5% CO_2 at 37 °C. The human corneal endothelial cell line HCEC-12 was immortalized (SV40-transfected) (113). For some articles in this habilitation thesis, they were obtained from DSMZ, Braunschweig Germany within a collaboration with Monika Valtink (Institute of Anatomy, Faculty of Medicine Carl Gustav Carus, TU Dresden, Germany). Similarly, SV40-immortalized HCEC were firstly created and characterized by Kaoru Araki-Sasaki et al. (114). These cells were kindly provided by Peter Reinach (Biological Sciences, the State University of New York, College of Optometry, New York, USA) within a collaboration. The same cell line as well as the spontaneously immortalized IOBA-NHC (HC_jEC) cell line was kindly provided by Friedrich Paulsen (Department of Anatomy II, University Erlangen-Nuremberg, Universitätsstraße 19, Erlangen, Germany). Furthermore, primary cultures of human corneal endothelium were additionally used and cultured as previously reported by Mergler et al. (110;115).

2.2 Measurements of intracellular Ca^{2+} concentration

Ca^{2+} as a classical second messenger is the most universal carrier of biological signals. It modulates cell life from its origin at fertilization to its end in the apoptotic process. Cells need Ca^{2+} for most of their important functions. In this habilitation thesis, fluorescence calcium imaging was used to investigate electrophysiological properties of the aforementioned cells types. This high-sensitive functional assay was firstly established by Grynkiewicz and Tsien et al. 1985 using the fluorescent dye fura-2, indo-1 and others (116). With this assay, it is possible to measure very small changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Here, fura-2/AM was used, which has specific photometric properties allowing an estimation of

$[Ca^{2+}]_i$. More specifically, fura-2/AM has two excitation wavelength (340 nm and 380 nm) and one emission wavelength (510 nm). Whereas the fluorescence signal at 340 nm is proportional to $[Ca^{2+}]_i$, the fluorescence signal at 380 nm has the opposite effect (Fig. 2.2).

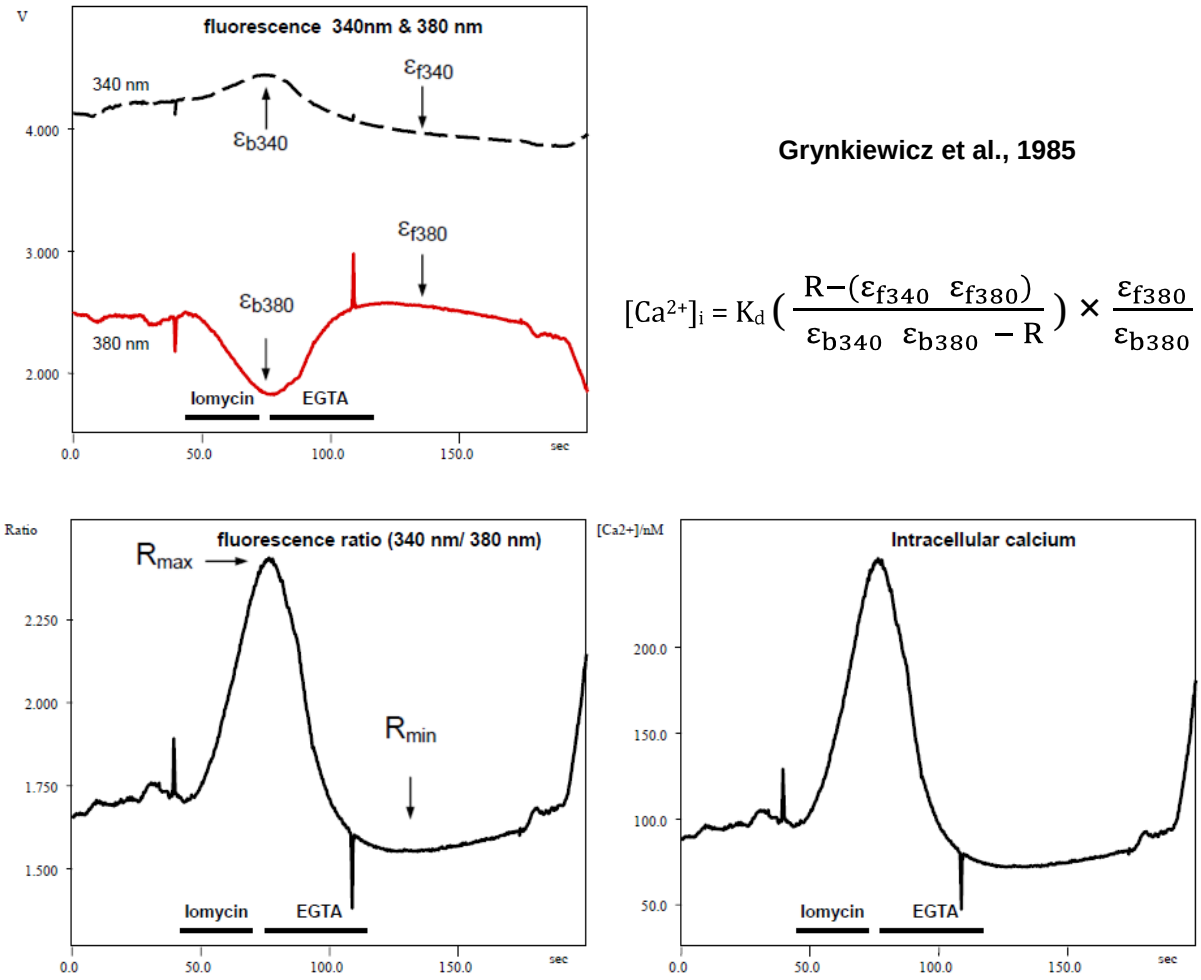

Fig. 2.2: Example of a calcium measurement (calibration) demonstrating the fluorescence ratio of R_{min} and R_{max} in neuroendocrine tumor cells. The ionophore ionomycin (1 µM) was used to generate a maximal fluorescence response signal (R_{max}) and the chelating agent EGTA (1 mM) was used to induce the lowest fluorescence response signal (R_{min}). The reagents were added at the time points indicated by bars. The parameters for the estimation of nM concentrations of $[Ca^{2+}]_i$ are shown in the left upper panel (upper dashed black trace versus lower solid red trace). The equation is according to Grynkiewicz et al., 1985 (116). K_d is the dissociation constant. The proportional factor ε_f corresponds to the basic property of the unbound dye whereas ε_b corresponds to the basic property of the dye binding to Ca^{2+}. R_{min} means the minimum fluorescence ratio f_{340}/f_{380}. R_{max} means the maximum fluorescence ratio f_{340}/f_{380} (Mergler 2000; unpublished data).

For the measurements, the two fluorescence response signals were measured alternately by rapid change of the excitation between the 2 wavelengths of 340 nm and 380 nm. Thereafter, a ratio of the both fluorescence response signals can be determined which is proportional to the change in $[Ca^{2+}]_i$. For the quantitative determination of $[Ca^{2+}]_i$ (nM), a calibration measurement was necessary (Fig. 2.2). The nM concentration of $[Ca^{2+}]_i$ and the fluorescence

ratio f_{340}/f_{380} (depending on time) was recorded by specific software for data acquisition and evaluation. In the investigations of this habilitation thesis, a quantitative calculation of $[Ca^{2+}]_i$ was not performed since changes in $[Ca^{2+}]_i$ were sufficient for qualitative analysis.

2.3 Measurements of ion channel currents

Using patch-clamp technique (117), very small ion channel currents can be detected. This opens a wide range of applications in the biology and in the medical basic research as well as drug discovery and development. Most cell-types of clinical relevance concerning biophysical investigation became accessible with it. Various diseases are due to a malfunction of definite ion channels or high expression of specific ion channels (78). In this habilitation thesis, whole-cell ion channel currents were measured using the planar patch-clamp technique (118) which has some advantages compared to conventional patch-clamping as demonstrated by Millligan et al., in Nat. Prot. 2009 (119).

2.3.1 Planar patch-clamp technique

About 10 years ago, automated planar patch-clamp has become available, which allows a rapid and efficient characterization of ion channels during voltage-clamp (120;121). The automated approach (Port-a-Patch©, Nanion, München) is based on planar patch-clamp chips. The glass pipette of a conventional patch-clamp recording set-up has been replaced by a thin sheet of flat glass with a small aperture in the middle (the planar chip) (Fig. 2.3.1).

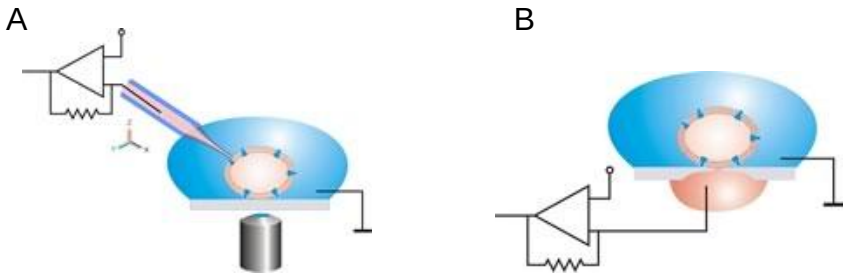

Fig. 2.3.1: **Patch-clamp techniques: (A)** Schematic view of a conventional patch-clamp experiment. The pipette is attached to an adherent cell using a micro manipulator. **(B)** Using the modern planar patch-clamping (automated patch-clamping), the cell will be attached to an aperture by negative pressure. This set-up excludes vibration-associated artifacts, which frequently occur using a conventional patch-clamp system (37).

Intracellular or extracellular perfusion of cells in this system allows a detailed characterization of the electrophysiology and pharmacology of various ion channel types. All path-clamp recordings in this habilitation thesis were carried out in the whole-cell configuration and relate to non-voltage-dependent Ca^{2+} channels such as the aforementioned TRPs since studies

of this large group of Ca^{2+} entry channels have significantly extended our knowledge about the molecular basis of sensory perception in animals (122). Due to their different activation mechanisms and biophysical properties, TRPs are working like receptors for environmental or endogenous stimuli or as molecular players in down-stream signaling (122).

2.3.2 Color contour plot analysis of whole-cell currents

To better evaluate ion channel currents Mergler developed a specific color contour plot method of analysis (123). This method of analysis was used for investigation of ion channel characteristics in ocular cells (unpublished), ocular tumor cells such as retinoblastoma (124) and other tumor cells (106;125). Certain differences between the effect of specific and unspecific Ca^{2+} channel modulators on whole-cell currents could be detected (123). Although most of this information can also be obtained by superimposing current-voltage curves at different times after the onset of the voltage step, such diagrams clearly provide a better visual summary. Since the data are normalized (pA/pF), it is possible to compare cell types and conditions more easily for electrophysiological analysis. The currents can be fitted into a three-dimensional cube, which is independent from current amplitudes, time range and current potentials. Therefore, it is possible to compare electrophysiological characteristics of different cell types under different conditions simultaneously. An example is shown in figure 2.3.2, in which the TRPV1 activator capsaicin led to a changed color pattern (106).

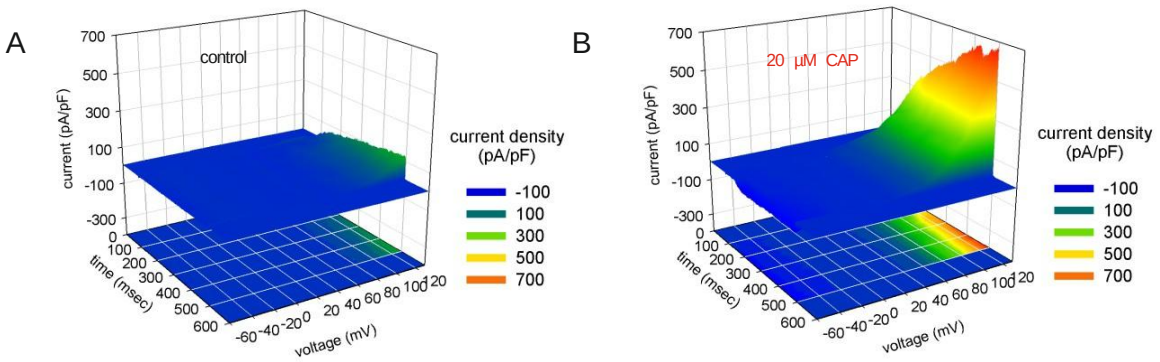

Fig. 2.3.2: **Identification of CAP-activated nonselective cation channels currents in HEK293 cells transfected with human TRPV1. (A)** Three-dimensional color diagram of whole-cell currents. The green areas (integrals) showed a minor basal cation channel current activity at control conditions (room temperature 21 °C). The normalized current amplitudes (current density; pA/pF) were divided into several color segments. **(B)** Three-dimensional color diagram of whole-cell currents. The red areas (integrals) show the effect of CAP on whole-cell channel currents in HEK293 cells transfected with human TRPV1. Currents (with leak subtraction) were recorded after stimulation from -60 mV to +130 mV (400 ms) from a holding potential of 0 mV in 10 mV steps (106).

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3 Objectives

Keratoconjunctivitis sicca or dry eye syndrome as well as corneal (endothelial) dystrophies are common disorders encountered in ophthalmological practice. Various therapy options have been established over the last years. Regarding corneal dystrophies, a surgical intervention such as a corneal transplantation (keratoplasty) or (more specific) DMEK (Descemet membrane endothelial keratoplasty) is required in worst cases. In this connection, the storage of donor corneas and the corresponding corneal endothelial cell density as well as cell vitally plays a crucial role in context with current corneal banking. At this point, the storage of donor corneas can still be improved since only cell counting and cell staining are the current standard methods for assessments. So far, cell biological processes such as apoptosis of corneal endothelial cells or the role of storage temperature was not fully clarified so far. Regarding DES, steroids and current immunosuppressants such as cyclosporine were established to suppress inflammation in response to tear film hypertonicity. Notably, hypertonicity is one crucial pathogenetic factor in the development of a (generally subclinical) inflammation of the ocular surface, the lacrimal gland and the tear film in DES. So far, current options are insufficient and the reasons for inflammatory processes in context with DES are not yet fully elucidated. In all these issues, receptor activity in ocular tissues is essential for maintaining normal ophthalmological functions since it transduces environmental stresses into cell signaling events controlling different physiological responses that offset declines in cellular function. This realization has prompted intense research efforts to more extensively characterize which receptors provide such control since such insight may lead to the identification of novel drug targets needed to treat ocular disease or to improve cell vitality.

Based on this background knowledge and the results of previous studies cited before, temperature sensitive receptors such as “thermo-TRPs” and putative endogenous modulators of these channels may be relevant targets. Therefore, the following aims are proposed:

1. **Characterization of thermo-TRP channel expression and function of human corneal endothelial cells (HCEC-12), human corneal epithelial cells (HCEC-SV40) and human conjunctival epithelial cells (IOBA-NHC).** Specifically, the heat- or capsaicin receptor TRPV1, the osmosensor TRPV4 and the cold- or menthol receptor TRPM8 have priority since they are well-investigated TRPs. The aforementioned cell models and high sensitive functional assays such as fluorescence calcium imaging and patch-clamping will be used.

2. **Identification of possible endogenous modulators of TRPs, which may be relevant for inflammatory or apoptotic processes.** The effects of certain hormones and agents on TRP channel expression and function in the aforementioned cells will be analyzed. Since an osmoprotectant such as L-carnitine showed a protective effect on HCEC and in DES using a murine model (126;127), its effect on Ca^{2+} regulation will be validated specifically in HC_jEC and HCEC. In addition, a possible effect of an endogenous thyroid hormone (TH) metabolite (T_1AM) will be investigated since this TH derivative induces numerous responses including a spontaneously reversible body temperature (128).

4 Results (own original articles)

4.1 TRPVs in human corneal endothelial cells (original article 1)

TRPV channels mediate temperature sensing in human corneal endothelial cells.

S. Mergler, M. Valtink, V. J. Coulson-Thomas, D. Lindemann, P. S. Reinach, K. Engelmann, and U. Pleyer. Exp.Eye Res. 90 (6):758-770, 2010.

http://dx.doi.org/10.1016/j.exer.2010.03.010

Author contribution: SM, MV and PSR designed the study, analyzed the data, wrote and edited the manuscript. KE and UP contributed with their expertise in ophthalmology and edited the manuscript. VJC-T and MV performed PCR analysis and immunohistochemistry. SM performed calcium measurements and planar patch-clamp recordings as well as plot analyses.

Using high-sensitive functional assays, TRP channels could be clearly demonstrated for the first time in HCE_nC. Initially, there were first indications about a possible TRP channel expression, which stem from previous studies of electrophysiological characterization of voltage-operated Ca^{2+} channels of the L-type in HCE_nC. Specifically, the L-type channel blocker nifidipine had a lower inhibitory effect on intracellular Ca^{2+} concentration than EGF (129). Therefore, this follow-up study focused on putative TRP channel expression in HCE_nC using specific protocols and solutions. In this article, an established HCE_n cell line named as HCEC-12 was used, which has also been exploited for transplantation purposes (130). Moreover, two morphologically distinct clonal cell lines derived from HCEC-12 were used. These were firstly established by Monika Valtink (TU Dresden) and named as HCEC-H9C1 and HCEC-B4G12 (131). All cell lines were probed for TRP channel members of the TRPV subfamily. As a result, TRPV1-3 functional activity could be demonstrated based on Ca^{2+} influxes and increases in whole-cell currents following heating the bath solution. They were minor differences between HCEC-12 and the cell clones. Overall, it was suggested that TRPV activity modulation by temperature triggers important homeostatic mechanisms contributing to the support of HCE_n function under different temperature levels. These findings motivated the author(s) of this article to probe for functional expression of further “thermo-TRPs” in HCE_nC, as shown in the following sections of this habilitation dissertation.

4.2 TRPM8 in human corneal endothelial cells (original article 2)

Functional significance of thermosensitive transient receptor potential melastatin channel 8 (TRPM8) expression in immortalized human corneal endothelial cells.

S. Mergler, C. Mertens, M. Valtink, P. S. Reinach, V. C. Szekely, N. Slavi, F. Garreis, S. Abdelmessih, E. Turker, G. Fels, and U. Pleyer. Exp Eye Res 116:337-349, 2013.
http://dx.doi.org/10.1016/j.exer.2013.10.003

Author contribution: SM, MV and PSR designed the study, analyzed the data, wrote and edited the manuscript. UP contributed with his expertise in ophthalmology and edited the manuscript. MV and SA performed PCR analysis. CM, SM, ET, VCS and NS performed calcium measurements. CM, VCS and SM performed planar patch-clamp recordings and plot analyses. GF performed cell preparations.

Previously, Mergler et al. described another thermo-TRP such as the osmosensor TRPV4 for the first time in HCEC-12 and the aforementioned clonal cell lines (132). As regulation of cell volume is essential for the function of HCE (95) and probably also for HCE_n, Mergler et al. probed for this TRP channel. Subsequently, TRPV4 could also be detected using physical and pharmacological approaches (e.g. moderate heating, hypotonic challenge, 4α-PDD). At that stage, TRPV1-4 was detected in HCE_nC. Therefore, the next question was whether also cold receptors such as TRPM8 and TRPA1 are functionally expressed in HCE_nC. This question may be relevant since donor corneas are preserved under different temperature conditions and may be dependent on thermo-TRP channel preservation. More specifically, donor corneas are preserved at a temperature level around 30 – 37 °C, which is preferred in Western Europe (133). In contrast, there are also other preservation techniques using hypothermic preservation media (134;135). Therefore, the author(s) of this article postulated that HCE_nC may be also composed to survive hypothermic challenge since it was shown that preservation of intact corneal thinness is better maintained at lower temperatures because of improved preservation of barrier function (136). In this context, this article describes putative functional TRPM8 expression in HCE_nC since this channel activates with moderate cooling below the physiological range of 28°C

(80;137). In a previous study, the author of this habilitation thesis already accumulated experience with the specific detection methods for this channel (99). Therefore, the same physical and pharmacological approaches were used in the study of HCE_nC as described in this article. Specifically, extracellular application of the high selective TRPM8 agonist like menthol (and also eucalyptol) increased intracellular Ca^{2+} influx and whole-cell currents in HCEC-12. In addition, the same applies to icilin activating both TRPM8 and TRPA1 (138). Overall, these detailed investigations indicate at least TRPM8 activity, but probably also TRPA1 in HCE_n. Incidentally, Mergler et al. have also used normal HCE_nC (primary cell cultures) to confirm functional cold receptor expression in these cells using icilin. More specifically, Mergler et al. have prepared a culture of normal HCE_nC from a human donor cornea and have used this culture for up to four passages for additional experiments. Since corneal temperature is estimated to reach the minimum of 33°C to 34°C depending on body and ambient temperatures (139), they also have performed calcium imaging experiments just below these temperatures (≈ 30 °C). As a result, functional cold receptor expression could be confirmed using icilin (96) as shown in this article. Therefore, this study also provide data on the functional state of TRPM8 at physiological temperatures. Apart from that, corneas that were kept in a specific medium at room temperature had relatively intact endothelium after several days (140). This suggests that tissue function and metabolic activity are restored at room temperature. In conclusion, Mergler et al. could demonstrate cold receptors in HCE_nC for the first time suggesting that HCE_n function can adapt to different ambient temperature levels through activation of this channel subtype.

Since the HCE is typically unable to be sustained for longer than 1 week in storage media (141), the investigation regarding preserving not only of HCE_nC but also of the HCE is relevant. Furthermore, there were also other reasons to investigate these TRPs in HCEC since the author of this habilitation thesis has established a close collaboration with Peter Reinachs' lab for many years (Biological Sciences, the State University of New York, College of Optometry, New York, USA). An important study in which thermo-TRPs were investigated in HCEC is shown in the next section.

4.3 Thermo-TRPs in human corneal epithelial cells (original article 3)

Thermosensitive transient receptor potential channels in human corneal epithelial cells. **S. Mergler**, F. Garreis, M. Sahlmuller, P. S. Reinach, F. Paulsen, and U. Pleyer. J. Cell Physiol 226 (7):1828-1842, 2011.
http://dx.doi.org/10.1002/jcp.22514

Author contribution: SM and PSR designed the study, analyzed the data, wrote and edited the manuscript. FP and UP contributed with their expertise in anatomy and ophthalmology, respectively, discussed data and edited the manuscript. FG performed PCR analysis and immunohistochemistry. SM and MS performed calcium measurements. SM performed planar patch-clamp recordings and plot analyses.

In this article, the aforementioned TRPs of the vanilloid subfamily were described in HCEC. Within the aforementioned collaboration, Zhang, Reinach, Mergler et al. already demonstrated 2007 that TRPV1 is expressed in HCEC. Moreover, they demonstrated that TRPV1 induces inflammatory cytokine release in corneal epithelium through mitogen-activated protein kinase signaling (111). This is an important result because of the following reasons: At first, TRPV1 can be activated by hypertonic stress in HCEC (142). Secondly, this can lead to inflammatory processes occurring in dry eye disease (143). Pan, Mergler and Reinach et al. concluded in their studies that TRPV1 receptors may play a role in mediating HCE inflammatory mediator secretion and subsequent hyperalgesia (111). In another preliminary study (2008), Pan, Mergler, Pleyer, Reinach et al. could demonstrate functional TRPV4 expression in HCEC and that its activity contributes to regulatory volume decrease (95).

Despite these preliminary findings, this article focused on a more electrophysiological approach because the membrane currents were not thoroughly studied using either selective agonists or temperature stimulation. In this article, TRPV1-4 functional expression could be confirmed in HCEC using planar patch-clamping. It was suggested that these thermo-TRPs confer temperature sensitivity at the ocular surface, which may protect the cornea against temperature-induced stress. Technically, an

alternative patch-clamp configuration is the aforementioned planar patch-clamping, which was documented to be advantageous (119). With this configuration, stable seals are easier to obtain due to lower access resistances. This improves the quality of the cell recordings. Even though this technique depends on suspending the cells in solution, the channel activities are unaltered from those measured when the cells are attached to a substratum.

A critical point of this article (and some previous ones) concerned the stress-induced temperature increases exceeding transiently the 50 °C level. This high temperature level specifically activates the heat and pain receptor TRPV2 (80). As a precaution, the author(s) of this article therefore checked for the morphology of these cells in the microscope during heating and documented it as shown in figure 1 of this article. As shown in this figure, there was only a minor decrease in the fluorescence signals whereas the cell morphology was mainly preserved. As the heat-induced current increases were reproducible, it was suggested that the cells remained viable. This indicates that transient exposures to approx. 50 °C did not have injurious effects on cell function.

In summary, this article evaluated detailed characteristics of the aforementioned thermo-TPRs, especially of the TRPV1. The basic finding of this study and the previous related ones by Mergler, Reinach et al was that osmosensitive TRPs like TRPV1 and TRPV4 probably have a role in the pathophysiology of the dry eye disease because of the aforementioned reasons. Therefore, another challenge is to evaluate possible endogenous modulators of TRPV1 to suppress at first hypertonic-stress induced inflammatory processes in these cells. Clinically, osmoprotection may be an alternative therapy for patients with dry eye.

A possible substance may be the osmoprotectant L-carnitine as mentioned in the working hypothesis in this habilitation thesis (chapter 3). A detailed study of the effect of L-carnitine in HC_jEC is presented in a follow-up study in the next section.

4.4 L-carnitine reduces cell shrinkage via TRPV1 (original article 4)

L-carnitine reduces in human conjunctival hypertonic-induced shrinkage through interacting with TRPV1 channels.

N. Khajavi, P. S. Reinach, M. Skrzypski, A. Lude, **S. Mergler**. Cell. Physiol. Biochem. 34:790-803 137, 2014. (Funded by DFG ME 1706/13-1).

DOI: 10.1159/000363043

http://www.karger.com/Article/FullText/363043

Author contribution: SM, NK, and PSR designed the study, analyzed the data, wrote and edited the manuscript. NK performed PCR analysis and immunohistochemistry. NK and AL performed calcium measurements. NK and AL performed planar patch-clamp recordings and plot analyses. NK and MS carried out the ELISA.

Previously, Mergler, Garreis et al. demonstrated functional expression of TRPV1 and TRPV4 for the first time in HC_jEC using the aforementioned IOBA-NHC cell line and the same electrophysiological approaches (112). The properties of these thermo-TRPs are similar to those in HCEC as described in the previous studies. Like HCE functional activity, HC_jE functional activity is essential to ocular health because of its barrier function and its contribution to the maintenance of surface hydration. As shown in HC_jEC (112) as well as in HCEC (142;144), TRPV1 can be activated by the same type of hypertonic stresses encountered in dry eye disease leading to declines in relative cell volume.

In this article, molecular biological assays as well as various functional assays were described and used to determine if changes in TRPV1 activity in HC_jE cells contribute to mediating described osmoprotective effects of L-carnitine against such a challenge. So far, the role of L-carnitine was not characterized in this cell type and osmoprotective effects were reported in other studies using HCEC and other cell types (126;127;145). Notably, Khajavi, Mergler et al. demonstrated that L-carnitine has an inhibitory effect on CAP- or hypertonic-stress induced Ca^{2+} increases. On the other hand, L-carnitine has diverse effects on ion channel regulation including Ca^{2+} channel blockade. This indicates that L-carnitine can regulate several kinds of ion channels or transporters involving volume regulation besides TRPV1. In accordance with this knowledge, it was necessary to examine the specific blockade of TRPV1 by L-

carnitine to exclude the possibility of different ion channels or transporters. As a result, the investigations clearly indicate that TRPV1 is one of the transport pathways with which L-carnitine interacts. Its interaction with TRPV1 is based on showing that the responses induced by procedures to stimulate TRPV1 are markedly attenuated by pre-exposure to L-carnitine. CAP is a well-accepted selective TRPV1 agonist (90) and its stimulation of Ca^{2+} influx and increases in whole-cell currents are markedly reduced in a similar fashion by either L-carnitine or by CPZ which is a well-accepted selective TRPV1 antagonist (90). Similarly, the well-accepted osmosensor role of TRPV1 in mediating increases in Ca^{2+} influx (142) were also blocked by either CPZ or L-carnitine further indicating that both of these agents elicit specific blockade of TRPV1. Finally, a putative (osmo)protective effect by L-carnitine failed if TRPV1 was "switched off" using TRPV1 silencing RNA.

In summary, this article showed that the osmoprotective role of L-carnitine depends on its suppression of TRPV1activation. These results are novel because literature investigations resulted that there are no studies showing that inhibition rather than TRPV1 activation elicits this response. In this context, the authors of this article were endeavored to investigate (further) endogenous TRP channel modulators, which would be able to suppress inflammatory-induced TRPV1 activation. Cannabinoid receptor 1 (CB1) which is G protein coupled was suggested as a possible target since an inhibitory effect on TRPV1 was shown (146). In this context, Mergler et al. could show in ocular tumor cells that activation of CB1 suppressed TRPV1-induded Ca^{2+} influx (124;147). Moreover, Yang and Reinach et al. recently demonstrated that CB1 suppressed TRPV1-induced inflammatory responses to corneal injury (148). Because CB1 is a G protein-coupled receptor, other ligands such as specific hormones may be relevant targets in this connection. 3-iodothyronamine (T_1AM), an endogenous thyroid hormone (TH) metabolite, is such a ligand generally binding GPCRs such as trace amine-associated receptor 1 (TAAR1) (149). Interestingly, application of this TH metabolite induces numerous responses including a spontaneously reversible body temperature decline (128). As such an effect is associated in the eye with increases in basal tear flow and thermosensitive TRPM8 channel activation (108), it was suggested to investigate a putative TRPM8 expression and T_1AM effect on HC_jEC as shown in the next section.

4.5 Thyronamine induces TRPM8 activation (original article 5)

Thyronamine induces TRPM8 channel activation in human conjunctival epithelial cells.

N. Khajavi, P. S. Reinach, N. Slavi, M. Skrzypski, A. Lucius, O. Strauss, J. Kohrle, and **S. Mergler**. Cell Signal. 27:315-325, 2015. (Funded by DFG ME 1706/13-1).

http://dx.doi.org/10.1016/j.cellsig.2014.11.015

Author contribution: SM, NK, and PSR designed the study, analyzed the data, wrote and edited the manuscript. OS and JK contributed with their expertise in physiology and endocrinology, respectively, discussed data and edited the manuscript. NK performed PCR analysis and immunohistochemistry. NK and NS performed calcium measurements. NK, AL, and NS performed planar patch-clamp recordings and plot analyses. NK and MS carried out the ELISA.

In this article, Khajavi, Mergler et al. determined in HC_jEC (IOBA-NHC) if T_1AM also acts as a cooling agent to directly affect TRPM8 activation at a constant temperature. Indeed, they found that T_1AM increased TRPM8 activation when the temperature level was constant. Specifically, T_1AM increased intracellular Ca^{2+} concentration as well as whole-cell currents similar as the cooling agent icilin. Therefore, they suggested that T_1AM directly interacts with TRPM8 or via a GPCR in order to activate this channel. Furthermore, the same may apply to an *in vivo* T_1AM effect, which may activate TRPM8 through an indirect effect subsequent to temperature lowering as well as by a direct interaction with TRPM8 independent of a temperature change.

To validate TRPM8 involvement, the authors of this article thought of silencing TRPM8 via silencing RNA as an alternative to pharmacological intervention. However, numerous studies have only resorted to this approach in cases where documented specific TRP channel antagonists are not available. Regarding TRPM8, the authors of this article thought that their results obtained with established TRPM8 blockers are an adequate documentation that T_1AM is a TRPM8 agonist since the inhibitory effects of BCTC-induced Ca^{2+} response patterns and whole-cell current responses are similar to those described in other studies by using TRPM8 silencing RNA. They stated that documentation of BCTC selectivity is not warranted because

BCTC was successfully used in their own previous studies regarding TRPM8 in HCE_nC (115). Furthermore, only BCTC was used in a Nature paper to document TRPM8-dependent cold thermoreceptors in the cornea *in vivo* (108). Finally, BCTC was identified as a full blocker of TRPM8 channels in the literature (150-152). Independently, Mergler et al. found that T_1AM activated TRPM8 linked Ca^{2+}influxes in various cell types since BCTC blocked the increases in TRPM8 activity evoked by icilin (unpublished data). Regarding TRPV1, Khajavi, Mergler et al. used CPZ as well as TRPV1 siRNA in the same cell line and found a close correspondence between their inhibitory effects on CAP-induced TRPV1 activation as shown in the previous original article of this habilitation thesis (153).

Taken together, Khajavi, Mergler et al. provide the first results describing the expression and function of the cold- and menthol receptor TRPM8 in HC_jEC. They concluded that T_1AM is like the cooling agent icilin since both substances directly elicit TRPM8 activation at a constant temperature. In addition, they could demonstrate a reverse link between changes in TRPM8 and TRPV1 activity since these cooling agents blocked TRPV1 activation by CAP. The content of this article is novel in the field of molecular endocrinology and calcium signaling. It is going to contribute to a better understanding of the pathophysiology of dry eye disease and aspects in endocrinology on molecule and cell level. This may provide a basis to develop novel therapies using thyronamines as possible endogenous modulators of thermo-TRPs.

5 Discussion

The results of the presented original articles of this habilitation thesis have clearly extended our knowledge about possible endogenous modulators of the aforementioned thermo-TRPs. Initially, there was a study by Mergler et al. about electrophysiological properties of voltage-operated Ca^{2+} channels (VOCCs) of the L-type in HCE_nC (129). Interestingly, Mergler et al. showed that oxidative stress using hydrogen peroxide (H_2O_2) led to an increase of intracellular Ca^{2+}, which could be more suppressed by EGF than by the L-type channel blocker nifedipine. These results indicate that not only L-type channels are involved but probably also other Ca^{2+} channels like TRPs since H_2O_2 associates with stress-sensitive TRPM2-mediated calcium influx (154-156). Moreover, extracellular application of the cooling agent icilin had a complex dose-dependent effect on intracellular Ca^{2+} in HCE_nC (129), indicating involvement of icilin-sensitive TRPM8 and/or TRPA1 channels (138). Based on the aforementioned findings, many studies followed, demonstrating that TRPs are not only expressed in HCE_nC, but also in HCEC as well as HC_jEC [review (3;12)]. Fig. 5 summarizes own data as well as data from collaboration partners and other research groups about TRP channel expression in the cornea (157).

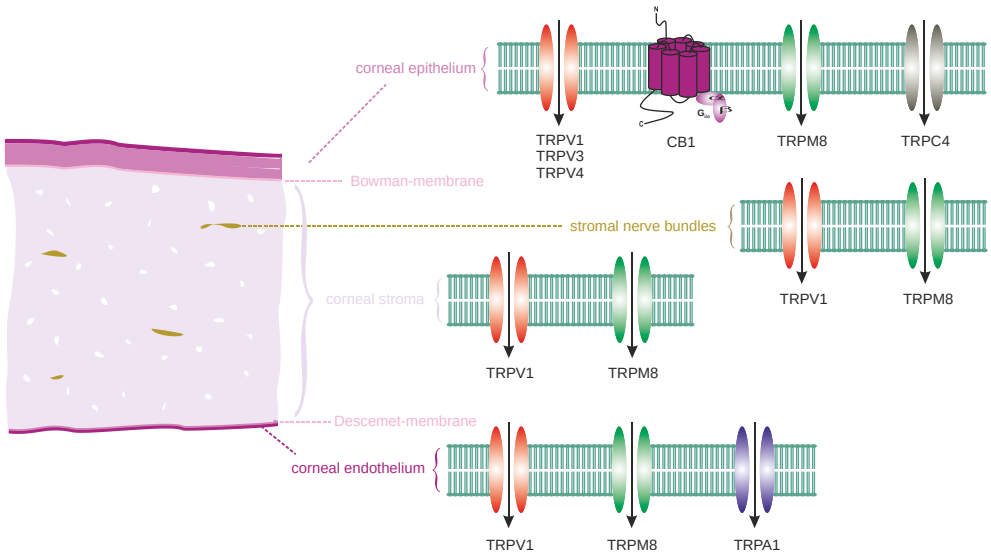


Fig. 5: TRP channels and cannabinoid receptor 1 (CB1) in cell layers of the human cornea: Corneal epithelium (157): TRPV1/3/4 (95;144;158), CB1 (148), TRPC4 (159), TRPM8 (Khajavi, Mergler et al., 2014, unpublished observation); corneal stroma: TRPV1 (160), TRPM8 (Türcker, Mergler et al., 2014, unpublished observation); corneal nerve fibers: TRPV1 (161), TRPM8 (108); corneal endothelium: TRPV1-3 (162), TRPV4 (132); TRPM8 (115), TRPA1 (115) (Reinach, Mergler et al. (157)).

5.1 Functional expression of TRPV1 and TRPM8

Initially, most of the studies that delineate TRP functional roles in the eye are in connection with their presence on neuronal elements. However, it is now evident that there is also TRP channel expression in *non-neuronal* ocular cells [6]. Specifically, there is functional TRPV1 and TRPM8 expression in HCE_nC (115;162), HCEC (111;144;163), and HC_jEC (112;164), concluded from its activation by CAP or a hypertonic challenge and suppression by CPZ on Ca^{2+} regulation regarding TRPV1. CAP and CPZ are established pharmacological tools for TRPV1 (90). The same applies to icilin and BCTC concerning TRPM8 (102). In addition, whole-cell current response patterns elicited by CAP or icilin are in line with aforementioned studies. Specifically, outwardly rectifying currents are similar to those described in TRPV1-transfected or TRPM8-transfected HEK293 cells as well as in tumor cells (86;99;106). This is a typical characteristic of these TRPs. In all of the present studies, the reversal potential was about 0 mV, which is consistent with TRPV1/TRPM8 behavior as non-selective cation channels. By replacement of the extracellular solution with Na-gluconate, the TRPV1-induced increases of whole-cell currents did not change. This negative effect indicates that Cl^- channel activity can be most likely excluded (144). Functional TRPM8 expression was also demonstrated by showing that temperature lowering below 20 °C induced Ca^{2+} transients, which were inhibited by the TRP channel blockers La^{3+} or BCTC. These effects are very similar to those described in the literature (86;99;106;124). Overall, there is functional TRPV1 and TRPM8 expression in the aforementioned ocular surface cells.

5.2 TRPV1-TRPM8 mediated down-stream signaling

As shown in the present studies concerning ocular surface cells, it turned out that these cells are equipped with receptors and TRPs that allow them to detect and respond to diverse chemical, mechanical, and thermal stimuli. The regulation of TRPs by G protein-coupled receptors (GPCRs) is generally known from literature (GPCRs-TRP channel axis) [review (165)]. It is also known that GPCRs and TRPs are co-expressed on sensory neurons and act as sensors of noxious irritants and inflammatory stimulants. The same applies to non-neuronal cells of the eye. GPRSs belong to a large family of signaling proteins and many drugs target GPCRs to treat pathophysiological conditions. Like sensory nerves, *non-neuronal* corneal cells also express TRPs, which also sense endogenous and exogenous chemical, mechanical, and thermal stimuli. These TRPs are also major downstream effectors of GPCR signaling. An example is given in figure 5.2.1 regarding putative TRPM8 activation by T_1AM (164).

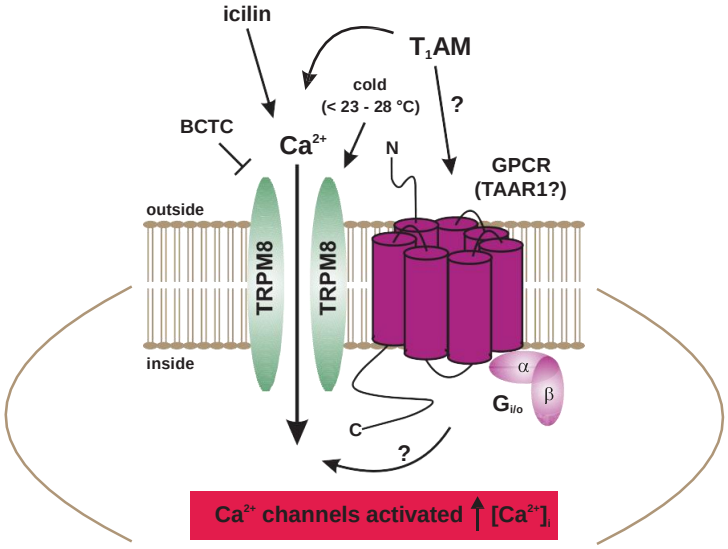


Fig. 5.2.1: **Suggested Ca^{2+} signal transduction pathway activated by T_1AM. A:** Ca^{2+} channels such as TRPs of the TRPM8 subtype (menthol receptor) can be selectively activated by cold (< 23 - 28 °C) or icilin and blocked by BCTC whereas a G-protein-coupled receptor (GPCR) coupled to Gi/o proteins could also be activated by T_1AM. T_1AM may also directly activate TRPM8 by a GPCR-independent mechanism (↑$[Ca^{2+}]_i$]) (164).

More specifically, it is suggested that TRPM8 is also linked with a GPCR such as hTAAR1 or beta adrenergic receptors (165). An interaction between TRPM8 and hTAAR1 is indeed reasonable, since earlier studies clearly showed that the hypothermic effects of T_1AM stem from a direct interaction with TAAR1 (166). On the other hand, interaction between TRPM8 and beta adrenergic receptors cannot be excluded since these receptors are expressed at a high level in different ocular tissue layers (167-169). An interesting finding is that the non-selective beta-adrenergic receptor antagonist timolol suppressed T_1AM induced Ca^{2+} increases in HC_jEC (unpublished observation). As shown in the present studies, there is clear evidence for a direct interaction between T_1AM and TRPM8. Therefore, the hypothermia induced by T_1AM may be attributable to co-activation of both beta-adrenergic receptors and TRPM8. Furthermore, it could be possible that co-activation is not located on the plasma membrane. Instead, it turned out that TRPM8 protein expression is probably also occurring in the perinuclear domain (164). Earlier studies also showed that TRPV1 expression is not plasma membrane delimited in HCEC as well as in some other tissues (111;170). In addition, T_1AM uptake into target cells has also been demonstrated (171;172). Another possible Ca^{2+} signal transduction pathways is shown in figure 5.2.2.

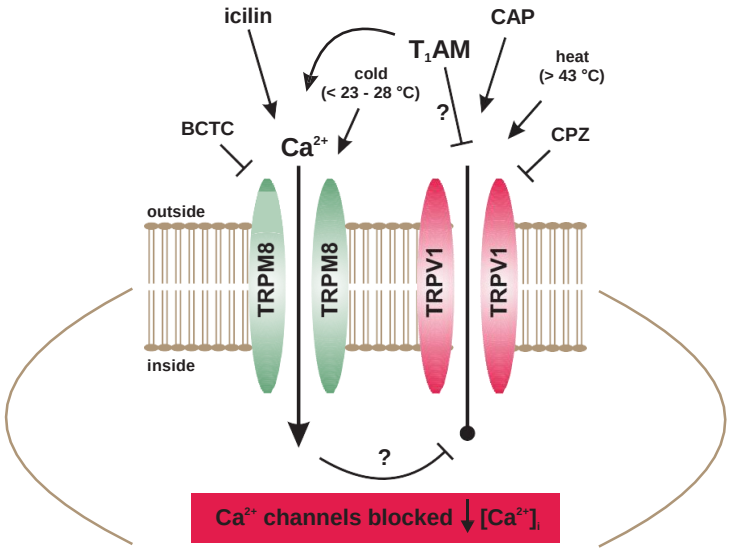


Fig. 5.2.2: **Suggested Ca^{2+} signal transduction pathway activated by T_1AM. A:** TRPs of the TRPV1 subtype (capsaicin receptor) can be selectively activated by heat (> 43 °C) or by CAP and blocked by CPZ. If TRPM8 is activated by T_1AM, T_1AM blocks TRPV1.Notably, T_1AM may also directly suppress TRPV1 by a GPCR-independent mechanism (↓$[Ca^{2+}]_i$]) (164).

There is indication that there is ab interplay between some TRPs like TRPV1 and TRPM8. For example, several studies demonstrated that TRPM8 activation leads to suppression of TRPV1 activity (107;124;147;173). As shown in figure 5.2.2, TRPM8 activation via T_1AM may lead to TRPV1 suppression whereas it is not clarified whether T_1AM may directly suppress TRPV1. Overall, such an interaction could provide a therapeutic option in a clinical setting to reduce TRPV1-induced inflammation and fibrosis subsequent to a severe ocular surface injury.

5.3 Putative role of thermo-TRPs in ocular surface cells

To date, there are clear indications that thermo-TRPs have a pathophysiological significance in eye surface tissue and layers. In the recent years, there are steadily increasing amount of reports about the putative role some thermo-TRPs in the cornea and conjunctiva. Some putative roles of certain thermo-TRPs are discussed in the following sections.

5.3.1 TRPV1 triggers the release of inflammatory mediators

CAP-induced TRPV1 activation not only triggers corneal epithelial cell proliferation, but also inflammatory processes. Specifically, this channel activation led to an increase of intracellular Ca^{2+} concentration and global mitogen-activated protein kinase (MAPK) activation, followed by increased release of interleukin-6 (IL-6) not only in HCEC but also in HC_jEC (111;164).

Therefore, TRPV1 in these ocular surface cells may be essential for inducing release of inflammatory mediators that could mediate the immune responses and injurious effects of noxious agents on tissue integrity and function (111). Interestingly, cannabinoid receptor 1 (CB1) is able to suppress TRPV1-induced inflammatory responses to corneal injury (148). A similar effect could be registered in eye tumor cells regarding Ca^{2+} regulation. More specifically, activation of CB1 led to suppression of TRPV1-induced Ca^{2+} increase in uveal melanoma cells (147).

5.3.2 TRPV1 and TRPV4 are osmosensitive ion channels

TRPV1 and TRPV4 are both osmosensitive ion channels in corneal and conjunctival cells. Whereas TRPV1 can be activated by hypertonicity, TRPV4 can be activated by hypotonicity. It was therefore suggested that these osmosensing TRPs serve as central sensors of systemic anisotonicity (174). In HCEC, hypertonic stress-elicited TRPV1 channel stimulation mediates increases in a proinflammatory cytokine IL-6. Therefore, selective drug modulation of either TRPV1 activity or its signaling mediators may yield a novel approach to suppressing inflammatory responses (142). On the other hand, TRPV4 also known as osmosensor can be activated by phorbol esters such as 4α-PDD or by exposure to hypotonicity (175). This led to an increase of intracellular Ca^{2+} concentration, which has been reported in HCE_nC and HCEC as well as in HC_jEC (95;112;132;144). In HCEC, it has additionally been shown that TRPV4 activation changed regulatory volume decrease (RVD) behavior (95). Therefore, it is suggested that TRPV1/TRP4 activation plays a role in context with osmolarity and that TRPV4 activation is essential for mediating a RVD response.

5.3.3 Possible role of TRPV1 and TRPM8 and corneal banking

Maintaining TRPM8 function in an eye bank setting may be dependent on TRPM8 activation during storage at temperatures below 23 °C (176;177). TRPM8 stimulation could result in graded shifts of voltage-dependent activation to more negative membrane voltages (86). As discussed before, this channel may also be regulated by GPCRs. For example, in non-corneal cells both activation of a GPCR and a nerve growth factor receptor inhibited menthol- and cold-induced TRPM8 activity (178). Using temperature sensitivity as a potential standard for channel identification in corneal endothelium may be more reliable than monitoring drug sensitivity, since many drugs used for TRP channel identification have a limited selectivity. In the context of corneal cell viability, it is important to note that the activity of certain TRPs is modulated by membrane receptor activity, e.g. EGFR (179). For TRPV1-4 (89;180-186) and

TRPM8/TRPA1 (100;103), a role in temperature sensing has been evidenced. Another study demonstrated an impact of temporary hyperthermia on corneal endothelial cell survival during organ culture preservation. Specifically, exposure for 12 h to 40°C as well as to 42°C induced no endothelial cell loss, whereas exposure to 44°C and 50°C lead to total necrosis of the endothelial cell layer of porcine corneas (187). Schroeter et al. (2015) concluded that temperatures above 42°C, as it might be the case during transports from the cornea bank to the ophthalmic surgeon, must be strictly avoided as they damage the endothelial cell layer (187). Since high expression of TRPV1 (specific temperature threshold ≈ 43 °C) was detected in human corneal endothelial cells, it is suggested that TRPV1-induced Ca^{2+} influx may contribute to such changes of corneal endothelium.

5.4 TRP-channel relevance to ocular function

The studies cited above have identified numerous receptors and mechanisms involved in the control of responses that counter losses in cellular function caused by tissue injury, touch, changes in ambient temperature, pH as well as osmolarity, taste, hormones and pathogen tissue infiltration. Furthermore, these studies have provided important insights, which can lead to the development of new drug targets for improving the treatment of different ocular diseases whose underlying pathophysiological consequences require clarification. In this context, there is increasing interest by drug companies in screening possible endogenous modulators of TRPs targeting certain ocular TRP receptor subtypes because some of them are promising targets for reversing pathophysiological changes that compromise ocular function. For example, in injury-induced cornea scarring, loss of TRP function is associated with a reduction in corneal stromal fibrosis (188). This example shows that evaluating the therapeutic value of drug targeting TRP activity still requires further investigation. Two interesting compounds mentioned in the result section of this habilitation thesis are discussed in the following sections.

5.4.1 Dry eye syndrome – osmoprotection as therapeutic principle

As mentioned in the introduction and generally known in ophthalmology, DES is one of the most regular eye disease compared to other eye diseases (189). Various therapies such as antiinflammatory therapy were established during the last years (143). In this context, it is known that an increased tear film osmolarity is associated with inflammation of the ocular surface in DES (189). As reviewed by Messmer, osmoprotection may therefore be a novel therapeutic option at which osmoprotective agents such as L-carnitine can suppress damage

on the ocular surface by increasing tear film osmolarity (189). In DES, however, ocular surface inflammation and dehydration may be due to dysfunctional TRPV1 and TRPM8 expression and/or conditions in this disease that promote their hyperactivation. With regards to TRPV1, it is activated by hypertonic conditions similar to those identified in some DES suffering patients. In vitro, such levels lead to increases in the expression of the same proinflammatory cytokines, chemokines and matrix metalloproteinases whose levels are also elevated in patient tears. TRPM8 is also implicated since its expression on afferent sensory corneal nerves and in the corneal cell layers contributes to maintenance of basal tear flow during thermal stress (108). In the present studies, changes in intracellular Ca^{2+} regulation and whole-cell currents in HC_jEC cells induced by L-carnitine in the presence and absence of CAP were registered. One crucial finding was the inhibitory effect of L-carnitine on hypertonicity-induced cell volume shrinkage in the presence of functional TRPV1 expression. This suggests that its osmoprotective effect is due to its interaction with TRPV1. Supposedly, TRPV1 inhibition by L-carnitine contributes to activation of L-carnitine transporters during exposure to an osmotic challenge, since this osmolyte reversed CAP- as well as hypertonicity-induced increases in intracellular Ca^{2+}. Overall, one strategy for an alternative treatment of patients with DES is addition of compatible solutes into topical formulations of artificial tears in order to compensate the hypertonic condition in dry eye syndrome. L-carnitine is one such compatible solute, due to its documented osmoregulatory activity.

5.4.2 Dry eye syndrome – novel options to suppress inflammation

Promising receptor targets whose selective modulation reduced inflammation (and also pain) are both, TRPV1 and TRPM8. Besides using the aforementioned osmoprotective agents such as L-carnitine (126;127;145;153), the use of anti-inflammatory agents can be another strategy for DES treatment, although most of the anti-inflammatory drugs do not address the underlying cause of inflammation (143). Recently, it was demonstrated that TRPV1 plays a role in the pathophysiology of inflammation through the EGFR signaling cascade. TRPV1 antagonists elicited suppression of injury-induced stromal TRPV1 activation, which reduced inflammation and fibrosis (142). As in non-corneal cells, TRPM8 activation leads to suppression of TRPV1 stimulation (190) (Fig. 5.2.1). Here, it is suggested that cooling agents may have a therapeutic value in reducing inflammation and fibrosis, since treatment of cells with the TRPM8 agonist icilin led to a suppression of the TRPV1-induced Ca^{2+} influx in retinal tumor cells and HC_jEC (124;164). First pilot studies using thyronamines (TAM) indicated that it may activate TRPM8 like icilin not only in HC_jEC but also in HCEC (163).

Therefore, this hormone may be an interesting alternative to activate TRPM8. Accordingly, there is a need for new TRPM8-specific ligands. Very recent studies indicate that 3-iodothyronamine (T_1AM), an *endogenous thyroid hormone (TH) metabolite* (191), may be such a possible TRPM8-specific ligand since it activated TRPM8 in HC_jEC (164), human hepatocellular carcinoma cells (Hep G2), neuroendocrine tumor cells (BON-1) and uveal melanoma cells (92.1) (unpublished observations by Mergler et al. 2014). Khajavi et al. could further demonstrate that T_1AM induces activation of TRPM8 in HC_jEC. In addition, pre-treatment of these cells with T_1AM suppressed TRPV1-induced Ca^{2+} influx and suppressed interleukin-6 (IL-6) linked inflammatory processes. Overall, this may be a possible option to suppress stress-induced inflammation occurring in dry eye disease.

5.5 Future directions and limitations of the studies

Ocular TRP functional expression is essential for mediating both adaptive and maladaptive responses to a wide variety of different environmental conditions that can influence maintenance of tissue homeostasis. There are still a lot of investigations necessary to elucidate the specific role and function of each thermo-TRP channel. For example, specific sites on TRP channel structures that account for each of the responses mediated by selective channel activity modulation are still not fully elucidated. Furthermore, site-specific mutations should be investigated, if such changes correspond with any of the pathophysiological responses of TRP-expressing cells. Finally, drug-induced reversal of any changes caused by disease must be selective without serious side effects. Judging from the increasing number of publications per year, TRP drug targeting may yet improve the management various diseases in a clinical setting.

5.5.1 Technical limitations

In connection with high-sensitive functional assays, such as fluorescence calcium imaging or patch-clamping, there is always a relatively high data scatter due to a more or less different signal-noise ratio. For example, the lowest T_1AM concentration at which Ca^{2+} responses could be detected was 300 nM. Fluorescence responses with lower concentrations were not detectable, limiting these investigations. On the other hand, novel protocols or novel technical proceedings can help to produce more reproducible and reliable data.

5.5.2 Cell cultures and cornea models

In general, extreme caution should be exercised when trying to extrapolate data obtained with the aforementioned cell lines to the normal situation, especially *in vivo*. Therefore, normal ocular cells were considered as controls. For example, HCE_nC (primary culture) was included in the studies regarding TRPM8 in HCE_nC to validate the data obtained from the HCEC-12 cell line (115). In this context, HCEC-12 is not transformed, but was immortalized by insertion of the Simian Virus 40 Large T antigen. The SV40 Large T antigen is an oncogene that provokes an ongoing proliferative capacity. The cells of this immortalized HCE_n cell line (HCEC-12) exhibit an *in vivo*-like, differentiated morphology (except for its proliferative capacity), which is otherwise usually lost during cell culture of normal HCE_nC (113). Moreover, transplantation of these immortalized human corneal endothelial cells onto recipient corneas led to the establishment of new endothelial layers which had the morphological and functional characteristics of the native ones in organ-cultured corneas (130). Therefore, the HCEC-12 cell line is widely accepted and was successfully used as a model for corneal endothelium in multiple studies dealing with improvement of corneal organ cultivation or corneal endothelial cell transplantation, and was extensively characterized (110;131;192-194). In addition, the HCEC-12 cell line is also described as a valid surrogate cell model of HCE_n as used in the literature (42;130;131). This was also confirmed in electrophysiological investigations (110;129). The same applies to HCEC-SV40 (114) as well as IOBA-NHC (195). As indicated before, it is known that cell lines may express different characteristics especially if they are used in different labs. Therefore, cells in the studies of this habilitation thesis were used at low cell passages and were checked for authenticity (e.g. HCEC-12). Nevertheless, differences between permanent cells and primary cultures cannot be excluded. Independently, preclinical studies concerning transcorneal absorption of ophthalmic drugs are tested generally using *ex vivo* animal corneas and *in vitro* corneal cell culture models. However, this leaves open the question of transferability to humans in an *in vivo* situation (196). At this point, little is known about the expression of transporter proteins and active drug transport in human and animal corneas as well as corneal cell culture models. Recently, Stephan Reichl et al. conducted an expression analysis of certain proteins/drugs in different *in vitro* and *ex vivo* corneal models, leading to a better understanding of the comparability of different corneal models regarding drug absorption and transferability to humans (196-199). Notably, they established two *in vitro* human corneal models: At first, they established a SV40 immortalized human corneal epithelial model (named HCE-T). Secondly,

they established a more organotypic hemicornea construct, which based on HCE-T (Fig. 5.5.2) (196;200).

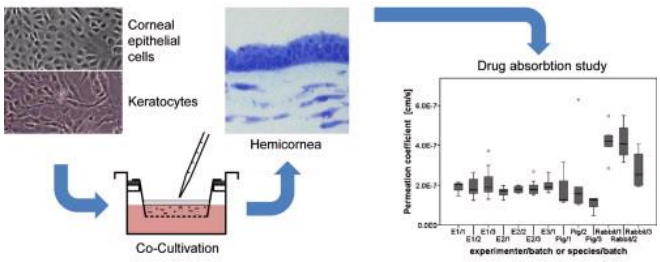
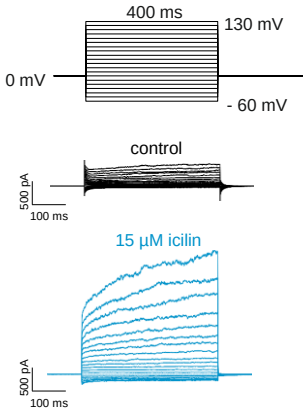


Fig. 5.5.2: **Development of a serum-free human cornea construct for in vitro drug absorption studies:** The influence of varying cultivation parameters on barrier characteristics [from Hahne and Reichl (200)].

In order to consider these cell models for electrophysiological investigations, the author of this habilitation thesis recently started a collaboration together with Monika Valtink (Institute of Anatomy, Faculty of Medicine Carl Gustav Carus, TU Dresden, Germany) and Stephan Reichl (Institute for Pharmaceutical Technology of the Technical University Braunschweig). First pilot electrophysiological data indicated that expression of TRPM8 could be confirmed (163) (Fig 5.5.3). Independently, transfer of data obtained from these models to an *in vivo* situation in humans should be performed with caution as discussed before (196).

Fig. 5.5.3: Icilin-induced outwardly rectifying whole-cell currents in HCE-T: Upper panel: Stimulation protocol. Cells were stimulated by 10 mV voltage steps from -60 mV to 130 mV at a holding potential of 0 mV. Lower panels: Whole-cell currents without icilin (black response pattern) and in the presence of the TRPM8/TRPA1 cooling agent icilin (blue response pattern). (Lucius, Mergler et al. 2014, unpublished data).



5.5.3 TRP channel pharmacology

Table 1 summarizes TRP channel subtype localization and pharmacology in ocular surface tissues and corresponding references.

Table 1: TRP channel pharmacology and ocular surface cell type localization

TRP channel subtype	Compound	Concentration (µM)	Efficacy	Expression localization	References
TRPC4	2-APB	100 µM	antagonist	RCEC	(159;201)
TRPV1	capsaicin	1 – 20 µM	agonist	HCEC, HCK HCE_nC HC_jEC)	(111;144) (160) (162)
TRPV1	capsazepine	1 – 10 µM	antagonist	HCEC HCK HCE_nC HC_jEC	(111;144) (160) (162) (112)
TRPV2	2-APB	200 - 400 µM	agonist	HCE_nC HCEC	(162) (202)
TRPV3	2-APB	200 µM	agonist	HCEC	(202)
TRPV4	4α-PDD,	5 µM	agonist	HCE_nC HCEC, HC_jEC	(132) (95;112)
TRPV4	GSK 1016790A	5 µM	agonist	HCE_nC	(132)
TRPM8	menthol	50 - 500 µM	agonist	HCNF HCENC	(108;109;152;203) (115)
TRPM8	icilin	2 – 60 µM	agonist	$HCK^{3,}$ $HCEC^{3}$ HCENC	(115;129) (112)
TRPM8	eucalyptol	3000 µM	agonist	HCENC	(115)
TRPM8	BCTC	10 µM	antagonist	HCENC	(115)
TRPA1	icilin	2 – 60 µM	agonist	HCENC TM	(115;129) (204)

[1]activation by hypertonic challenge.
[2]activation by hypotonic challenge.
[3]Mergler et al (unpublished data)
HCEC = human corneal epithelial cells
RCEC = rabbit corneal epithelial cells
HC_jEC = human conjunctival epithelial cells
HCK = human corneal keratocytes (stroma)
HCE_nC = human corneal endothelial cells
HCNF = human corneal nerve fibers
TM = Trabecular meshwork

Regarding the above-listed TRP channel modulators, a critical point is the specificity of these drugs for TRP channel identification [review (90)]. For example, CAP also triggers TRPV1-independent effects in non-corneal cells (205). Therefore, it is not just reliable for TRPV1 activity. The same applies to CPZ, which is commonly used as a TRPV1 antagonist. This cautionary note is indicated since CPZ also has effects in $TRPV1^{-/-}$ mice (206). Another drug such as icilin is known as a potent and efficient TRPM8 agonist, but it also activates TRPA1 (138). Even though another TRPM8 agonist, menthol, is more selective than icilin, the latter agent is a more efficacious and potent TRPM8 agonist (96;207). On the other hand, BCTC is an established TRPM8 blocker (102) since its inhibitory effects on intracellular Ca^{2+} transients are similar to those described in other studies, in which TRPM8 gene silencing was examined (170;208). Notably, BCTC was used for TRPM8 inhibition and led to identify TRPM8 involvement in mediating lacrimation *in vivo*, which was based on the inhibitory effects on this response (108). Furthermore, TRPM8 selectivity of BCTC is consistent in a number of studies (150-152). Overall, although some TRP channel modulators were well-established, there is a remaining uncertainty in TRP channel pharmacology. To investigate certain TRPs, silencing them via “small interfering RNA” (siRNA) can therefore be a complement to pharmacological intervention.

6 Summary

The dry eye syndrome is the prevalent eye disease, which regularly occur in eye clinic practice. It is a field of increasing complexity and several new treatment agents are developed and tested in corneal cell culture models as well as in cornea constructs in recent years. At this point, an increased tear film osmolarity plays an important role in connection with (generally subclinical) inflammation of the ocular surface and the tear film in this disease. Therefore, protective drugs or modulation of putative osmosensitive receptors may help to prevent (theoretically) an increased tear film osmolarity from injuring the ocular surface. Other ocular surface diseases, which are more or less insidious, are corneal (e.g. stromal) dystrophies. Corneal transplantations, mainly penetrating keratoplasty or DMEK, are frequently performed to treat extensive corneal dystrophies. In this connection, the human corneal endothelium plays a major role, since its various functions are crucial for corneal performance.

Characteristics and pathophysiological properties of cells in the cornea and conjunctiva are determined by calcium-dependent cellular mechanisms that mediate regulation of intracellular calcium levels. Maintenance of intracellular Ca^{2+} levels at orders of magnitude below those in the extracellular environment is required for cell viability. TRPs contribute to such control of Ca^{2+} through modulating their time dependent opening and closing behavior. Such regulation is required for Ca^{2+} to serve as a second messenger for mediating receptor control of numerous life sustaining responses. In this context, intracellular calcium is significantly regulated by TRPs. As there is an association between aberrant TRP channel expression and human diseases, these regulators of Ca^{2+} influx in connection with any endogenous modulators may be potential drug targets in a clinical setting.

Based on the results of this habilitation thesis, performed experiments and various literature investigations, there are the following insights:

1. Functional expression of TRPV1 and TRPM8 could be demonstrated in human corneal and human conjunctival cells using highly sensitive functional assays, such as fluorescence calcium imaging and planar patch-clamping. Typical characteristics of these TRPs could be confirmed. Specific investigations were carried out to elucidate further characteristics and pharmacological aspects.

2. TRPV1 and TRPM8 trigger down-stream signaling. Specifically, TRP channel activation is connected with GPCRs (GPCRs-TRP channel axis). Two different Ca^{2+} signal transduction pathways, which are activated by thyronamines (T_1AM) could be suggested. First, T_1AM is activating a GPCR, thereby increasing intracellular Ca^{2+} via TRPV1. At this point, (beta)-adrenergic receptors were suggested to be involved, since pilot experiments indicated that a corresponding receptor blocker suppressed Ca^{2+} influx. Second, an interplay between TRPV1 and TRPM8 was suggested, which led to a suppression of Ca^{2+} influx.

3. Based on own data and data from collaborations, several possible roles of TRPs could be suggested. First, TRPV1 triggers release of inflammatory mediators as well as mediators for corneal epithelial cell proliferation. Specifically, TRPV1-induced activation increases intracellular Ca^{2+}, which is linked to an increase in IL-6 release. Second, TRPV1 and TRPV4 are osmosensitive channels in ocular cells. Selective drug modulation of either TRPV1/TRPV4 activity or its signaling mediators may yield a novel approach to suppressing inflammatory responses. Finally, a possible role of TRPV1 and TRPM8 in cornea banking was suggested. Donor cornea storage below room temperature (hypothermic corneal preservation) sustains TRPM8 function. In addition, temperature sensitivity can alternatively be used for channel identification in corneal endothelium, because known TRPM8 modulators have a limited selectivity. Furthermore, it was suggested that TRPV1 is very likely involved when corneal endothelial cells are exposed to temperatures above 43 °C, e.g. during transport of the cornea from the cornea bank to the ophthalmic surgeon, because higher temperatures damaged the endothelial cell layer.

4. Due to the aforementioned acquisition of results, a TRP-channel relevance to ocular function could be suggested: With regard to dry eye syndrome, an inhibitory effect of the osmoprotectant L-carnitine on hypertonicity-induced cell volume shrinkage in the presence of functional TRPV1 expression was demonstrated. This suggests a link between L-carnitine and TRPV1 since hypertonicity also activates TRPV1.

5. Another option to suppress inflammation (and also pain) in dry eye syndrome is based on results with the aforementioned thyronamine (T_1AM). Specifically, T_1AM may be as a possible TRPM8-specific ligand, because it activated TRPM8 in HC_jEC. Moreover, pre-treatment of these cells with T_1AM suppressed a TRPV1-induced Ca^{2+} influx and suppressed IL-6 linked inflammatory processes.

6. Finally, future directions and limitations of these studies were discussed. Concerning TRPM8 characteristics, it was demonstrated that electrophysiological data obtained from the HCEC-12 cell line could also be confirmed using normal HCE_nC (primary culture). Furthermore, first electrophysiological data in connection with a recently initiated collaboration regarding the development of *in vitro* human corneal models (cornea construct) confirmed TRPM8 channel expression for the first time in a SV40 immortalized human corneal epithelial model (HCE-T).

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8 Abbreviations

Abbreviation	Meaning
ATD	aqueous tear deficient
4-αPDD	4α-Phorbol 12,13-didecanoate (TRPV4 agonist)
BCTC	N-(4-tert.butyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide
$[Ca^{2+}]_i$	intracellular calcium concentration
CAP	capsaicin (TRPV1 agonist)
CB1	cannabinoid receptor type 1
CCE	capacitative calcium entry
CPZ	capsazepine (TRPV1/TRPM8 antagonist)
DES	dry eye syndrome
DMEK	Descemet membrane endothelial keratoplasty
EDE	evaporative dry eye
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
f_{340}/f_{380}	fluorescence ratio between 340 and 380 nm
fura-2/AM	fura-2/acetoxymethyl ester
GPCRs	G-protein coupled receptors
GSK 1016790A	glaxosmithkline 1016790A (novel and potent TRPV4 agonist)
HCE	human corneal epithelium
HCE-T	SV40 immortalized human corneal epithelial model
HCEC	human corneal epithelial cells (HCE-T)
HCE_n	human corneal endothelium
HCE_nC	human corneal endothelial cells
HC_jE	human conjunctival epithelium
HC_jEC	human conjunctival epithelial cells
HP	holding potential
hTAAR1	human trace amine-associated receptor 1
I-V plot	current voltage plot
IL-6	interleukin-6
KCS	keratoconjunctivitis sicca
L-type	long-lasting Ca^{2+} channel
MAPK	mitogen-activated protein kinase
MGD	meibomian gland disease
NADA	N-Arachidonoyl dopamine (agonist of CB1 and TRPV1)
ORAI1	calcium release-activated calcium channel protein 1
RTX	resiniferatoxin
RVD	regulatory volume decrease
siRNA	small interfering ribonucleic acid
SOCs	store operated channels
STIM1	stromal interaction molecule 1
T_1AM	3-Iodothyronamine
TAM	thyronamines
thermo-TRPs	temperature-sensitive TRPs
TRPs	transient receptor potential channels
TRPA	ankyrin receptor
TRPC	canonical receptor
TRPM	melastatin receptor
TRPML	mucolipins
TRPN	mechanosensory channel
TRPP	polycystins
TRPV	vanilliod receptor
SV40	simian virus 40
VOCCs	voltage-operated Ca^{2+} channels

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Die vorliegenden Studien wurden an der Klinik für Augenheilkunde am Campus Virchow-Klinikum der Charité Universitätsmedizin Berlin angefertigt. Mein Dank gilt allen, die zum Gelingen dieser Arbeit beigetragen haben.

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Aus CHARITÉ am Puls vom Mai 2014
Bericht von Claudia Peter, Foto Wiebke Peitz
Charité - Universitätsmedizin Berlin

Die Musik der Zellen

Wenn Zellen eine Melodie spielen, dann sind Ionenkanäle die Instrumente. Sie sitzen wie Poren an den Zellmembranen und sind durchlässig für winzige Ströme, die Kommunikationsaufgaben erledigen, also praktisch die „Töne" dieser Melodie sind. Dr. Stefan Mergler von der Klinik für Augenheilkunde am Campus Virchow-Klinikum erforscht sie seit 15 Jahren und ist immer noch fasziniert. „Vor Kurzem konnten wir als erste Arbeitsgruppe weltweit nachweisen, dass eine bestimmte Gruppe von Ionenkanälen in Hornhautzellen vorhanden ist", berichtet er. „Die Hornhaut ist das durchsichtige Fenster des Auges. Fehlfunktionen können bis zur Erblindung führen. Die Erforschung der Ionenkanäle der Hornhaut kann daher ein wichtiger Ansatzpunkt für die Behandlung weit verbreiteter Hornhauterkrankungen sein."

In den Laboren der Forschungsabteilung der Augenheilkunde unter der Leitung von Prof. Dr. Olaf Strauß, in denen Mergler forscht, findet also Grundlagenforschung statt, die beileibe nicht nur für die Augenheilkunde wegweisend ist. Er kooperiert sowohl mit der Gastroenterologie als auch mit der Endokrinologie an der Charité. „Prof. Dr. Josef Köhrle, der Leiter der Experimentellen Endokrinologie, hat vor Kurzem eine neue Gruppe von Schilddrüsenhormonen entdeckt", berichtet Mergler. „Wir haben begonnen, die Wirkung dieser Hormone auf verschiedene Ionenkanäle in Hornhautepithelzellen zu testen. „Und siehe da – es gibt messbare Veränderungen." Darüber hinaus forscht Mergler in einer weiteren Kooperation mit Prof. Dr. Mathias Strowski und Dr. Carsten Grötzinger von der Gastroenterologie auch an Tumorzellen des Auges. „Wir konnten bestimmte Ionenkanäle in Melanomzellen der Aderhaut nachweisen, die in gesunden Aderhautzellen nicht vorhanden sind", erläutert Mergler. „Beide Arbeitsgruppen entwickelten so ein gemeinsames Verständnis auf dem Gebiet der Tumorforschung."

Merglers Werdegang ist etwas untypisch für einen Forscher an der Charité. Nach dem Studium der Physik und Biologie wollte der ambitionierte Marathonläufer und Violinist eigentlich Lehrer werden. Geblieben ist die Lust an der lebensnahen Vermittlung komplexer biologischer Zusammenhänge. Jahraus, jahrein hängt an der Tür seines Schreibraums das Werbeplakat der jeweils letzten Langen Nacht der Wissenschaften. „Da geht es bei mir zu wie im Taubenschlag. Es macht großen Spaß, den Menschen zu erklären, wofür ihre Steuergelder ausgegeben werden."

Merglers zentrale Tätigkeit ist die „Patch-Clamp-Technik": Er misst winzig kleine Ströme und studiert damit das Verhalten von Zellen. Durch elektrische Signale oder andere Prozesse im Zellinneren werden sie zu unterschiedlichen „Tätigkeiten" angeregt. Manche sorgen dafür, dass wir physikalische Außenreize wie Licht, Schall und Berührung wahrnehmen können. Andere steuern die Muskeln oder den Stofftransport zwischen den Organen.

Ionenkanalkrankheiten (Channelopathies) sind vor etwa 20 Jahren erstmals beschrieben worden. Es sind Erkrankungen der Ionenkanäle oder der Proteine, die diese regulieren. In kranken Zellen können Ionenkanäle unerwünschte Prozesse ausführen. „Sie lassen sich durch Zugabe von Wirkstoffen herunterregulieren oder gar stoppen", sagt Mergler. „Wenn wir die Klaviatur dieser Reaktionen erst einmal beherrschen, dann kann das ein Schlüssel für die Linderung oder Heilung vieler Krankheiten sein."

Bei der Langen Nacht der Wissenschaften am 10. Mai führt Dr. Stefan Mergler am Campus Virchow-Klinikum jeweils um 19:00, 21:00 und 23:00 Uhr 30 Minuten lang seine Zellforschung vor.

Der Biophysiker Dr. Stefan Mergler verwendet das kleinste Patch-Clamp-Setup der Welt, das sich im Forschungslabor der Klinik für Augenheilkunde am CVK befindet

10 Erklärung

Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,
- mir die geltende Habilitationsordnung bekannt ist.

……. ……..

Datum Unterschrift

Erklärung

Hiermit erkläre ich, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und die Habilitationsschrift unter Beachtung der dort enthaltenen Grundsätze erstellt wurde.

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