Physiological characterization of chemosensory mechanisms in mice

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1. Introduction

In animals, environmental chemical cues play a crucial role not only for the survival of an individual, but for the whole species. Chemodetection is essential for finding food (including quality control), predator recognition (triggering escape behavior), mate choice and, accordingly, reproduction. Chemical senses comprise the sense of smell (olfactory system) and the sense of taste (gustatory system). Together, these are responsible for the detection of a vast range of molecular signals. The gustatory system mainly detects water-soluble, non-volatile molecules that each elicit either of five distinct perceptual qualities: sweet, bitter, sour, salty and umami (Zhang et al., 2003; Mombaerts, 2004a; Chandrashekar et al., 2006; Yarmolinsky et al., 2009). In contrast, the olfactory system detects an enormous structural complexity of mainly volatile substances. Intraspecific chemical communication is mediated by pheromones, providing information about social hierarchy, sex and age as well as the health and endocrine state of an individual. Moreover, pheromones can trigger stereotyped behaviors and alter endocrine state, e.g. by accelerating puberty or synchronizing estrus in females (Novotny et al., 1999; Brennan & Zufall, 2006).

Several anatomically distinct olfactory subsystems evolved to accomplish these diverse olfactory tasks in rodents: the main olfactory epithelium (MOE), the vomeronasal organ (VNO), the Grueneberg ganglion (GG) and the septal organ of Masera (SO) (Tian & Ma, 2004; Breer et al., 2006; Ma, 2007; Brechbühl et al., 2008) (Fig.1.1). Using these dedicated tissues, the olfactory subsystems detect in part overlapping sets of chemosignals (Spehr et al., 2006b).

Fig.1.1: Schematic viewing of the mouse olfactory system. Location of the distinct olfactory subsystems composed of the main olfactory epithelium (MOE), vomeronasal organ (VNO), Grueneberg ganglion (GG) and septal organ (SO) in the periphery, and the main olfactory bulb (MOB) and accessory olfactory bulb (AOB) as a part of the central nervous system. Modified from Spehr et al., 2006.

1.1 The main olfactory system

The main olfactory system consists of the main olfactory epithelium (MOE) in the periphery and the main olfactory bulb (MOB), where initial central processing takes place (Fig 1.2). The MOE is located in the posterior-dorsal nasal cavity, where it lines both several endoturbinates and the nasal septum. Bipolar canonical olfactory sensory neurons (OSNs) reside in the MOE, extending their cilia into the olfactory mucus. For odor detection, members of the odorant


receptor (OR) superfamily are expressed in the ciliary membranes (Buck & Axel, 1991). OSNs project an unmyelinated axons through the basal lamina and cribriform plate to the MOB. Neurons expressing the same OR send their axons into one or a few spherical structures, called glomeruli, in each hemisphere of the MOB (Vassar et al., 1994; Ressler et al., 1994; Mombaerts et al., 1996) (Fig. 1.2). In those structures, OSN axon terminals synapse with MOB projection neurons, mitral cells, which project to higher brain regions such as the piriform cortex (Stettler & Axel, 2009). The axonal expression of ORs is required for convergence of axons and to target a specific glomerulus forming a topographic map in the MOB (Wang et al., 1998).

![Fig 1.2: Schematic diagram of organization and location of the MOE components in the rodent nose.](image)

The OSNs are situated in the MOE, from where they project their axons through the cribriform plate into glomerular structures in the MOB. In those neuropil structures, OSN axons form synapses with the OB projection neurons, i.e. mitral cells, which, in turn, target higher brain regions. CP, cribriform plate; GL, glomerulus; MC, mitral cell; MOB, main olfactory bulb; MOE, main olfactory epithelium; OSN, olfactory sensory neuron. Modified from Spehr et al., 2006.

## 1.1.1 Anatomical structure and cells of the main olfactory epithelium

The MOE mainly comprises three different cell types: OSNs, responsible for odor detection, sustentacular cells, also called supporting cells (SCs), ensure metabolic support for OSNs and tissue stability, and basal cells (BCs), serving as progenitor cells for newborn OSNs (Fig. 1.3). Additionally, microvillous cells have been described as MOE cell type with yet unclear functional properties. These microvillous cells express IP$_3$ receptors as well as TRPM5 channels (Lin et al., 2008; Pfister et al., 2012). Whether or not they possess an axonal process or if there are different subpopulations of microvillous cells is controversially discussed (Carr et al., 1991; Elsaesser et al., 2005).

By contrast, the role of basal cells in the olfactory epithelium is less enigmatic. Because the nasal cavity is directly exposed to environmental and mechanical stress factors the MOE requires regular regeneration. Therefore, olfactory cells are replaced throughout life by progenitor BCs (Graziadei & Graziadei, 1979). The group of BCs comprises two main populations, the globose and the horizontal basal cells (GBCs and HBCs, respectively) (Schwob, 2002). While GBCs constitute the pool of direct OSN progenitors with a high proliferation rate (Caggiano et al., 1994), HBCs comprise a more conserved pluripotent progenitor type, where proliferation is induced by lesion of the MOE (Carter et al., 2004).
1.1.1.1 Olfactory sensory neurons (OSNs)

OSNs are bipolar neurons with one apical dendrite that terminates in a small globular bulge at the top, the dendritic knob. Olfactory cilia emanate from the dendritic knob containing signaling molecules for odorant detection in their membrane. Olfactory cilia are distinct cellular compartments segregated by the basal body that serves as a gate for ciliary entry (Ying et al., 2014). Though OSN cilia have the (9+2) microtubule configuration, normally found in motile cilia, olfactory cilia are nonmotile (Menco, 1984; Jenkins et al., 2009). At the basal OSN soma, a thin unmyelinated axon projects directly to the OB. Targeted axonal wiring to a specific glomerulus requires the expression of a given OR as well as axon guidance molecules expressed in a graded manner along the dorsomedial-ventrolateral axis in the olfactory epithelium, such as Robo-2, Neuropilin-2 and adhesive molecules Kirrel2/Kirrel3 (Norlin et al., 2001; Feinstein et al., 2004; Serizawa et al., 2006; Cho et al., 2007; Imai & Sakano, 2007).

Since, the olfactory system has to detect an enormous structural complexity of mainly volatile substances an immense repertoire of receptors is required. A multigene family of ~1100 functional olfactory receptors (ORs) is expressed in olfactory sensory neurons (OSNs) of mice in a mutually exclusive manner (Buck & Axel, 1991; Zhang et al., 2004). This founded the ‘one neuron - one receptor’ hypothesis (Serizawa et al., 2004), which, however, is still far from being proven (Mombaerts, 2004b; Fuss et al., 2013). The spatial distribution of OSNs expressing the same OR is not arbitrary. OSNs can be classified into two groups in mammals distinguished by the OR expression of either class I or class II OR genes (Niimura & Nei, 2007). Phylogenetically ancient class I OR genes are solely expressed in the most dorsal regions of the MOE (Zhang et al., 2004), whereas more recent class II ORs are expressed throughout the dorsal and ventral MOE. OSNs expressing class I or II ORs project their axons to glomeruli in distinct determined spatial regions of the OB (Bozza et al., 2009).

Chemoreceptors expressed by sensory neurons in the MOE

In the MOE, three main types of chemosensory receptors have been identified. Receptors, expressed in ciliary OSNs include two groups of G protein–coupled receptors (GPCRs), the ORs and the TAARs, as well as the receptor guanylyl cyclase isoform D (GC-D) (Spehr & Munger, 2009). Three main GPCR families can be classified by their amino-acid sequences:
the rhodopsin receptor-like family (A), the secretin receptor-like receptor family (B) and the metabotropic glutamate receptor family (C) (Bockaert & Pin, 1999; Joost & Methner, 2002). All these receptors have in common a central core domain constituted of seven transmembrane helices (TM I-VII) connected by three intracellular and three extracellular loops (Baldwin, 1993). A change in conformation of this seven transmembrane (7TM) domain is responsible for receptor activation. ORs and the TAARs belong to the family A of GPCRs. They possess a relatively short extracellular N (amino)-terminus and an intracellular C (carboxyl)-terminus. TM III-V show regions of high variability, thus providing a binding pocket for ligands (Zhang & Firestein, 2002; Mombaerts, 2004a).

**Canonical odorant receptors (ORs)**

In OSNs, one of ~1100 ORs is expressed in a mutually exclusive monoallelic manner (Buck & Axel, 1991; Magklara et al., 2011). OR monoallelic expression is critical to the establishment of the functionally distinct neuronal populations required for odor discrimination and axonal projection to the OB. Following the ‘one neuron- one receptor’ rule, this expression pattern is regulated by a negative feedback mechanism controlled by the OR gene product (Serizawa et al., 2003). In the absence of expression of a specific functional OR protein, promoter activation is insufficient to restrict / ‘shut-down’ expression from additional OR loci in a given neuron. Insertion of an OR coding region restores monoallelic expression. Thus, monoallelic OR expression is achieved through a feedback mechanism in which the OR protein itself attends to abolish expression of other OR genes (Lewcock & Reed, 2004).

OR genes can be divided into two phylogenetic classes in mammals: class I and class II OR genes (Niimura & Nei, 2007). More ancient class I OR genes, also referred to as fish-like receptor genes, compose up to 10 % of intact OR genes in mice (Zhang et al., 2004). In contrast, class II ORs comprise the much larger group of ORs in the MOE. It is currently not known if there are systematic functional differences between class I and II ORs. OSNs expressing class I or II ORs project their axons to glomeruli in distinct determined spatial regions of the OB (Bozza et al., 2009).

ORs are able to detect a broad but selective ligand spectrum mostly via hydrophobic and van der Waals interactions (Katada et al., 2005; Oka et al., 2006). Olfactory signaling via ORs can be even able of inhibiting OSNs upon ligand binding (Ukhanov et al., 2010).

**Trace-amine-associated-receptors (TAARs)**

Recently a second class of chemosensory receptors was identified in the MOE in mice: the trace-amine-associated-receptors (TAARs) (Liberles & Buck, 2006). It seems that the expression of TAARs and ORs is mutually exclusive, because coexpression in OSNs has not been identified (Liberles & Buck, 2006). TAARs are family A GPCRs that are phylogenetically related to serotonin and dopamine receptors (Borowsky et al., 2001). In the mouse, there are
15 different TAAR genes, 14 of which are expressed in the MOE (Liberles & Buck, 2006). TAARs selectively respond to volatile amines and some TAARs even detect odorants that could serve as social cues and/or predator-derived signals (Ferrero et al., 2011, 2012). Thus, components such as 2-Phenylethylamine produced in urine of many carnivores, i.e. predators, may serve as warning substances for mice (Ferrero et al., 2011). When all olfactory TAARs are deleted, coding of such aversive odorants is non-redundant (Dewan et al., 2013). The majority of TAARs are mapped to a cluster of glomeruli located between the previously characterized DI and DII domains for class I or class II OR expressing OSNs, respectively, in the dorsal olfactory bulb (Pacifico et al., 2012). The TAAR projection is associated with a distinct subset of OSNs that is biased to choose TAARs over other ORs, when a given TAAR is deleted (Pacifico et al., 2012).

Receptor guanylyl cyclase D
An additional family of receptors expressed in a small subset of OSNs has been identified: the receptor guanylyl cyclase D (Fülle et al., 1995). In contrast to ORs and TAARs, GC-D does not belong to the 7TM GPCR family. These proteins comprise one extracellular receptor domain, a single transmembrane segment, an intracellular regulatory domain, and one C-terminal cyclase intracellular catalytic domain (Gibson & Garbers, 2000). While the intracellular domains are more conserved, their extracellular domains are less similar, reflecting different ligand-binding specificities. In GC-D specific OSNs, several proteins that could participate in a cGMP-mediated signal transduction cascade are expressed. Activation of the intracellular cyclase domain results in the elevation of intracellular cGMP (Leinders-Zufall et al., 2007), subsequently regulating the cGMP-stimulated phosphodiesterase 2 (PDE2) and the cGMP-gated channel subunit A3 (CNGA3) (Juilfs et al., 1997; Meyer et al., 2000). Moreover, GC-D neurons lack many of the proteins required for cAMP-mediated odor transduction by canonical OSNs (Juilfs et al., 1997; Meyer et al., 2000). Consistent with a function as a chemosensory receptor, GC-D is expressed most predominantly in the cilia of olfactory neurons (Juilfs et al., 1997; Duda et al., 2004).

In GC-D neurons, uroguanylin serves as a ligand that promotes the acquisition of food preferences (Leinders-Zufall et al., 2007; Arakawa et al., 2013) and the detection of carbon disulfide mediates food-related social learning (Munger et al., 2010). GC-D expressing OSNs are also involved in detection of near-atmospheric concentrations of CO₂ (Hu et al., 2007). Additionally, GC-D-expressing neurons have been demonstrated to target necklace glomeruli in the OB (Leinders-Zufall et al., 2007; Cockerham et al., 2009).

1.1.1.2 Sustentacular cells (SCs)
Sustentacular cells (SCs), also referred to as supporting cells, comprise another major population of MOE-resident cells. SCs usually have an oval shaped cell body with a length up
to 20 µm and width of 10 µm. Topologically, SCs are situated along the dendritic apical surface of the OE, where their microvilli reach into the olfactory mucus (Breipohl et al., 1974; Getchell, 1977; Vogalis et al., 2005a). Most of them possess a thick foot-like process towards the basal region of the OE. SCs are able to secrete mucus, mediated by secretary granules found in the upper part of the cytoplasm (Okano & Takagi, 1974; Getchell et al., 1987). They seem to be responsible for the metabolism of noxious chemicals (Hegg et al., 2003) and can even function as phagocytes for dead cells, including OSNs (Suzuki et al., 1996). SCs are involved in the regulation of extracellular ionic gradients, i.e. by buffering K⁺. In the frog olfactory epithelium, for example, SCs express big conductance Ca²⁺-activated K⁺ (BK) channels (Trotier, 1998), which are activated by increases in the intracellular Ca²⁺ concentration([Ca²⁺]). It was also recently shown that nucleotides such as ATP and adenosin act on both SCs and on OSNs via purinergic receptors (Hegg et al., 2003; Hegg & Lucero, 2006). ATP is released by olfactory neurons and evokes a cytosolic calcium increase in sustentacular cells. Consequently, the phosphorylation level of the transcription factor CREB is significantly increased, suggesting a mechanism of intercellular communication via ATP (Czesnik et al., 2006; Dooley et al., 2011).

It was also shown that SCs are electrically coupled by gap junctions (Vogalis et al., 2005b). Furthermore, SCs generate oscillatory Ca²⁺ transients in response to purinergic signaling. A G-protein-dependent phospholipase C (PLC) pathway contributes to this purine-evoked increases in intracellular Ca²⁺ via release from intracellular stores, i.e the endoplasmatic reticulum (ER) (Hegg et al., 2009).

SCs are also capable of generating voltage-gated Na⁺ and K⁺ currents that could support action potential firing, but the Na⁺ current was largely inactivated at the SC resting potential in slices due to the presence of an outwardly rectifying resting ‘leak’ conductance (Vogalis et al., 2005a).
1.1.2 Physiological properties of OSNs

1.1.2.1 Signaltransduction in OSNs

Olfactory ligand detection induces a signal transduction cascade that converts the chemical energy of OR stimuli binding into electrical signals. One OR can recognize multiple odorants and a single odorant can be recognized by several receptors (Krautwurst et al., 1998; Malnic et al., 1999; Spehr & Leinders-Zufall, 2005). Today the individual mechanisms of olfactory perception in the MOE of mammals are generally well understood. Upon binding of an odorant to the OR binding site, a change in conformation leads to the activation of a membrane-associated olfactory-specific guanosine-5'-triphosphate (GTP)-binding protein, the Go∗ protein (Jones & Reed, 1989; Belluscio et al., 1998). Subsequently, this results in the dissociation of the trimeric Go∗ protein into an active GTP-bound Go∗ subunit and the Gβγ-complex (Jones & Reed, 1989; Belluscio et al., 1998). Go∗, in turn, stimulates the type III adenylyl cyclase (ACIII), which is highly expressed in olfactory cilia, to transform adenosine triphosphate (ATP) to cyclic adenosine 3',5'-monophosphate (cAMP) (Bakalyar & Reed, 1990; Wong et al., 2000). The ciliary increase of the second messenger cAMP (Takeuchi & Kurahashi, 2003), causes the activation of a cyclic nucleotide-gated (CNG) channel (Dhallan et al., 1990; Liman & Buck, 1994; Pifferi et al., 2006). The cAMP molecules bind to the intracellular CNG channel subunit A2 (CNGA2) (Lin et al., 2004). This leads to the CNG channel opening enabling an influx of cations, Na+ and Ca2+ in particular (Leinders-Zufall et al., 1997; Kleene, 2008). Ca2+ ions entering through the CNG channel activate Ca2+-activated Cl−-channels (CaCCs) which conduct a Cl− outward current. Anoctamin 2 (ANO2) was only recently identified as the ciliary CaCC in OSNs (Stephan et al., 2009; Rasche et al., 2010; Pifferi et al., 2011). Heterologously expressed ANO2 was shown to exhibit close similarity to channel properties of the native olfactory CaCC (Stephan et al., 2009). Thus, indicating that ANO2, a member of the anoctamin protein family is involved in the olfactory signal transduction cascade. It was shown that endogenous ANO2 is indeed specifically expressed in the ciliary layer of mature olfactory neurons (Rasche et al., 2010). In most central neurons, Cl− channels mediate inhibitory responses based on the electrochemical gradient for Cl− ions, normally leading to a Cl− entry into the cell through open channels. In OSNs though, a relatively high intracellular Cl− concentration is maintained due to the action of membrane pumps, promoting an excitatory Cl− outward current that amplifies the odor response (Kleene, 1993; Kaneko et al., 2004; Reisert et al., 2005; Yang & Delay, 2010; Hengl et al., 2010) (Fig. 1.5). Subsequently, the influx of cations and efflux of anions leads to a net positive charge on the membrane resulting in a depolarizing receptor potential (Firestein, 2001). However, the impact of Cl− efflux on olfactory signaling is still controversial as a recent study reported that Ca2+-activated Cl− currents mediated by ANO2 channels are dispensable for olfaction (Billig et al., 2011).
1.1.2.2 Adaptation mechanisms

OSNs respond to an adequate stimulus with a transient depolarization of the resting membrane potential that, at sufficient excitation, induces action potential (AP) firing. Upon sustained stimulus exposure of constant intensity, OSNs show a characteristic reduction of electrical signals, designated as adaptation (Reisert & Matthews, 1999). Generally, the term ‘sensory adaptation’ is often described as a temporary decrease of a stimulus-induced neuronal response as a result of prolonged or repetitive stimulation (Torre et al., 1995; Reisert & Matthews, 1999). Adaptation is primarily mediated by a Ca\(^{2+}\)–calmodulin (CaM) complex-dependent inhibition of the CNG channel (Kurahashi & Menini, 1997; Reisert & Matthews, 1999). The olfactory CNG channel is a heterotetrameric protein that comprises three different types of subunits: two CNGA2 subunits, one CNGA4 and one CNGB1\(\beta\) subunit (Bönigk et al., 1999; Zheng & Zagotta, 2004; Pifferi et al., 2006). The CNG subunits A4 and B1\(\beta\) (a B1 splice variant) play the most important modulatory role. While the CNGA4 subunit shows increased sensitivity for cAMP (Bradley et al., 1994; Liman & Buck, 1994), thus promoting the excitatory response, both subunits A4 and B1\(\beta\) contain binding sites for the Ca\(^{2+}\)-CaM complex (Munger et al., 2001; Song et al., 2008). Thus, the A4 and B1\(\beta\) subunits regulate the inhibitory negative feedback mechanism exerted by Ca\(^{2+}\) ions (Liu et al., 1994; Kaneko et al., 2006). Surprisingly, a recent study showed that Ca\(^{2+}\)-free apocalmodulin is constitutively bound to the olfactory CNG channel even at resting (low) Ca\(^{2+}\) levels (Bradley et al., 2004). As the resting [Ca\(^{2+}\)] in olfactory cilia is reported to be ~40 nM (Leinders-Zufall et al., 1998), Ca\(^{2+}\)-CaM mediated inhibition of the olfactory CNG channel should only occur during odor responses because
inhibition requires an [Ca\textsuperscript{2+}] of at least 100 nM (Bradley et al., 2004, 2005). During an odor response intracellular [Ca\textsuperscript{2+}] can be triggered up to micromolar levels (Bradley et al., 2005). However, the Ca\textsuperscript{2+}–CaM modulation of CNG channel activity is not the only Ca\textsuperscript{2+}–CaM-dependent adaptation mechanism. Ca\textsuperscript{2+}–CaM also reduces cAMP levels by activating a phosphodiesterase (PDE1C2) (Borisy et al., 1992) and by inhibiting the adenylyl cyclase through Ca\textsuperscript{2+}–CaM-dependent protein kinase II (CaMKII)-mediated phosphorylation (Leinders-Zufall et al., 1999; Bradley et al., 2005). Therefore, the odor-induced intracellular rise in [Ca\textsuperscript{2+}] through the CNG channel in OSN cilia is responsible for both the excitation and adaptation of the neuron (Menini, 1999; Matthews & Reisert, 2003).

1.1.2.3 The physiological role of Ca\textsuperscript{2+}
In neuronal cells, Ca\textsuperscript{2+} ions are of great importance as second messenger elements, mediating various intracellular events. For this reason, cells maintain a low level of cytoplasmic Ca\textsuperscript{2+}, thus establishing a steep Ca\textsuperscript{2+} concentration gradient between the inside and the outside. Resting [Ca\textsuperscript{2+}] is maintained at a concentration of 100 - 200 nM, approximately 20,000-fold lower than in the extracellular space (1-2 mM) (Pietrobon et al., 1990; Carafoli, 1994). Therefore, cells must extrude, compartmentalize or chelate Ca\textsuperscript{2+} ions. Two principle mechanisms are responsible for Ca\textsuperscript{2+} entry into the cells. The first mechanism of Ca\textsuperscript{2+} influx depends on voltage-gated calcium channels (VGCCs), which are activated upon membrane depolarization (Restrepo et al., 1993). Especially during spike generation, low voltage-activated Ca\textsuperscript{2+} T-type currents (I\textsubscript{CaT}) as well as high voltage-activated Ca\textsuperscript{2+} L-type currents (I\textsubscript{CaL}) contribute to AP discharge in OSNs (Kawai et al., 1996). These channels are highly selective for Ca\textsuperscript{2+} and, at resting membrane potential, VGCCs conduct ~10\textsuperscript{6} Ca\textsuperscript{2+} ions per second (Clapham, 2007). A second way of Ca\textsuperscript{2+} entry is comprised by ligand-gated ion channels, such as the CNG channel. CNG channel opening in OSNs results in influx of Na\textsuperscript{+} and Ca\textsuperscript{2+}, therefore providing less specificity and a minor conductance of Ca\textsuperscript{2+} than VGCCs (Leinders-Zufall et al., 1998; Dzeja et al., 1999; Boccaccio & Menini, 2007). Two mechanisms mainly provide a low resting [Ca\textsuperscript{2+}] by extrusion of elevated cytosolic Ca\textsuperscript{2+}. The first candidate is the plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA) (Carafoli, 1992; Saidu et al., 2009). PMCA\textsubscript{s} are shown to have a high affinity and low capacity for Ca\textsuperscript{2+} ions (Carafoli, 1991; Weeraratne et al., 2006). PMCA\textsubscript{s} also contribute to olfactory response termination by calmodulin binding to the pump in the presence of elevated Ca\textsuperscript{2+}, thus providing a regulatory feedback mechanism for Ca\textsuperscript{2+} extrusion (Vorherr et al., 1990; Antolin et al., 2010). Second, Ca\textsuperscript{2+} removal from cytosolic compartments is accomplished by Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (NCX). These antiporters eject one Ca\textsuperscript{2+} ion for the entry of three Na\textsuperscript{+} ions comprising a low-affinity and high-capacity extrusion mechanism (Reisert & Matthews, 2001a; Clapham, 2007). NCX is thought to be responsible for rapid extrusion especially during AP generation in neurons (Hilgemann et al., 2006). As mentioned above (1.1.2.2), cytosolic Ca\textsuperscript{2+} is not only removed
from this compartment, but can also be chelated by associating to various proteins in the cytosol just as CaM or CaMKII (Matthews & Reisert, 2003; Dougherty et al., 2005). Ca\textsuperscript{2+}–CaM, for example, reduces the sensitivity of CNG channels by shifting the EC\textsubscript{50} for cAMP activation from 7 to 25 µM, as cytosolic Ca\textsuperscript{2+} concentrations exceed 1 mM (Bradley et al., 2004, 2005). Additionally, Ca\textsuperscript{2+} is removed from the cytosol by sequestration in internal storage organelles such as the endoplasmic reticulum (ER) and mitochondria (Michelangeli et al., 2005). Constant Ca\textsuperscript{2+} leakage from the ER is counteracted by active Ca\textsuperscript{2+} uptake via the smooth sarco-/endoplasmic reticular Ca\textsuperscript{2+}ATPases (SERCA) within the ER membrane (Pozzan et al., 1994; Zufall et al., 2000). A second important class of Ca\textsuperscript{2+} storage organelles are mitochondria (Rizzuto et al., 1994). Mitochondrial Ca\textsuperscript{2+} uptake occurs even at submicromolar resting cytosolic [Ca\textsuperscript{2+}], but reaches maximal rates at micromolar concentrations (Rizzuto & Pozzan, 2006; Szabadkai et al., 2006).

1.2 The accessory olfactory system

The VNO, the peripheral sensory structure of the accessory olfactory system (AOS), was first described by Ludvig Jacobson in 1813 as a nasal organ found in a variety of mammals (Jakobson, 1813; Doving & Trotier, 1998). Jacobson intensively studied the organ’s anatomy, but its physiological function remained elusive. Today, we know that some primates, including old world monkey and humans, lack a functional VNO (Bhatnagar & Meisami, 1998; Zhang & Webb, 2003) and that the main physiological role of the VNO is pheromone detection (Zufall & Leinders-Zufall, 2007). Pheromones are water-soluble molecular signals, defined in 1959 by Karlson and Lüscher as ‘substances secreted to the outside of an individual and received by a second individual of the same species in which they release a specific reaction, for example, a definite behavior or developmental process’ (Karlson & Lüscher, 1959).

1.2.1 Anatomy of the AOS

The AOS consists of the VNO in the periphery and the accessory olfactory bulb (AOB) in the central nervous system. The VNO is composed of two cylindrical tubes located above the palate in the anterior nose. In mice, it is connected to the nasal cavity via a duct filled with aqueous fluid. During conspecific social investigation, stimuli are pumped through this duct into the VNO lumen, which requires a vascular pumping mechanism, probably driven by a large lateral blood vessel (Fig. 1.6) (Halpern, 1987). Luminal stimuli then reach vomeronasal sensory neurons (VSNs), which reside in a crescent-shaped medial sensory epithelium (Liberles, 2014). VSNs are bipolar neurons that extend a single apical dendrite that terminates in a dendritic knob with several microvilli. The microvillar membrane contains the vomeronasal receptors, responsible for odor detection. VRs are monoallelically expressed in subsets of
sensory neurons of the VNO sensory epithelium (Rodriguez et al., 2002). Apical and basal VSNs are divided into two different types depending on the receptor type and signaling molecules they express. Vomeronasal receptor 1 (V1R)-expressing neurons are located in the apical layer, whereas vomeronasal receptor 2 (V2R)-expressing VSNs are found in the basal part of the sensory epithelium. The VSN axons are bundled in an ascending vomeronasal nerve that projects to the AOB. In the AOB, these axons form synaptic connections with second-order mitral cells in spatially defined spherical structures termed glomeruli (Dulac & Torello, 2003). Contrary to the MOS, individual glomeruli can receive input from more than one type of VSN (Belluscio et al., 1999; Rodriguez et al., 1999; Wagner et al., 2006). However, projections from apical and basal VSNs are segregated in distinct AOB domains (Jia & Halpern, 1996; Del Punta et al., 2002). While VSNs expressing V1Rs project to the rostral part of the AOB, V2R-expressing neurons send their axons to the caudal AOB (Fig. 1.6). This organization generates a “labeled line” of information processing (Luo & Katz, 2004). AOB mitral cells directly target third-order neurons in higher brain regions, including the medial amygdala (MeA), the bed nucleus of the striaterminalis (BNST) and the nucleus of the accessory olfactory tract (NAOT) (Dulac & Wagner, 2006). Neurons from those regions further project to the medial preoptic area (MPOA) and the ventromedial hypothalamus (VMH) (Petrovich et al., 2001).

![Fig. 1.6: The accessory olfactory system. Chemical cues are detected by the vomeronasal organ (VNO). It is located at the anterior base of the nasal septum and consists of a pair of bilateral tubes. Each tube comprises a lateral blood vessel (BV) and a medial sensory epithelium, which contains 2 types of sensory neurons: apical vomeronasal sensory neurons (VSNs) (red) and basal VSNs (green) differing by their receptor expression. Their axons project to the accessory olfactory bulb (AOB). Apical neurons project to the rostral part (red), whereas basal VSNs innervate the caudal part (green) of the AOB. Modified from Spehr et al., 2006.]

1.2.2 Chemoreceptors in the VNO
In the VNO, sensory neurons detect stimuli via different receptor types. There are three main types of chemoreceptors: V1Rs, V2Rs and formyl peptide receptors (FPRs). All of these receptors belong to the GPCR family (Chamero et al., 2012). Moreover, a few ORs were also shown to be expressed in the VNO (Lévai et al., 2006).

**Vomeronasal receptor type 1 (V1R)**
V1Rs comprise a large GPCR family of 187 potentially functional members in mice (Dulac & Axel, 1995; Chamero et al., 2012). Each V1R is monoallelically expressed in a subset of apical
sensory neurons of the VNO sensory epithelium (Rodriguez et al., 2002). They are coexpressed with the G-protein alpha subunit Gαi2 (Berghard & Buck, 1996; Jia & Halpern, 1996). V1Rs belong to the rhodopsin-like family A of GPCRs with a relatively short extracellular N-terminus. The members of the V1R family genes can be divided into 12 subfamilies, V1Ra – V1Rl, each comprising multiple members except for families k and l, with only a single member (Dulac & Axel, 1995; Rodriguez et al., 2002). Furthermore, they are closely related to type 2 taste receptors (T2Rs) (Rodriguez et al., 2002).

V1R-expressing VSNs show ultrasensitive threshold detection near ~10 pM for some small urinary volatile molecules that have been attributed putative pheromonal function (Leinders-Zufall et al., 2000). Among those molecules was 2-heptanone, which was later shown to activate V1Rb2-expressing neurons, constituting the first identified receptor-ligand pair in the VNO (Boschat et al., 2002).

Vomeronasal receptor type 2 (V2R)

V2Rs are members of the metabotropic glutamate family C of GPCRs, which possess a large and highly variable extracellular N-terminus (Ryba & Tirindelli, 1997; Herrada & Dulac, 1997; Matsunami & Buck, 1997; Yoshinaga et al., 2013). V2R-expressing VSNs are located in the basal region of the sensory epithelium and coexpress the G-protein alpha subunit Gαo (Berghard & Buck, 1996). The V2R family comprises ~140 members that can be divided into 4 subclasses, i.e. V2RA - V2RD (Ryba & Tirindelli, 1997; Chamero et al., 2012). Many V2R pheromone receptors are coexpressed with the non-classical M10 and M1 families of the MHC class I multigene family. Referring to the VSN-specific expression of these genes they were termed H2-Mv genes (Ishii et al., 2003; Loconto et al., 2003). H2-Mv-expressing VSNs were only recently shown to contribute to pheromone detection, mediating aggressive and sexual behavior (Leinders-Zufall et al., 2014). Coexpression of V2Rs was also shown for a specific member of the V2RC subfamily, the putative coreceptor V2R2 (Martini et al., 2001).

Members of the V2R family primarily detect large, nonvolatile molecules. To access the VNO, these macromolecular stimuli require direct contact between the nose and stimulus source. Such chemosignals, shown to mediate social interaction between individuals, are e.g. MHC peptides (Leinders-Zufall et al., 2004), exocrine gland secreting (ESP) peptides (Kimoto et al., 2005) as well as major urinary proteins (MUPs) (Hurst et al., 2001).

Formyl peptide receptors (FPRs)

FPRs belong to the large family A of GPCRs. Depending on the individual receptor type, FPR neurons coexpress the G-protein subunit Gαi or Gαo (Liberles et al., 2009). FPRs normally play an important role in the innate immune system by recognizing formylated peptides secreted by bacteria or mitochondria at sites of infection or tissue damage (Migeotte et al., 2006; Ye et al., 2009). The FPR family consists of seven functional members in mice, forming a gene cluster
on chromosome 17, named FPR1, FPR-rs1, FPR-rs2, FPR-rs3, FPR-rs4, FPR-rs6, and FPR-rs7 (Fpr-rs5 is a pseudogene) (Rivière et al., 2009). While 2 of the 7 mouse Fpr genes, Fpr1 and Fpr-rs2 are expressed by neutrophils or immune cells and play a role during immune responses (Migeotte et al., 2006), the presence of Fpr-rs1, Fpr-rs3, Fpr-rs4, Fpr-rs6 and Fpr-rs7 transcripts was exclusively observed in vomeronasal tissue (Rivière et al., 2009; Liberles et al., 2009). Vomeronasal FPRs are expressed in subsets of sensory neurons that do not express V1Rs or V2Rs and were activated by inflammation- and disease-associated ligands including N-formyl-methionyl-leucyl-phenylalanine (fMLF), the antimicrobial peptide CRAMP and lipoxin A4. Accordingly, vomeronasal FPRs were hypothesized to contribute to the assessment of health status during social communication (Rivière et al., 2009; Chamero et al., 2012).

1.2.3 Physiological properties of VSNs

1.2.3.1 Signaltransduction in VSNs

The signaling pathway in VSNs differs from the canonical MOE pathway and is not yet completely understood. The following signal transduction cascade model has been proposed by several groups (Spehr et al., 2002; Chamero et al., 2012). First, binding of a cognate ligand to either V1R or V2R receptors leads to G_{ai} or G_{oo} activation, respectively (Berghard & Buck, 1996; Chamero et al., 2011). Subsequently, the activated G-protein provokes catalytic activity of a phospholipase Cβ (PLCβ), shown to function as a crucial stimulus response element (Holy et al., 2000). PLCβ activity results in the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP$_2$), leading to an increase in the intracellular concentrations of the second messengers inositol-1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG) (Harteneck et al., 2000). Both, IP$_3$ and DAG, as well as polyunsaturated fatty acids (PUFAs), such as arachidonic acid, have been suggested to contribute to transient receptor potential channel C 2 (TRPC2) activation. TRPC2, an unspecific cation channel conducting Na$^+$ and Ca$^{2+}$, is highly expressed in VSN microvilli (Liman et al., 1999). TRPC2-deficient mice have severe deficits in social behavior, such as male-male aggression or sexual behavior (Stowers et al., 2002; Leypold et al., 2002). The opening of TRPC2 channels results in elevated cytosolic [Ca$^{2+}$] and, consequently, in CaCC activation, leading to an amplifying Cl$^-$ outward current in stimulus-induced responses (Yang & Delay, 2010; Kim et al., 2011). Recently, ANO1 and ANO2 have been identified to be highly expresses in the apical surface, i.e. microvillous layer of the VNO sensory epithelium (Dibattista et al., 2012). This expression suggests a role for those channels in VSN signaling and, thus, they are candidates for vomeronasal CaCCs.
Fig. 1.7: Schematic diagram of the signal transduction cascade in VSNs. Topographically separated VSNs express distinct repertoires of ligand receptor and signaling molecules. Apical VSNs (red) coexpress V1R receptors and the G-protein subunit Gαi, whereas in basal neurons (green) V2R and the G-protein subunit Gαo are coexpressed. After activation of either Gαi or Gαo, phospholipase C β (PLCβ) is activated to catalyze phosphatidylinositol 4,5-bisphosphate (PIP₂) turnover to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Polysaturated fatty acids (PUFAs) are assumed to be a side product and to function also as signaling molecules. DAG, in turn, is thought to open the transient receptor potential C2 (TRPC2) channel, enabling the influx of cations and subsequent activation of the ANO2 Cl⁻ channel, leading to a Cl⁻ efflux, thus enhancing a depolarizing receptor current. Modified from Spehr et al., 2006.

However, the contribution of Ca²⁺-activated Cl⁻ currents to stimulus-induced responses is controversially discussed (Billig et al., 2011). As in the MOE, a Ca²⁺-CaM feedback mechanism mediates sensory adaptation in the VNO by inhibiting pheromone-sensitive ion channels (Spehr et al., 2009). As Ca²⁺-dependent interaction of other TRP channel family members with calmodulin by specific Ca²⁺-CaM-binding sites has been shown, TRPC2 is hypothesized to be the interacting ion channel (Warr & Kelly, 1996; Trost et al., 1999; Spehr et al., 2009).

1.2.3.2 Physiological role of Cl⁻

Cellular Cl⁻ and hence Cl⁻ channels, have long been less investigated compared to other ion channels, i.e. Na⁺, K⁺, Ca²⁺ and H⁺ cation channels. This was probably based on findings in the first investigated cell types (i.e. skeletal muscle cells and erythrocytes) concerning Cl⁻ studies, where Cl⁻ was usually in electrochemical equilibrium across cell membranes. Thus, Cl⁻ channels putatively provided no capability to execute important, Cl⁻-driven physiological mechanisms in cells (Duran et al., 2010). The finding that Cl⁻ is actively transported in squid axons first demonstrated an important physiological role for Cl⁻ (Keynes, 1963). Another important discovery revealed that the inhibitory action of GABA was caused by an increased Cl⁻ conductance resulting in inhibitory postsynaptic potentials (Coombs et al., 1955). Today it is clear that, in most cells, Cl⁻ is actively transported and is not at electrochemical equilibrium. Mechanisms that control Cl⁻ flux therefore do contribute to various signaling processes. For example, epithelial cells, immature neurons, as well as sensory neurons express transporters,
which pump Cl⁻ into the cells resulting in high intracellular Cl⁻ concentration (Haas & Forbush, 2000; Schöbel 
 et al., 2012). By contrast, most mature neurons express transporters that pump 
Cl⁻ out of the cell, thus, providing a low intracellular Cl⁻ concentration for the typical 
hypermultiplying action of GABA (Duran et al., 2010). Most of these transporters are members 
of the cation-coupled Cl⁻ cotransporter (CCC) family (Russell, 2000). CCCs mediate the 
coupled movement of Na⁺ and / or K⁺ to that of Cl⁻ ions across the plasma membrane. Eight 
CCCs have been identified so far: two Na-K-Cl cotransporters (NKCC), four K-Cl 
cotransporters (KCCs), one Na-Cl cotransporter (NCC) and one CCC interacting protein 
(Isenring & Forbush, 2001). Most of these transporters share a common 12 transmembrane 
domain structure (Hebert et al., 2004). Other active transport proteins that participate in 
neuronal Cl⁻ homeostasis include Na⁺-dependent and Na⁺-independent anion exchangers 
(NDAE and AE, respectively), which exchange Cl⁻ for HCO₃⁻ ions (Romero et al., 2000; 
Grichtchenko et al., 2001). While NKCC, NCC and AE contribute to intracellular Cl⁻ accumulation, KCCs and NDAEs are responsible for Cl⁻ extrusion (Payne et al., 2003; Duran 
et al., 2010).

In the peripheral olfactory system, several of these transporters have been identified. PCR 
experiments on rat olfactory epithelium cDNA showed that mRNA for NKCC1, NCC, and KCC1 
is expressed (Kaneko et al., 2004). Especially NKCC1 has been shown to be required for the 
excitatory amplifying Cl⁻ outward current during olfactory signaling in OSNs (Reisert et al., 
2005; Nickell et al., 2007). Notably, KCC2 expression is lacking in the MOE (Kaneko et al., 
2004). In CNS neurons, this protein maintains a low [Cl⁻] (Rivera et al., 1999; Stein et al., 
2004). Both the lack of KCC2 expression and high expression of Cl⁻ accumulating NKCC1 
indicate elevated levels of intracellular [Cl⁻] and therefore a depolarizing Cl⁻ outward currents 
in OSNs. A crucial role for this Cl⁻ current is attributed to a particular class of Ca²⁺- activated 
Cl⁻ channels (CaCCs): the anoctamins (Galietta, 2009; Hartzell et al., 2009). The mammalian 
anoctamin gene family has 10 members that are well conserved across species. Anoctamins 
possess an eight-transmembrane domain structure with cytoplasmic N and C termini and a 
putative pore region between transmembrane domains 5 and 6 (Duran et al., 2010). In sensory 
neurons of both the MOE and the VNO, members of the ANO family have been identified and 
proposed to play a crucial role during stimulus-induced responses (Yang & Delay, 2010; Pifferi 
et al., 2011; Kim et al., 2011; Dibattista et al., 2012).

1.2.4 Pheromones and behavior

The term pheromone originates from the Greek word “pherein” (to carry or transfer) and 
“hormon” (to stimulate or excite) and was introduced by Karlson and Lüscher in the late 1950s 
(Karlson & Lüscher, 1959). One of the first identified pheromones was bombykol, a compound 
released by the female silkworm moth Bombyx mori to attract mates (Butenandt et al., 1959).
Generally, two types of pheromones can be distinguished: releaser pheromones, which elicit an immediate behavioral response and primer pheromones that mediate more slowly developing and longer-lasting changes to endocrine state or development. An example for a releaser pheromone is 2-methylbut-2-enal, which is present in rabbit milk. This molecule elicits stereotyped nipple-search behavior in rabbit pups and is thus of vital importance for them to locate the nipples for suckling (Schaal et al., 2003). Typical primer pheromones are α- and β-farnesene and 2,3-Dehydro-exo-brevicomin (DB), which accelerate puberty in pre-pubertal female mice by promoting uterus growth (Novotny et al., 1999).

Farnesenes and DB belong to a group of small volatile urinary molecules. Together with other compounds like 2-heptanone, 2,5-dimethylpyrazine and 2-sec-butyl-4,5-dihydrothiazole (BT), they were identified to be enriched in male or female mouse urine by Novotny and colleagues (Novotny et al., 1985, 1999). These pheromones evoke high-affinity responses in both MOE and apical V1R-expressing sensory neurons (Leinders-Zufall et al., 2000). Some of these compounds such as DB and BT were also linked to intermale aggression (Novotny et al., 1985).

Mouse urine is not only the source of small volatile compounds, but also contains high levels of nonvolatile macromolecules, the majority of which are major urinary proteins (MUPs) (Shaw et al., 1983). MUPs are 19-24 kD members of the lipocalin family, which have a β-barrel structure bearing a central hydrophobic binding pocket that has been shown to carry small organic ligands and therefore might deliver urinary volatile pheromones through hydrophilic environments such as urine or the VNO lumen (Timm et al., 2001; Sharrow et al., 2002; Chamero et al., 2007). Different members of this protein family have been shown to be involved in distinct social behaviors, such as aggression (Chamero et al., 2007), individual recognition (Hurst et al., 2001), sexual attraction (Roberts et al., 2010) and even puberty acceleration, known as the Vandenbergh effect (Mucignat-Caretta et al., 1995; Novotny et al., 1999). This effect is mediated via basal V2R-expressing VSNs and requires Gαo expression (Chamero et al., 2011).

Another class of macromolecules found in mouse urine that activates V2R neurons are major histocompatibility complex class I (MHC class I) peptide ligands (Leinders-Zufall et al., 2004, 2009). Their classical main function is to present intracellular peptides from cytosolic pathogens to immune cells (Morrison et al., 1986). The MHC polypeptides AAPDNRETF and SYFPEITHI, which bind to the H-2b MHC haplotype of C57BL/6 mice or H-2d haplotype of BALBc mice, respectively, stimulate electrical and calcium responses in both VNO and MOE sensory neurons (Leinders-Zufall et al., 2004; Spehr et al., 2006a). Behavioral studies in mice show that MHC genotype can determine individual recognition as well as mate choice by assessing the degree of relatedness of a potential mate to reduce inbreeding and maximize offspring fitness (Brennan, 2004; Brennan & Kendrick, 2006). Vomeronasal detection of MHC
peptides mediates the formation of an olfactory memory that is required for mate recognition in the context of selective pregnancy failure, termed Bruce effect (Bruce, 1959; Brennan, 2004, 2009; Kelliher et al., 2006). The Bruce effect is induced when the odor of a new male, but not that of a familiar male is presented to a recently copulated female mouse. The female can effectively establish an olfactory memory of her mate and distinguish his odor from that of a stranger. An unfamiliar male's odor induces a pregnancy block during a sensitive period of around 4 h immediately after mating by inhibiting the implantation of fertilized eggs into the uterine wall (Brennan, 2004). Urine is not the only source of murine pheromones. Other bodily secretions such as saliva, fluids from exocrine glands, anogenital glands, and vaginal regions are efficient sources of pheromones. One important pheromone class is the family of exocrine gland-secreting peptides (ESPs), 7 - 10 kDa peptides that are secreted in mouse saliva and tears. In mice, the ESP family consists of 38 members which are clustered on chromosome 17 (Kimoto et al., 2007). In recent studies, ESPs were shown to be expressed in a sex- and strain-specific manner and to be capable of activating V2R-expressing sensory neurons in the VNO (Kimoto et al., 2005, 2007). Of special interest is a male-specific ESP that is secreted from the extraorbital lacrimal gland into tear fluid: ESP1 (Kimoto et al., 2005). ESP1 was among the first pheromones which could be linked to a specific vomeronasal receptor and to a highly stereotyped behavior. ESP1 is detected by V2Rp5-expressing VSNs and enhances female sexual receptive behavior, i.e. lordosis, a posture in which the female curves the spine (Haga et al., 2010). ESP1-secreting males are sexually more successful than males that do not secrete ESP1 (Haga et al., 2010). This finding indicates that dedicated functions for other ESP family members are possible. For example, only recently Liberles and colleagues identified ESP22 as a juvenile-specific peptide secreted into tear fluid of prepubescent mice (Ferrero et al., 2013). Studies on this peptide are, in part, subject of this thesis.

1.3 Mitochondria

Mitochondria are cytosolic organelles involved in multiple functions such as generating most of the energy in animal cells via oxidative phosphorylation, production of reactive O₂ species (ROS), Ca²⁺ homeostasis or apoptotic cell death. Mitochondria have retained extranuclear mitochondrial DNA (mtDNA) from their evolutionary origin as endosymbiotic proteobacteria (Thrash et al., 2011). The human mitochondrial genome of only 16 kb encodes a few proteins of the electron transfer chain (ETC) such as cytochrome oxidase subunits and cytochrome b (Andersson et al., 2003). Mitochondria are composed of multiple compartments comprising an outer and inner membrane enclosing an intermembrane space and the matrix. This compartmentalization is important for the key role of mitochondria: producing most of the
energy for cellular ATP-dependent processes. While the outer membrane is permeable for ions and small molecules, the inner membrane is nearly impermeable. Ion transport, however, is mediated by several ion channels and transporters. Among these are proteins of the ETC, consisting of four distinct complexes: complex I (NADH ubiquinone oxidoreductase), complex II (succinatedehydrogenase), complex III (ubiquinol cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase) (Saraste, 1999; Lenaz et al., 2006; Hosler et al., 2006). Oxidative phosphorylation is the process in which electrons are passed along these molecules and transferred to oxygen. These electrons are generated from NADH (reduced nicotinamide adenine dinucleotide) and FADH$_2$ (reduced flavin-adenine dinucleotide), which are metabolites from cytosolic glycolysis and the Krebs cycle in the mitochondrial matrix. During oxidative phosphorylation, protons are pumped across the inner mitochondrial membrane resulting in an electrochemical H$^+$ gradient (Saraste, 1999; Hosler et al., 2006). Thus, energy is preserved across the inner mitochondrial membrane. This gradient drives ATP synthase (ATPase, complex V) to produce ATP from ADP (adenosine 5'-diphosphate) and phosphate (Elston et al., 1998).

Mitochondria are also involved in Ca$^{2+}$ homeostasis. They can directly influence the Ca$^{2+}$ concentration in the cytosol by importing Ca$^{2+}$ via the mitochondrial Ca$^{2+}$ uniporter (MCU) or by transporting Ca$^{2+}$ from the interior of the organelle into the cytosol via 2 Na$^+$ / Ca$^{2+}$ (NCX) or 2 H$^+$ / Ca$^{2+}$ (HCX) exchangers (Crompton et al., 1978; Fiskum & Lehninger, 1979; Kirichok et al., 2004; Jiang et al., 2009; Walsh et al., 2009). Driving force for Ca$^{2+}$ uptake via MCU is the electrochemical gradient across the inner mitochondrial membrane. Mitochondrial Ca$^{2+}$ sequestration upon cell stimulation is fast, likely due to exposure to Ca$^{2+}$ microdomains (Graier et al., 2007). Such microdomains are thought to be based on close proximity of mitochondrial Ca$^{2+}$ uptake sites and Ca$^{2+}$ release sites of the endoplasmic reticulum (ER) (Rizzuto & Pozzan, 2006; Pozzan & Rudolf, 2009; Rizzuto et al., 2009).

Different drugs or protonophores reduce the mitochondrial Ca$^{2+}$ uptake ability. The reversible protonophore FCCP (p-[trifluoromethoxyl]-phenyl-hydrazone) dissipates the negative inner mitochondrial membrane potential ($\Delta\Psi_m$), disrupting the Ca$^{2+}$ driving force and therefore Ca$^{2+}$ sequestration ability (Rizzuto et al., 1994; Szabadkai et al., 2006). Direct inhibition of mitochondrial Ca$^{2+}$ uptake is provided by MCU blockers such as Rutenium Red (RuR) and its derivative RuR360 (Rizzuto et al., 1994, 2009).

Mitochondrial network morphology depends on the size, shape, position and dynamics of mitochondria. Mitochondrial dynamics result from fission and fusion that constantly remodel the mitochondrial network (Rafelski, 2013). While fission describes separation of one mitochondrial tubule, during fusion two mitochondria are link to form a longer tubule or a branch (Nunnari et al., 1997). Those processes greatly influence mtDNA maintenance and are required to properly organize and distribute the mtDNA throughout the mitochondrial network.
Mitochondria are highly motile organelles, able of anterograde as well as retrograde transport to accumulate in particular subcellular regions of high metabolic activity or Ca\textsuperscript{2+} buffering demands (Morris & Hollenbeck, 1993; McCarron et al., 2013). Mitochondria travel along cytoskeletal elements involving both microtubules and actin filaments (Simon et al., 1995; Fehrenbacher et al., 2004; Frederick & Shaw, 2013). Within the microtubule network, organelle trafficking occurs with the help of motor and linker proteins of the kinesin and dynein families of microtubule-binding ATPases (Romagnoli et al., 2003). This way, mitochondria are transported along axons and dendritic regions in cultured hippocampal neurons (Ligon & Steward, 2000).

### 1.4 Voltage-gated ion channels

Voltage-gated ion channels are well known for their functional roles in excitable tissues. Voltage-dependent ionic currents across biological membranes were first identified by Hodgkin and Huxley in 1952. Hodgkin and Huxley showed a crucial role of voltage-gated sodium and potassium currents in the generation and propagation of action potentials in the squid giant axon (Hodgkin & Huxley, 1952). Voltage-gated ion channels are activated upon a change of membrane potential exceeding a specific threshold and typically conduct one or a few specific sorts of ions. Among the most important ion channels are voltage-gated Na\textsuperscript{+} channels (Na\textsubscript{v}), voltage-gated K\textsuperscript{+} channels (K\textsubscript{v}), voltage-gated Ca\textsuperscript{2+} channels (Ca\textsubscript{v}) and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Lai & Jan, 2006).

**Voltage-gated Na\textsuperscript{+} channel**

Voltage-gated Na\textsuperscript{+} channels are activated by stimulus-dependent membrane depolarization. Their activation frequently initiates an action potential by fast regenerative depolarization (Bean, 2007). Na\textsubscript{v} channels are formed by a 260 kDa single polypeptide that consists of four domains (I–IV), each of which has six α-helical transmembrane segments (S1–S6) associated with one or more auxiliary β-subunits (β 1/3 and β 2/4) of 33-36 kDa (Hartshornes & Catterall, 1984). The fourth transmembrane segment S4 of each domain contains positively charged arginines that are primarily responsible for voltage sensing. The \textit{S5-pore loop-S6} region forms the pore domain through which sodium ions flow (Yang & Horn, 1995; Catterall, 2000). The β-subunits, β1/3 and β2/4, are single transmembrane proteins that have an immunoglobulin-like extracellular domain that co-assembles with the Na\textsubscript{v} α-subunit (Yu & Catterall, 2003; Catterall et al., 2005a). Nine α-subunits (Na\textsubscript{v}1.1-Na\textsubscript{v}1.9) have been functionally characterized. A tenth related isoform (Na\textsubscript{x}) was only recently shown to function as a concentration-sensitive Na\textsuperscript{+} channel with a threshold of ~150 mM for extracellular Na\textsuperscript{+} concentration (Catterall et al., 2005a; Noda & Hiyama, 2014).
Different channel subtypes can be distinguished by pharmacological properties of the α-subunits. The main discrimination depends on tetrodotoxin (TTX) sensitivity (England & Groot, 2009). Genes encoding Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, and Na\textsubscript{v}1.7, clustered on chromosome 2 in mice, are blocked by nanomolar concentrations of TTX, whereas Na\textsubscript{v}1.5, Na\textsubscript{v}1.8, and Na\textsubscript{v}1.9 are located on chromosome 3 and show TTX resistance (Goldin et al., 2000; Yu & Catterall, 2003). Na\textsubscript{v}1.4 and Na\textsubscript{v}1.6 are also TTX-sensitive and are expressed on chromosome 17 or 12, respectively, in mice (Yu & Catterall, 2003). The current through Na\textsubscript{v} channels can be mainly divided into fast transient Na\textsuperscript{+} (I\textsubscript{NaT}) and slow persistent Na\textsuperscript{+} currents (I\textsubscript{NaP}). While persistent subthreshold TTX-sensitive sodium currents are supposed to play a role in driving the pacemaking of many central neurons (Crill, 1996; Taddese & Bean, 2002; Do & Bean, 2003), transient TTX-sensitive I\textsubscript{NaT} are most important in AP generation in peripheral as well as central neurons.

Voltage-gated K\textsuperscript{+} channel

During the termination phase of an AP cells are typically repolarized by K\textsuperscript{+} ion efflux via voltage-gated K\textsuperscript{+} channels (Bean, 2007). Based on sequence homology, K\textsubscript{v} channels have been divided into four main families (KV1–KV4) (Trimmer & Rhodes, 2004). Like Na\textsubscript{v} channels, they assemble as four subunits with six α-helical transmembrane segments (S1–S6) each. The S4 comprises the main voltage-sensing segment and the loop connecting S5/S6 helices form the K\textsuperscript{+}-selective pore (Choe & Jolla, 2002; Long et al., 2005; Catterall, 2010).

A large subfamily of K\textsubscript{v} channels is comprised by Ca\textsuperscript{2+}-activated K\textsubscript{v} (K\textsubscript{Ca}) channels. Those channels are expressed in neuronal and non-neuronal tissues including epithelia, smooth muscle, and sensory cells (Berkefeld et al., 2010). Upon increased intracellular [Ca\textsuperscript{2+}], K\textsubscript{Ca} channels conduct an efflux of K\textsuperscript{+} at rest, which feeds back onto cytosolic [Ca\textsuperscript{2+}] via re-/hyperpolarization of the membrane potential (Fakler & Adelman, 2008). K\textsubscript{Ca} channels have been classified mainly into two subtypes based on their biophysical properties: big conductance K\textsubscript{Ca} (BK\textsubscript{Ca}) channels, which exhibit a large conductance and are gated cooperatively by both membrane depolarization and [Ca\textsuperscript{2+}] elevations, and small conductance K\textsubscript{Ca} (SK\textsubscript{Ca}) channels solely ligand-gated by [Ca\textsuperscript{2+}] (Sah, 1996; Berkefeld et al., 2010). BK\textsubscript{Ca} channels have single-channel conductances of 100–200 pS and are blocked by low concentrations of tetraethyl ammonium chloride (TEA) (Coetzee et al., 1999). BK\textsubscript{Ca} channels comprise seven transmembrane domains (S0-S6), which is unique among K\textsuperscript{+} channels, placing the short amino-terminus extracellularly and the large carboxyl-terminus at the intracellular side of the membrane, where it contains four additional hydrophobic segments (S7- S10) (Meera et al., 1997; Swartz, 2004). By contrast, SK\textsubscript{Ca} channels (~10 pS) share the tetrameric six-transmembrane domain of voltage-gated cation channels, but lack the typical features of the voltage-sensing S4 segment (Stocker, 2004; Adelman et al., 2012). Consequently, the gating of SK\textsubscript{Ca} channels is solely driven by changes in [Ca\textsuperscript{2+}], with
submicromolar concentrations being sufficient to effectively gate the channels (Köhler et al., 1996; Hirschberg et al., 1998). Apamin, a neurotoxin from the honey bee, is a specific blocker of SK<sub>Ca</sub> channels (Grunnet et al., 2001).

**Voltage-gated Ca<sup>2+</sup> channel**
Voltage-gated calcium (Ca<sub>v</sub>) channels mediate Ca<sup>2+</sup> influx in response to membrane depolarization and regulate an immense diversity of intracellular processes. Like the α-subunits of sodium channels, the α-subunits of Ca<sub>v</sub> channels consist of four homologous domains (I–IV) with six transmembrane segments (S1–S6) each. The S4 segment also serves as the voltage sensor and the pore loop between S5 and S6 determines ion conductance and selectivity (Catterall et al., 2005b). Auxiliary subunits are an intracellular β-subunit, a transmembrane, disulfide-linked subunit α<sub>δ</sub> complex and a γ-subunit (Hofmann et al., 1994). Ca<sub>v</sub> channels are divided into 3 main subfamilies by different α-subunits and physiological as well as pharmacological properties: Ca<sub>v</sub>1, Ca<sub>v</sub>2 and Ca<sub>v</sub>3 (Dolphin, 2009). Depending on their gating properties, they are further divided into high voltage-activated Ca<sub>v</sub> channels (Ca<sub>v</sub>1 and Ca<sub>v</sub>2) and low voltage-activated Ca<sub>v</sub> channels (Ca<sub>v</sub>3).

The Ca<sub>v</sub>1 family conducts L-type Ca<sub>v</sub> currents, which are characteristic for the distinct members (Ca<sub>v</sub>1.1 - Ca<sub>v</sub>1.4) (Catterall, 2011). These currents typically require a strong depolarization for activation, are long-lasting and blocked by organic antagonists, e.g. dihydropyridines (Tsien et al., 1986). N-type, P/Q-type, and R-type calcium currents also require strong depolarization for activation and are attributed to Ca<sub>v</sub>2 channels (Ca<sub>v</sub>2.1 - Ca<sub>v</sub>2.3) (Mintz et al., 1992; Catterall et al., 2005b). They are typically blocked by specific polypeptide toxins from snail and spider venoms such as ω-conotoxin, ω-agatoxin and SNX-482 (Olivera et al., 1994; Randall & Tsien, 1995). T-type calcium currents (Nilius et al., 1985) can be assigned to members of Ca<sub>v</sub>3 channel family (Ca<sub>v</sub>3.1 - Ca<sub>v</sub>3.3). These channels are activated by weak depolarization and display transient kinetics (Nilius et al., 1985; Perez-Reyes et al., 1998). There is no specific blocker known for those channels (Catterall et al., 2005b).

**Hyperpolarization-activated cyclic nucleotide-gated channels**
Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels were first identified as channels mediating pacemaker currents that control rhythmic activity of cardiac pacemaker cells (Brown et al., 1977; Yanagihara & Irisawa, 1980; DiFrancesco, 1981). HCN channels comprise a subfamily of proteins within the superfamily of pore-loop cation channels. In mammals, the HCN channel family consists of four members (HCN1-4) that are expressed in heart and nervous system (Kaupp & Seifert, 2001; Whitaker et al., 2007). Like Kv channels, they are organized in four independent α-subunits each containing six α-helical segments (S1–S6) and an ion-conducting pore loop between S5 and S6 as well as a voltage sensor formed by a charged S4-helix carrying nine arginines (Vaca et al., 2000; Much et al., 2003). The
hyperpolarization-activated inward current produced by HCN channels is known as $I_h$. Carried by Na$^+$ and K$^+$ ions, $I_h$ depolarizes the membrane potential (Kamondi & Reiner, 1991; DiFrancesco, 1993). $I_h$ is slowly activated by membrane hyperpolarization to potentials negative to -55 mV near the resting potential of most cells (Biel et al., 2009). Moreover, activation of $I_h$ is facilitated by cAMP. The sensitivity to cAMP is mediated the cytosolic C-terminus, which contains a cyclic nucleotide-binding domain (CNBD). Binding of cAMP to the CNBD leads to faster channel opening and shifts the threshold activation potential to more positive voltages (DiFrancesco & Tortora, 1991; Zagotta et al., 2003; Wahl-Schott & Biel, 2009). Among cardiac sinoatrial node cells and various neurons, HCN channels were found in in the MOE, VNO and GG and in central neurons of the MOB (Maccaferri et al., 1993; Holderith et al., 2003; Dibattista et al., 2008; Mobley et al., 2010; Angelo & Margrie, 2011; Liu et al., 2012).
Aims

In most vertebrates, the detection of environmental chemicals is mediated by peripheral olfactory organs of high complexity. The remarkable sensitivity and range of these olfactory subsystems enables organisms to detect and discriminate thousands of compounds of immense structural diversity. In mice, the MOE is the largest olfactory structure containing millions of sensory neurons that detect mostly airborne odorants that enter the nasal cavity through the nostrils. Another subsystem, the VNO, plays an essential role in pheromone detection. Pheromones are conspecific chemical cues that control innate social behaviors by providing information about sex, age, receptivity, and social hierarchy and by regulating an individual’s endocrine state. While animal behavior is largely controlled by chemosensory input, some of the underlying mechanisms remain poorly understood.

The overall aim of my thesis is to gain more profound insight into sensory signaling mechanisms in the mouse olfactory system. I focus on basic physiological processes that control olfactory detection. In two independent lines of research, I aim to investigate physiological processes in a neuronal and non-neuronal MOE cell type, as well as different physiological aspects of vomeronasal signaling. Additionally, I intend to establish a new Ca$^{2+}$ imaging approach in VSNs using targeted single neuron electroporation. To address these different issues I will use acute tissue preparations of either the MOE or the VNO and employ both electrophysiological and live-cell fluorescence imaging techniques.

First, I will focus on a functional role of mitochondrial Ca$^{2+}$ signaling in odor-activated OSNs. Using patch-clamp recordings in acute MOE tissue slices, I aim to examine the role of mitochondria in regulating the odor response input-output signal. Furthermore, I will investigate mitochondrial mobility in OSNs upon odor stimulation using fluorescence recovery after photobleaching (FRAP).

Second, I aim to provide an in-depth electrophysiological characterization of SCs. I aim to describe the general biophysical properties of these cells and, in addition, I will investigate potential electrophysiological responses to odor stimulation.

Third, in collaboration with Prof. Dr. Stephen D. Liberles and his group (Harvard Medical School), I will analyze the physiological impact of a previously undescribed age-dependent mouse pheromone (ESP22). I plan to use both Ca$^{2+}$ imaging and electrophysiological recordings to examine the detection of ESP22 by the VNO.

Fourth, my research will focus on the role of Cl$^-$ in VSN signaling. In collaboration with Thomas Gensch (Forschungszentrum Jülich), I will use 2-photon fluorescence lifetime imaging microscopy (FLIM) to examine both the physiological Cl$^-$ concentrations and their potential stimulus-dependent changes in VSN knobs and vomeronasal SCs.
2. Materials and Methods

2.1 Chemicals

4-Aminopyridin  
Agar  
peqGold Agarose  
Adenosin 5’-triphosphate magnesium salt (MgATP)  
Alexa Fluor® 488 sodium salt  
Alexa Fluor® 555 Hydrazide, Tris (Triethylammonium) Salt  
Apamin  
Barium chloride  
Bis(2-hydroxyethyl)-2-aminoethanesulfonic Acid (BES)  
Cadmium chloride  
Calcium chloride  
Carbonyl cyanid 4-(trifluoromethoxy) phenyl-hydrazone (FCCP)  
Cesium chloride  
Cesium hydroxide  
Concanavalin A  
Ethylene glycol tetraacetic acid (EGTA)  
Ethanol  
Glucose  
Guanosin-5’triphosphate sodium salt (NaGTP)  
Fluo-4, AM, cell permeant  
Fluo-4, pentapotassium salt  
(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)  
Magnesium chloride  
Magnesium gluconate  
Magnesium sulfate
MitoTracker® Deep Red FM  
Life Technologies

N-(Ethoxycarbonylmethyl)-6-methoxyquinolinium Bromide (MQAE)  
Sigma-Aldrich

Oligomycin  
Sigma-Aldrich

Papain  
Sigma-Aldrich

Pluronic F-127  
Life Technologies

Potassium chloride  
Sigma-Aldrich

Potassium gluconate  
Sigma-Aldrich

Ru 360  
Merck

Rutenium Red  
Sigma-Aldrich

Sodium chloride  
Sigma-Aldrich

Sodium gluconate  
Sigma-Aldrich

Sodium hydrogencarbonate  
Sigma-Aldrich

Tetraethylammonium chloride (TEA)  
Sigma-Aldrich

Tetrodotoxin (TTX)  
Alomone Labs

Vanillin  
VWR

2.2 Solutions

Physiological extracellular solution (E_{\text{regular}}):

(pH = 7.3, osmolarity = 300 mosm):

145 mM NaCl
5 mM KCl
1 mM CaCl$_2$
1 mM MgCl$_2$
10 mM HEPES
Glucose to adjust osmolarity (0.18 g / mosm)

Extracellular solution (oxygenated, 95% O$_2$ / 5% CO$_2$) (E$_{\text{oxy}}$):

(pH = 7.3, osmolarity = 300 mosm):

120 mM NaCl
25 mM NaHCO$_3$
5 mM KCl
1 mM CaCl$_2$
1 mM MgSO$_4$
5 mM BES
Glucose to adjust osmolarity (0.18 g / mosm), pH value adjusts at oxygenated state
Extracellular solution (for VNO-slices; oxygenated, 95% O₂ / 5% CO₂) (E₄NO oxy):
(pH = 7.3, osmolarity = 300 mosm):
120 mM NaCl
25 mM NaHCO₃
5 mM KCl
1 mM CaCl₂
1 mM MgSO₄
5 mM BES
10 mM Glucose
Glucose to adjust osmolarity (0.18 g / mosm), pH value adjusts at oxygenated state

TEA (Tetraethylammonium) 1 mM extracellular solution (E₄TEA):
(pH = 7.3, osmolarity = 300 mosm)
144 mM NaCl
1 mM TEACl
5 mM KCl
1 mM CaCl₂
1 mM MgCl₂
10 mM HEPES
Glucose to adjust osmolarity (0.18 g / mosm)

TEA (Tetraethylammonium) 30 mM extracellular solution (E₄TEA30):
(pH = 7.3, osmolarity = 300 mosm)
115 mM NaCl
30 mM TEACl
5 mM KCl
1 mM CaCl₂
1 mM MgCl₂
10 mM HEPES
Glucose to adjust osmolarity (0.18 g / mosm)

TEA (Tetraethylammonium) 25 mM extracellular solution (for Ca²⁺ channel investigation) (E₄Ca):
(pH = 7.3, osmolarity = 300 mosm)
105 mM NaCl
25 mM TEACl
5 mM BaCl₂
1 mM MgCl₂
10 mM HEPES
Glucose to adjust osmolarity (0.18 g / mosm)

Physiological intracellular solution (symmetric [Cl⁻]):
(pH = 7.1, osmolarity = 290 mosm)
143 mM KCl
1 mM EGTA
0.3 mM CaCl₂
2 mM KOH
10 mM HEPES
1 mM MgATP
0.5 mM NaGTP
(→ 110 nm free Ca²⁺)
Glucose to adjust osmolarity (0.18 g / mosm)

**Intracellular solution (gluconate-based, 15 mM Cl⁻) (for SC V_reset investigation):**

(pH = 7.1, osmolarity = 290 mosm)
128 mM KGluconate
15 mM KCl
1 mM EGTA
0.3 mM CaCl₂
2 mM KOH
10 mM HEPES
1 mM MgATP
0.5 mM NaGTP
(→ 110 nm free Ca²⁺)
Glucose to adjust osmolarity (0.18 g / mosm)

**Intracellular solution (CsCl) (for Ca²⁺ channel investigation):**

(pH = 7.1, osmolarity = 290 mosm)
133 mM CsCl
10 mM NaCl
1 mM EGTA
0.3 mM CaCl₂
2 mM CsOH
10 mM HEPES
1 mM MgATP
1 mM NaGTP
(→ 110 nm free Ca²⁺)
Glucose to adjust osmolarity (0.18 g / mosm)

**Agar (10 %):**

10 g Agar / 100 ml A. dest.

**Agarose (4 %):**

4 g peqGold Agarose / 100 ml E_regular

**2.3 Consumables**

Borosilicate glass capillary tubes with filament
(1.05 x 1.5 x 100 mm)  
Science Products
Cell culture dishes (35 x 10 mm)  
Cell culture dishes (145 x 20 mm)  
Cell strainer (70 μm nylon)  
Cover glasses (round, 50 mm)  
Eppendorf tubes, 0.5 ml  
Eppendorf tubes, 1.5 ml  
Filter IC Acrodisc 13 mm syringe filter with 0.2 μM Supor (PES) membrane (for intracellular solutions)  
Filter Filtropur S 0.2 (for extracellular solutions)  
Gloves, GentleSkin, XS, sensitive  
Pasteur pipets, 3 ml  
Syringe, 10 ml  
Syringe BD Plastipak, 1 ml  
Syringe BD Discardit™ II, 10 ml  
Tubes 50 ml / 15 ml

2.4 Equipment
Centrifuge mini spin  
Hamilton Syringe  
Hot plate magnetic stirrer  
Microforge MF-830  
Micropipette puller PC-10 vertical two-step puller  
Osmometer osmomat 030  
pH Meter (Five Easy FE 20)  
Vibratome VT 1000 S  
Water bath  
Set-up 1 (Electrophysiology in the MOE and FRAP)  
Amplifier EPC-10  
Camera Leica DFC360 FX CCD

Falcon  
Falcon  
BD Falcon  
Menzel–Gläser (Thermo Scientific)  
Eppendorf (Diagonal)  
Eppendorf (Diagonal)  
Life Sciences  
Sarstedt  
Meditrade  
VWR  
Braun Melsungen AG  
BD Bioscience  
BD Bioscience  
Sarstedt

Eppendorf AG  
Hamilton Company  
Snijders  
Narishige  
Narishige  
Gonotec  
Mettler Toledo  
Leica Microsystems  
Memmer  
HEKA Elektronik  
Leica Microsystems
Camera Image EM 9100-13 CCD  
Camera Orca R2  
Fluorescence Illumination System EL6000  
Laser Merge Module, dual output (Laser wavelengths: 405 nm, 488 nm, 642 nm)  
Micromanipulator Luigs-Neumann Device SM-5  
Microscope Leica DM 6000 FS  
Objectives  
- 10x (HCX APO L U-V-I 10x/0.30)  
- 63x (HCX APO L U-V-I 63x/0.90)  
Oscilloscope (DSO 1002A)  
Perfusion system 8-in-1  
Recording chamber Slice mini chamber  
Trigger interface TIB-14 S  
Vibration-cushioned table  
VT-HAWK multi point confocal scanner (integrated FRAP capability)  
Set-up 2 (Ca²⁺ imaging and electroporation in the VNO)  
CCD Camera Leica DFC360 FX  
Confocal laser scan microscope SP5 (Leica DM 6000 CSF)  
Fluorescence Illumination System EL6000  
Objective 20x (HCX APO L20x/1.00 W)  
Laser Module (Laser wavelengths: 458 nm, 477 nm, 488 nm, 514 nm, 633 nm)  
Micromanipulator Luigs-Neumann Device SM-5  
Axoporator 800A  
Set-up 3 (Cl⁻ imaging / FLIM in the VNO)  
Nikon A1 MP Multiphoton Confocal  
Objective 25x (APO LWD 25x/1.10 W)  
Laser module Mai Tai® DeepSee™
Vibration-cushioned table (Fill and Forget™)  Newport

2.5 Software

Corel Draw 16.2  Corel Corporation
Fitmaster 2.67  HEKA Elektronik
Igor Pro 6.3  Wavemetrics Inc.
Leica Application Suite (LAS)  Leica Microsytems
Leica AF6000 confocal imaging software  Leica Microsytems
MS Office 2013  Microsoft
NIS-Elements AR 4.13  Nikon
Patchmaster 2.67  HEKA Elektronik
PPT, Patcher’s Power Tools (Igor Application Software)  developed by: Mendez and Würriehausen (2005)
Mendeley Reference Manager  Mendeley Ltd.
SpAcAn, Sponaneous Activity Analysis (Igor Exchange)  developed by: Dugué and Rousseau (2010)
SPCImage  Becker & Hickl
VoxCell Scan imaging software  VisiTech International

2.6 Mouse strains

C57BL/6 strain  Charles River
mOR-EG strain  kindly provided by Dr. Kazushige Touhara (The University of Tokyo)
OMP-GFP strain  kindly provided by Dr. Peter Mombaerts (MPI for Biophysics, Frankfurt)
Methods

2.7 Animal and tissue preparation

All animal procedures were in compliance with local and European Union legislation on the protection of animals used for experimental purposes (Directive 86/609/EEC) and with recommendations put forward by the Federation of European Laboratory Animal Science Associations (FELASA). C57BL/6, mOR-EG and OMP-GFP mice were housed in groups of both sexes at RT on a 12 h light/dark cycle with food and water available ad libitum. We did not observe obvious gender-dependent differences in electrophysiological recordings.

2.7.1 Acute coronal MOE slices

C57BL/6 and mOR-EG pups (P0-P5) of both sexes were sacrificed by decapitation. The skin, eyes, lower jaw, teeth and the palate were removed. The nose was washed in oxygenated ice-cold solution and air bubbles were removed from the nasal cavity by gentle pressure. The intact nose was then embedded in 4% low-melt agarose (~37°C) and coronal slices (150-300 μm) were cut in ice-cold oxygenated solution (Eoxy) using a vibratome (VT1000S; speed: 5.5 a.u., frequency: 7.5 a.u. = 75 Hz; amplitude: 0.4-0.8 mm). Slices were transferred to a submerged, chilled, and oxygenated storage chamber until use.

2.7.2 Acute coronal VNO slices

Adult male or female C57BL/6 mice were sacrificed by cervical dislocation or by brief exposure to CO2 followed by decapitation using sharp surgical scissors. The lower jaw, fur and skin, front teeth and palate of the mouse head were removed allowing access to the vomeronasal capsule. The VNO was dissected from the cartilage and embedded in 4% low-gelling temperature agarose. Coronal slices (200 – 250 μm) were cut on a vibratome (VT1000S; Fig. 2.1: (A) Dorsal (left) and ventral (right) view of a prepared skull of a neonatal mouse (p 5). (B) Acute coronal vibratome slice of the anterior nose and (C) of the posterior nose, revealing several endo-turbinate to enlarge the epithelial surface; dr, dorsal roof; et, endoturbinate; fh, frontal hemisphere; MOE, main olfactory epithelium; nc, nasal cavity; pa, palate; ph, parietal hemisphere; se, septum; vb, vomerbone; VNO, vomeronasal organ; scale bars as indicated.
speed: 5.5 a.u.; frequency: 7.5 a.u. = 75 Hz; amplitude: 0.6 mm) in ice-cold oxygenated extracellular solution. The acute slices were transferred to a submerged, chilled and oxygenated storage chamber until use.

2.7.3 *En face* preparation of the VNO sensory epithelium

Adult (≥ 8 weeks age) female OMP-GFP mice were sacrificed and decapitated. The fur, lower jaw, front teeth and palate were removed from the mouse head. The skull was cut sagittally ~1-2 mm lateral to midline. The hemisected head was transferred to a 35 mm culture dish and embedded in agar (10%). After removing the lateral cartilaginous capsule surrounding the vomeronasal organ, the non-sensory tissue was carefully removed to gain access to the luminal surface of the VNO sensory epithelium. The preparation was then submerged with $E_{\text{regular}}$ solution at RT.

2.7.4 Dissociation of olfactory sensory neurons

Male and female mOR-EG mice of adult age were decapitated. The skull was sagittally hemisected and the septum was removed to gain access to the main olfactory epithelium. The olfactory epithelium was dissected and placed into a Ca$^{2+}$-free solution containing 0.09 mg/ml papain, followed by incubation in the enzyme solution for 15 min at 37°C. The olfactory epithelium was then transferred to $E_{\text{regular}}$ solution for gentle trituration with fire-polished Pasteur pipettes. Dissociated cells were subsequently filtered through a 70 μm nylon mesh cell strainer into fresh $E_{\text{regular}}$ solution. Single cells were attached to concanavalin A-coated 35 mm Petri dishes.

2.8 Electrophysiology

2.8.1 Patch-clamp technique

The study of physiological membrane properties is accomplished by the patch-clamp technique. It allows the measurement of ion currents across biological membranes and recordings of cellular membrane potentials. Therefore, it provides a method to analyze the function and activity of ion channels in cell membranes. This technique was developed by Erwin Neher and Bert Sakmann between 1973 and 1976 (Neher & Sakmann, 1976; Hamill *et al.*, 1981).

The principal of the patch-clamp technique is based on a tight seal between the patch pipette (or recording electrode) and the cellular membrane. Therefore, a fire-polished borosilicate glass pipette with a fine tip diameter (1-2 μm) is filled with electrolyte and imposed on the recording electrode. The patch pipette is positioned towards the cell membrane and slight positive pressure is used during this process to avoid clogging of the small tip. In close
proximity to the cell surface this positive pressure leads to an inversion of the membrane and an increase in pipette resistance, which is an adequate indicator for the right position of the pipette at the cell. Removal of positive pressure results in a movement of the cell membrane towards the patch pipette. Sometimes, this process already establishes a tight (high resistance) seal (1-20 GΩ) of the lipid membrane to the pipette tip. Otherwise the membrane can be sealed to the patch pipette via slight negative pressure, leading to the ‘cell-attached’ configuration (Fig. 2.2). From this configuration, various other possible configurations can be created such as ‘whole-cell’, ‘inside-out’ or ‘outside-out’ modes.

If the patch of membrane under the pipette tip is ruptured by a short suction pulse, then the pipette solution and the electrode make direct electrical contact with the cytoplasm, forming the whole-cell configuration. Thus, current conductance as well as voltage can be measured over the whole membrane.

The outside-out patch is obtained by simply pulling away the patch pipette from a whole-cell configuration. Thereby, a part of the membrane is ruptured and, due to the properties of the phospholipids, folds back to form a patch covering the pipette. This recording conformation enables conductance measurements through single or a few ion channels.

In cell-attached configuration, fast pipette retraction leads to inside-out recording mode. This results in a membrane vesicle, which is attached to the pipette tip. The vesicle can be destroyed by exposure to air. This generates a patch with the cytosolic side of the inner membrane leaflet exposed to the bath solution. In contrast to outside-out configuration, it is now possible to apply different stimuli to the intracellular membrane site.

One central component of the patch-clamp technique is the amplifier, which is composed of two devices. A preamplifier (‘headstage’), which includes the recording electrode and is used...
for measurements of ion currents as well as membrane potentials, and a main device with the central processing unit. The basic principle underlying the ‘voltage-clamp’ patch clamp technique is the constant maintenance of a predetermined membrane potential. Without this ‘clamping’ of the membrane potential, receptor currents would lead to membrane depolarization, which would impair selective measurements of defined ionic currents. The amplifier performs voltage measurement and current injections simultaneously using a single electrode. Therefore, a current is injected, which is inverted proportional to the difference between the measured receptor current and a given holding potential. Normally, beginning deviations are corrected that fast, that the membrane potential is equal to the holding potential at any time.

2.8.2  Electrophysiological recordings from OSNs in acute MOE tissue slices
For electrophysiological recordings, MOE slices from mOR-EG mice were transferred to a recording chamber (Luigs & Neumann) and fixed with stainless steel strung with 0.1 mm thick lycra threads. Cells were continuously superfused with fresh oxygenated extracellular (E_{ox}) solution at room temperature. To visualize single cells in a tissue slice an upright fixed-stage light microscope (Leica DM 6000 FS) with infrared-optimized differential interference contrast (IR-DIC) optics was used, equipped with following water immersion objectives: 10x (HCX APO L U-V-I 10x/0.30) and 63x (HCX APO L U-V-I 63x/0.90). GFP-expressing neurons were identified via a Fluorescence Illumination System (Leica EL6000) and a GFP-optimized fluorescence filter cube (Leica L5). To monitor the cells, a CCD-camera (Leica DFC360 FX CCD) controlled by Leica Application Suite (LAS) software was used.

Fig. 2.3: Patch-clamp set-up (1) for recordings in acute tissue slices of the MOE. (A) Image of the upright fixed stage light microscope (Leica DM6000 FS) with a Leica CCD-camera (CCD) used for visualization of cells in the MOE. The microscope is equipped with peripheral experimental devices such as an amplifier headstage (probe) and a perfusion system on the other site, for a precise stimulus application. Both probe and perfusion are mounted on a Luigs-Neumann device (LN) for micromanipulator controlled movement. Additionally, this setup is equipped with a multi point confocal scanner (vt hawk) from visitech Int. with an integrated FRAP capability. For FRAP experiments the high sensitivity CCD camera ImageEM was used. Inaddition, this setup features a high resolution Orca R2
CCD camera. (B) Devices for electrophysiological recordings include an oscilloscope (osc.), a Luigs-Neumann control box (LN), an EPC-10 amplifier (amp.) and a TIB-14 trigger interface (TIB).

Patch pipettes with a resistance of 4-6 MΩ were pulled from borosilicate glass capillaries with filament and fire-polished ends by PC-10 vertical two-step micropipette puller and were fire-polished via MF-830 microforge. Patch pipettes were filled with intracellular solution and mounted on an Ag/AgCl electrode connected to an EPC-10 headstage. To avoid clogging of pipette tips, slight positive pressure was applied before dipping pipettes into bath solution. In mOR-EG mice, neurons expressing the Olfr73 gene coexpress GFP and the mOR-EG receptor. This receptor is activated by eugenol (EG) or vanillin. Somata from identified green-fluorescent OSNs were ‘patched’ from a basal direction. Pipettes were advanced towards individual neurons under optical control using Luigs & Neumann micromanipulators. An EPC-10 amplifier controlled by Patchmaster 2.67 software was used for data acquisition. Both, pipette (Cfast) and cell membrane capacitance (Cslow) as well as series resistance (Rs) were monitored and automatically compensated throughout the experiment. In whole-cell experiments, only neurons exhibiting stable access resistances were used for analysis. Theoretical liquid junction potentials were calculated using JPCalcW software and corrected offline. An agar bridge containing 150 mM KCl connected the reference electrode with the bath solution.

Data were obtained by using whole-cell experiments in voltage-clamp or current-clamp mode, respectively, or by loose-seal experiments in voltage-clamp mode. Signals were low-pass filtered (analog 3- and 4-pole Bessel filters in series) with an effective corner frequency (-3 dB) automatically adjusted to \( \frac{1}{5} - \frac{1}{3} \) of the sampling rate (5 – 20 kHz, depending on protocol). Between recordings, cells were kept at a holding potential (Vhold) of -70 mV. Data were analyzed offline using FitMaster 2.20 (HEKA Elektronik) and Igor Pro 6.04 (WaveMetrics) software.

To prevent dialysis of intracellular components via the patch pipette during spike recordings, action-potential-driven capacitive currents were recorded from the soma of identified OSNs in the loose-patch cell-attached configuration using patch pipettes filled with physiological extracellular solution (Eregular) (seal resistances of 50 - 120 MΩ obtained by gentle suction). Spikes were analyzed offline using SpAcAn (Spontaneous Activity Analysis), a collection of Igor Pro functions (written by Guillaume Dugué and Charly Rousseau) for the detection and analysis of spontaneously occurring events by employing a threshold detection algorithm. Peristimulus time histograms (PSTH; bin width, 1 s) were generated and plotted as a function of time. These histograms are used to visualize the rate and timing of neuronal spike discharge in relation to an external stimulus or event. Inter-stimulus intervals were 30 s. PSTH peak versus baseline significances were determined by a paired two-tailed t-test.
Application of extracellular solutions or drug-/stimuli-containing solutions was achieved by an air pressure-driven 8-in-1 perfusion system consisting of a custom-built valve bank connected to a ‘perfusion pencil’ (AutoMate Scientific). To apply different solutions in an exactly time-locked manner a TIB-14 S trigger interface was used and controlled by the Patchmaster 2.67 software. Individual voltage step protocols are described in the results section.

2.8.3 Electrophysiological recordings from sustentacular cells in acute MOE tissue slices

Coronal MOE slices of 200-300 µm thickness were prepared from C57BL/6 mice. If not stated otherwise equipment and procedures were the same as outlined in 2.8.2.

Patch pipettes were made from borosilicate glass capillaries with filament and fire-polished ends and pulled to have resistances of 4-6 MΩ. They were filled with intracellular solution containing the fluorescent dye Alexa 488 (20 µM). Cells that were filled with dye were visualized using a Fluorescence Illumination System (Leica EL6000) and a Leica L5 filter cube (excitation: bp 480/40, suppression: bp 527/-30) to observe the morphology of SCs.

Sustentacular cells (SCs) were patched by positioning the tip of the pipette adjacent to the apical surface of the OE using Luigs & Neumann micromanipulators. The electrode was then slowly advanced towards the surface and positive pressure was applied until contact with the outer cell layer was established. Following a high resistance seal (1-20 GΩ) the membrane was ruptured to allow the whole-cell configuration. After gaining whole-cell access, $R_{\text{input}}$ of SCs increased from initially 50-100 MΩ to 100-300 MΩ. Cells were kept at a holding potential ($V_{\text{hold}}$) of -50 mV.

2.8.4 Electrophysiological recordings from VSNs in acute VNO tissue slices

Vomeronasal organ dissection and preparation of acute tissue slices was performed as described under 2.7.2. For electrophysiological recordings, VNO slices were transferred to a recording chamber and anchored with stainless steel strung with 0.1 mm thick lycra threads. Cells were continuously superfused with fresh oxygenated extracellular solution ($E_{\text{VNO oxy}}$). If not stated otherwise equipment and procedures were the same as mentioned in 2.8.2.

Action-potential-driven capacitive currents were recorded from the somata of VSNs located deep in the sensory epithelium’s basal layer close to the basement membrane. Recordings were performed in the loose-patch cell-attached configuration, to prevent dialysis of intracellular components via the patch pipette (seal resistances of 50 - 120 MΩ obtained by gentle suction). Spikes were analyzed using Igor Pro functions (SpAcAn, G. Dugué and C. Rousseau). Inter-stimulus intervals were 30 s. Neuronal responses were classified according the following criteria: a) discharge was time-locked to stimulus presentation (responses occurred during and/or up to 3 s after stimulation onset); b) spike patterns clearly deviated
from prior baseline activity (frequency histograms (1 s bin width) were calculated over repeated trials and responses were evaluated according to a $\Delta f \geq 2 \times SD f_{\text{baseline}}$ criterion).

2.9 Confocal imaging

Confocal microscopy is an optical imaging technique, which aims to overcome the limitations of conventional wide-field light microscopy that delivers only two-dimensional images. Confocal microscopy adds a third dimension. The constitutional principle of confocal imaging, developed and patented in 1957 by Marvin Minsky, is based on point illumination of a restricted spot in the same focal plane ($\text{confocal}$) (Minsky, 1988; Egger & Petran, 1967; Brakenhoff et al., 1985). Together with a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus light, this leads to improved optical resolution. As shown in Fig. 2.3, the pinhole aperture excludes a high proportion of light. Therefore, only extremely bright light sources and / or fluorescent specimens can be used. This difficulty is overcome by the use of laser illumination. Using point illumination, a complete image of a specimen at the highly restricted focal depth can only be taken by scanning the specimen (Egger & Petran, 1967; Brakenhoff et al., 1985). Therefore, a confocal laser scanning microscope (CLSM) contains a scanning unit that comprises the electronics to drive two mirrors to produce a focused scanning beam together with a photomultiplier tube detector (PMT) (White et al., 1987; Fine et al., 1988; Paddock, 2008). This scanning process results in a digital ‘monochrome’ intensity image.

![Schematic drawing of principle confocal scanning microscope.](image)

**Fig. 2.3:** Schematic drawing of principle confocal scanning microscope. Only emissions from in-focus plane (---) is able to pass through the pinhole of the imaging aperture. Fluorescence from out-of-focus plane (---) is reduced by the imaging aperture (from Shotton & White, 1989).
2.9.1  **Ca\textsuperscript{2+} imaging in VNO slices via confocal imaging**

VNO slices were transferred to an upright confocal Leica Microsystems microscope CFS SP5 equipped with a 20x (water immersion, HCX Apo, high numerical aperture) objective. The VNO slices were incubated with the Ca\textsuperscript{2+}-sensitive reporter dye Fluo-4 AM (10 μM; 0.1% Pluronic F-127 was added for better Fluo-4 uptake into cells; in E\textsubscript{VNOoxy}) for 30 min at RT, washed in E\textsubscript{regular} and briefly exposed to stimuli (10 s) using a custom-built multi-barrel perfusion system. Fluo-4 was excited using the 488 nm line of an argon laser and emitted fluorescence was captured using a 500–550 nm filter. Changes in cytosolic Ca\textsuperscript{2+} were monitored over time at 1 Hz frame rate. Offline analysis of time-lapse experiments was performed using LAS-AF software. All cells in a given field of view were marked as individual regions of interest (ROIs), and the relative fluorescence intensity for each ROI was calculated and processed as a function of time.

2.10  **Single-cell electroporation**

Electroporation is a physical technique, originally developed for targeting gene transfer to individual cells in intact tissue (Neumann & Rosenheck, 1972; Neumann et al., 1982; Haas et al., 2001). Using modified patch electrodes and common electrophysiological equipment, this technique can be adapted for use with other charged transfer materials, such as fluorescence dyes. In general, single-cell electroporation is applicable to various systems and tissues where cells can be targeted with a micropipette (Bestman et al., 2006). Based on the observation that biomembranes show an enhanced permeability upon short electric impulses without lasting damage to the membrane structure, the technique leads to a transient exchange of material across the perturbed membrane (Sale & Hamilton, 1968; Lindner et al., 1977). The increase in permeability is due to hydrophilic pore formation within a fraction of a microsecond (Kinosita & Tsong, 1977). Once the transmembrane potential reaches a critical value, a reversible pore is formed in the lipid part of the membrane (Benz & Zimmermann, 1981).

A biological membrane is a co-operatively stabilized organization of lipids and proteins, which contains dynamic, locally limited structural 'defects'. These local disorders are candidate structures for the onset of electric field-induced perturbations. The presence of an electric field would favor charge and dipole configurations that leads to larger dipole moment components in the field direction. This, in turn, leads to pore formation, stabilized by a favorable lipid head group dipole interaction with the external electric field (Neumann et al., 1982).

2.10.1  **Electroporation in VNO slices**

An Axoporator A800 headstage and a bath electrode (silver electrode) with a distinct grounding were attached to a conventional up-right fixed-stage setup. Solutions and tissue were prepared
as described (see 2.7.2). VNO slices were transferred to the stage of an upright confocal microscope and superfused with oxygenated extracellular solution. Pipettes with a resistance of 5-10 MΩ (optimal ca. 7 MΩ) were pulled from glass capillaries with filament and fire-polished ends. A Hamilton syringe was used to fill pipettes with dye solution. Pipettes were then placed on the electrode of the Axoporator headstage. Fresh Alexa hydrazide 633 or fluo-4 pentapotassium salt (cell impermeable) was dissolved in 150 mM KCl (previously sterile filtered) to a stock solution of 10 mM. For electroporation, fluo-4-pp stock solution was diluted to 500 µM in sterile filtered extracellular solution and centrifuged before filling to the Hamilton syringe. Dye solution was protected from light and kept on ice. Residual dye solution was always reused.

Before pipettes were dipped into the extracellular solution (bath solution only, no specimen to avoid unspecific loading by dye exposure), minimal positive pressure was applied to avoid clogging of the pipette. Under optical control, pipettes were controlled for dye leakage. Therefore, an offset potential is applied until no dye leakage from the pipette tip occurred. The offset voltage is of opposite charge compared to the fluorescence dye (Alexa hydrazide 633 is negatively charged → positive offset voltage; Fluo-4 is positively charged → negative offset voltage).

Once the offset was adjusted correctly (dye diffusion from the pipette tip is hardly detectable), the slice chamber was placed onto the microscope. Pipettes were advanced towards individual neurons in the VNO basal layer. Positive pipette pressure was never removed during the whole process. In close proximity to a cell, a rectangular voltage pulse of ±7 V for 300 ms was set. Positive or negative pulse amplitude is set depending on dye charge so that the dye is ejected from the pipette tip (Alexa hydrazide 633 is negatively charged → negative voltage pulse; Fluo-4 pentapotassium salt is positively charged → positive voltage pulse). The patch pipette was removed from the cell without disrupting the membrane and more cells can be electroporated with the same pipette. There is no need to change pipette until it is visibly plugged.

2.11 Two-Photon fluorescence lifetime imaging microscopy (2P-FLIM)

Two-Photon fluorescence microscopy was invented about 25 years ago by Winfried Denk and coworkers (Denk et al., 1990). Conventional confocal fluorescence microscopy is limited by the use of pinholes for 3D resolution. To obtain better 3D resolution, pinholes need to be narrowed but simultaneously the number of detected photons is decreased (Shotton & White, 1989). Since increasing excitation power to compensate for the loss of signal (pinhole effect) leads to phototoxicity and photobleaching, the application of fluorescence microscopy to living tissue is limited. Furthermore, 2PM provides an increased imaging depth to analyze cells in vivo in deeper tissue layers. In 2PM, two low-energy photons of approximately twice the
wavelength are used to excite a fluorescence molecule. Here, absorption depends crucially on the use of a pulsed laser with pulse durations of femto- or picoseconds (Denk et al., 1990). Due to the focusing of the laser beam in the microscope, fluorophores are excited almost exclusively in a small diffraction limited focal volume (Zipfel et al., 2003; Svoboda & Yasuda, 2006). The result of such localized excitation is three-dimensional contrast and resolution. Because of the nature of nonlinear excitation used in 2PM, scattered excitation photons are too dilute to cause substantial out-of-focus fluorescence. This is based on the fact that only in the focal plane two photons converge, and the excitation wavelength is achieved. Out of the focus only single photons of the higher wavelength, thus less energetic, reach the tissue. This is also an argument for the reduced phototoxicity. Even in deep tissue planes, excitation is still mostly limited to a tiny focal volume. As a consequence, pinholes are not necessary, which leads to a large increase of detected photons. Since localization of excitation is also a direct consequence of the nonlinear nature of 2PM, all fluorescence photons, even scattered, constitute useful signal.

FLIM is a technique that maps the spatial distribution of the lifetimes within microscopic images of living cells (Becker, 2012; Ishikawa-Ankerhold et al., 2012). It is based on the fact that fluorophores are not only characterized by their excitation and emission spectra, but also by their unique lifetime. The fluorescence lifetime $\tau$ is the characteristic time of an exponential decay function that describes the time course of the emission after the excitation of a fluorescent probe. $\tau$ is a measure for the average time that the fluorophores reside in the excited state. Beside others, time-resolved fluorescence imaging in single cells was first performed in the laboratories of König, Lakowicz and Kusumi (Bugiel et al., 1989; Lakowicz et al., 1992; Oida et al., 1993).

FLIM experiments are independent from excitation light intensity variations or local concentration of the fluorophores and are insensitive to moderate levels of photobleaching. These experiments are implemented for mapping spatial variations of the lifetime in response to changes in the environment. Depending on the fluorophore used the fluorescence lifetime can encode the information for pH or ion concentrations (Biskup & Gensch, 2014) or protein-protein interactions, refractive index or viscosity (Suhling et al., 2005).

### 2.11.1 Fluorescence lifetime and collisional quenching using Cl'-sensitive MQAE

In our experiments, 6-methoxy-quinolyl acetoethyl ester (MQAE) was used as a fluorescent reporter of intracellular Cl'. MQAE molecules reach the excited state upon absorption of a single ultraviolet photon ($\lambda = 375$ nm) or, alternatively, the absorption of two infrared photons ($\lambda = 750$ nm) (Fig. 2.4 A). Here, I used two-photon excitation. Moreover, the infrared light used for two-photon excitation causes no detectable photodamage, even with the relatively long observation times of up to 80 sec. UV light, however, needed for one-photon excitation is
expected to cause considerable photodamage to biological cells if used for prolonged durations at the required intensities.

**Fig. 2.4:** Principle of two-photon excitation and collisional quenching. (A) Instead of one high energy photon, two infrared photons are used for excitation, causing much less observable photodamage. (B) During collisional quenching the fluorescence lifetime $\tau$ depends on kinetic constants of fluorescence ($k_f$), nonradiative processes ($k_{nr}$) and quenching ($k_q$); Q, quencher molecule.

MQAE as a fluorescence indicator for live cell imaging has several advantages. It is not only excitable by two photons, but is also nontoxic and easily entering cells and tissues by diffusion. MQAE is membrane permeable. MQAE accumulation results from intracellular cleavage by unspecific esters at sites of esterification. Consequently, MQAE molecules become membrane impermeable. The molecule has one of the highest Cl$^-$ sensitivities among quaternary nitrogen based fluorescent compounds (Verkman, 1990). The Cl$^-$ sensitivity is caused by efficient collisional quenching (also called dynamic quenching; Fig. 2.4 B; (Biskup and Gensch, 2014)). During collisional quenching the MQAE molecules form short-lived (picoseconds) encounter complexes with the Cl$^-$ ions. The photophysical properties of the fluorescent dye in this transient complex are much different from that of the dye alone. In the case of MQAE, the rate constant for non-radiative deactivation ($k_{nr}$) in the encounter complex with Cl$^-$ is orders of magnitudes higher than for the non-complexed fluorophore. Non-radiative deactivation manifests in relaxation of the excited electron without emitting a photon (fluorescence) instead releasing heat. This causes in turn a reduction of the fluorescence proportional to the Cl$^-$ concentration because collision probability is enhanced with a higher Cl$^-$ concentration. Therefore, fluorescence lifetime $\tau$ depends on kinetic constants of fluorescence ($k_f$), nonradiative processes ($k_{nr}$) and quenching ($k_q$) and is described by the following equation: $\tau = 1 / (k_f + k_{nr} + k_q + Q)$. In other words, the higher the Cl$^-$ concentration, the shorter the lifetime $\tau$. It was also shown that charge-transfer processes are playing a central role for the quenching mechanism (Jayaraman & Verkman, 2000).

Collisional quenching is quantitatively described by the Stern–Volmer equation (Biskup and Gensch, 2014) that is valid for both the fluorescence intensity and the fluorescence lifetime. Here, the Cl$^-$ dependency of lifetime $\tau$ is described by the following Stern–Volmer relation: $\tau_0/\tau = 1 + K_{SV} [Cl^{-}]$, where $\tau_0$ is the fluorescence lifetime in Cl$^-$-free solution, and $K_{SV}$, the Stern–Volmer constant, which is a measure of the Cl$^-$ sensitivity of MQAE.
2.11.2 *En face* Cl\(^{-}\) imaging of the VNO sensory epithelium via 2P-FLIM

For FLIM measurements, the VNO *en face* preparation was used as outlined in 2.7.3. The laterally opened VNO was submerged in 7 mM MQAE dissolved in oxygenated extracellular solution. 1 - 2 ml solution were carefully added from the side of the dish (never directly on top of the vomeronasal sensory epithelium since it is very sensitive). After incubation at room temperature for 30 min, the MQAE solution was removed, the preparation was then washed once and fresh non-oxygenated extracellular solution was carefully added. Next, the specimen was immediately transferred to the stage of a two-photon laser scanning fluorescence microscope (Nikon, A1 MP). The water immersion objective (25x, APO LWD 25x/1.10 W had to be adjusted carefully in the transmission mode to locate the epithelium. To identify the knobs of the sensory epithelium more precisely, I used OMP-GFP mice which express eGFP in all mature sensory neurons (Potter *et al.*, 2001). First, I imaged the sensory epithelium with light suitable for two-photon excitation of EGFP (\(\lambda = 910\) nm) and chose a region with many knobs in the focal plane. Depending on the preparation, the amount of knobs in one plane was variable. Knobs could also be identified by measuring their size (\(\sim 2\ \mu m\)). After a suitable position in the sample was found the excitation light was switched to 750 nm to acquire the MQAE FLIM image. Pooled (male / female) mouse urine (dilution, 1:100 in \(E_{\text{regular}}\)) was used for stimulation of a large VSN subset.

2.11.3 Calibration of fluorescence lifetime in cells of the VNO sensory epithelium

Because MQAE measurements display only relative changes of fluorescence intensity and lifetime a quantitative calibration with determined Cl\(^{-}\) concentrations is required to define a certain chloride concentration in cells. MQAE molecules are not only quenched by Cl\(^{-}\) but also by other ions, amino-acid residues and small cytosolic molecules. Therefore, MQAE properties depend on intracellular milieu and are consequently different for distinct cell types. In contrast to fluorescence intensity, fluorescence lifetime is independent of dye concentration and cell volume and is therefore constant for one cell type. For this reason, fluorescence lifetimes are not to be compared between different cell types. Each cell type requires calibration to gain absolute values of [Cl\(^{-}\)] and to allow comparison of intracellular Cl\(^{-}\) levels.

In our experiments, calibration was carried out by incubation of the *en face* preparation in different bath solutions with defined [Cl\(^{-}\)]. Two K\(^{+}\)-based calibration solutions with either high [Cl\(^{-}\)] or approximately 0 Cl\(^{-}\) were mixed to adjust defined Cl\(^{-}\) concentrations. Here, I first used a high [Cl\(^{-}\)] of 140 mM or 0 mM Cl\(^{-}\) in our calibration solution. Additionally, we also tried another approach for quantitative calibration, in which we used VNO slices to achieve better access for ionophores to the cells. To equilibrate the intra- and extracellular [Cl\(^{-}\)] I used a double ionophore technique employing tributylin (10 \(\mu M\)) and nigericin (10 \(\mu M\)) and a high K\(^{+}\)-based solution (Krapf *et al.*, 1988; Chao *et al.*, 1989). Tributylin is a Cl\(^{-}\)/OH\(^{-}\) exchanger, whereas
nigericin constitute a K⁺/H⁺ antiporter. Outward directed transport of OH⁻ leads to cytosolic acidification. To antagonize the intracellular change of the pH value, nigericin is required. It ensures stabilization of the physiological intracellular pH value by the outward transport of protons. The high [K⁺] in the calibration solution is therefore required as a driving force for this process. However, this technique did not lead to satisfactory results. Treatment with the ionophores did not produce uniform Cl⁻ levels in the sensory epithelium of the VNO as it did in OSNs in the MOE (Kaneko et al., 2004). Though, such an incidence has been reported before. In 2007 Gilbert and colleagues showed a functional calibration of dorsal root ganglia (DRG) in mice (Gilbert et al., 2007) and determined absolute [Cl⁻] values in DRG neurons. One year later Funk and coworkers were not able to reproduce this in rat DRG neurons (Funk et al., 2008) and instead could only observe relative changes of [Cl⁻].

2.12 **Fluorescence recovery after photobleaching (FRAP)**
FRAP was developed in the 1970s by Axelrod and co-workers as a technique to study protein mobility in living cells by measuring the rate of fluorescence recovery at a previously bleached site (Axelrod et al., 1976; Koppel et al., 1976). Originally, the FRAP technique was utilized as a method to measure diffusion in cellular membranes using lipophilic or hydrophilic fluorophores, like fluorescein, coupled to proteins and lipids (Liebman P A & Entine G, 1974; Edidin et al., 1976). In a typical FRAP experiment, fluorescent molecules are irreversibly photobleached in a small area of the cell by high intensity illumination with a focused laser beam. Photobleaching is a photochemical process in which the fluorochrome’s ability of repetitive excitation and photon emission is permanently interrupted by irreversible destruction of the fluorochrome. Subsequently, diffusion of the surrounding intact, non-bleached fluorescent molecules into the bleached area leads to recovery of fluorescence with a particular velocity, which is recorded at low laser power (Reits & Neefjes, 2001). This process is a result of the diffusional exchange between the fluorochromes. In FRAP experiments, the images are analyzed and processed to generate a kinetic plot of photobleaching by displaying the temporal fluorescence changes in the bleached region of the cell. From such plots the mobile fraction and immobile fraction and corresponding time can be calculated. Therefore, FRAP provides important insights into the properties and interactions of molecules within the cellular environment.

2.12.1 **FRAP in OSNs from MOE-slices and dissociated OSNs**
Tissue or cells were prepared as described in 2.7.1 and 2.7.4. For labeling mitochondria in living cells, the cell-permeant MitoTracker® probes were used, which contain a mildly thiol-
reactive chloromethyl moiety. To label mitochondria for FRAP experiments here, cells were incubated with 500 nM MitoTracker® Deep Red FM for 30 min at RT (Zhou et al., 2010). MitoTracker® probes passively diffuse across the plasma membrane and accumulate in active mitochondria regardless of mitochondrial membrane potential. MitoTracker® Deep Red FM has its excitation maximum at 644 nm and emission maximum at 665 nm. Here, this probe was excited with a 642 nm laser beam (laser power <20 %) (Shim et al., 2012).

After loading, residual dye containing solution was removed and replaced by fresh extracellular solution. Slices or cells were then placed onto an upright microscope (Leica DM6000 FS; water immersion objective (63x / 0.9)) and superfused with E\textsubscript{oxy}. GFP-expressing OSNs from mOR-EG mice, either dissociated or from MOE-slices, were optically identified. FRAP experiments were performed using a multi-beam confocal system with an integrated FRAP module (VT-HAWK; VisiTech Int., Sunderland, UK) under control condition (extracellular solution) or during / after stimulation with 100 μM vanillin (60 s before and after bleaching). Regions of interest (ROI) in dendritic knobs were selected and bleached with an intense laser beam (488 nm; 3 s maximum output). Fluorescence recovery in the ROI was measured over time. Relative fluorescence intensities were measured before odor application (pre-stimulus; to establish a stable baseline), during odor application (stimulation) and after odor application (post-stimulus). The relative mobile fraction and the monoexponential recovery time constant τ were calculated for each measurement.

### 2.13 Data Analysis

If not stated otherwise, results are presented as means +/- SEM and statistical analyses were performed using paired or unpaired t-tests (as dictated by experimental design). In fluorescence or life time imaging experiments, ROIs were defined to encircle cellular compartments (soma, knob) based on visually identified morphological features of dye-loaded cells. In Ca\textsuperscript{2+} imaging experiments an increase in fluorescence intensity, and in loose-patch recordings an increase in spike frequency was judged as a stimulus-dependent response if the following two criteria were both fulfilled: 1) the peak intensity / frequency value exceeded a given threshold that was calculated as the average baseline intensity / frequency before stimulation plus an intensity value corresponding to twice the baseline intensity / frequency standard deviation (\(f_{\text{peak}} > f_{\text{baseline}} + 2 \times \text{SD (}f_{\text{baseline}}))\); 2) the increase in light intensity / spike frequency was observed within 10 s after stimulus application.

Decay time constants (τ) were calculated by fitting individual traces to monoexponential or biexponential functions \(y(x) = y_0 + A \exp \{-((x-x_0) / \tau)\}\) or \(y(x) = y_0 + A_1 \exp \{-((x-x_0) / \tau_1)\} + A_2 \exp \{-((x-x_0) / \tau_2)\}\), respectively. Dose-response curves and activation / inactivation curves were fitted by the Hill equation: base + (max – base) / \(1 + [x_{\text{half}} / x]^{\text{rate}}\).
3. Results

3.1 Mitochondrial Ca\(^{2+}\) sequestration plays a key role in olfactory signaling in mice

Ionized Ca\(^{2+}\) is a key component of a variety of sensory signaling pathways in OSNs. Odor binding leads to odorant receptor activation in the olfactory cilia and to a subsequent Ca\(^{2+}\) influx via cyclic nucleotide-gated (CNG) channels (Reed, 1992; Zufall et al., 1994). Ca\(^{2+}\), in turn, then triggers a number of secondary cellular responses. One crucial mechanism regulated by Ca\(^{2+}\) is sensory adaptation (Reisert & Matthews, 1999). Ca\(^{2+}\) forms a complex with calmodulin leading to a negative-feedback modulation of the CNG channel. Remarkably, Ca\(^{2+}\)-free calmodulin (apocalmodulin) is permanently coupled with modulatory CNG channel subunits at low Ca\(^{2+}\) concentrations (<100 nM) (Bradley et al., 2004). Hence, only small changes in intracellular [Ca\(^{2+}\)] are sufficient to affect CNG channel sensitivity and, consequently, the shape of the primary odor response. Therefore, the resting cytosolic Ca\(^{2+}\) concentration has to be tightly controlled. In many cells of different species a relatively low cytosolic Ca\(^{2+}\) level is maintained by compartmentalization of both, the endoplasmic reticulum or mitochondria (Kann & Kovács, 2007) and as well by Ca\(^{2+}\) extrusion via plasma membrane Ca\(^{2+}\) ATPases (Antolin et al., 2010) and Na\(^{+}\)/Ca\(^{2+}\) exchangers (Clapham, 2007).

Previous experiments in our working group (performed by Daniela Flügge (Fluegge et al., 2012)) revealed mitochondrial Ca\(^{2+}\) uptake upon odor stimulation using mitochondrial bioluminescence Ca\(^{2+}\) imaging. Here, we investigated the role of mitochondrial Ca\(^{2+}\) mobilization in OSNs during odor-triggered stimulation using electrophysiological recordings from single OSNs. We measured the functional consequences of mitochondrial perturbation on the odor-mediated primary receptor current and the sensory output signal. Mitochondrial Ca\(^{2+}\) uptake function was impaired upon Δψ\(_m\) dissipation by the protonophore FCCP or MCU inhibition by either RuR or Ru360, its related compound. Employing the patch clamp technique, Olfr73 (mOR-EG)-expressing OSN in acute MOE slices from mOR-EG mice were activated with vanillin, which is a specific ligand for this receptor (Oka et al., 2004). Vanillin was focally applied to the MOE and induced robust responses. Based on mOR-EG/GFP co-expression, vanillin-sensitive neurons were identified by green fluorescence (Oka et al., 2006). Combining this with mitochondrial mobility assays, we report activity-dependent mitochondrial translocation to dendritic knob compartments upon odor stimulation regulating the olfactory input-output gain control.

3.1.1 Mitochondrial Ca\(^{2+}\) uptake shapes the odor-mediated primary receptor current in OSNs ensuring a broad dynamic range

Odor-induced responses in OSNs are characterized by membrane depolarization caused by primary Ca\(^{2+}\) currents through CNG channels and secondary Cl\(^{-}\) currents via ANO2 channels.
(Stephan et al., 2009). Because mitochondria play an essential role in cytosolic Ca\(^{2+}\) homeostasis, we here studied the mitochondrial influence on odor-induced response properties.

Recordings were performed in whole-cell voltage-clamp configuration from optically identified Olfr73-expressing OSNs in acute MOE slice preparations (Fig. 3.1 (A)). During whole-cell recordings, millimolar ATP concentrations were dialyzed into the cell and the membrane potential (V\(_{m}\)) was set to a physiological value of -70 mV. Cells were patched at the basal region of the soma and stimuli were applied to the apical cilia. First, experiments were performed to obtain the stimulus-response (S-R) function for vanillin. Because detection thresholds are extremely low and highly variable between OSNs, we varied stimulus strength by increasing the odor pulse duration. Vanillin (300 nM) pulses were applied sequentially from 40 ms to 3 s. Response amplitudes from representative original current traces showed a gradual increase, reaching a saturating maximum at 1 s duration under control conditions (Fig. 3.1 (B), top). When Ru360 (1 µM), FCCP (1 µM) or RuR (1 µM) were administered via the patch pipette responses saturated at a shorter stimulus duration of only 360 ms (Fig. 3.1 (B), bottom). During prolonged stimulation (3 s), the response currents exhibited progressive reduction, a characteristic of sensory adaptation. Because of desensitization effects, experiments were obtained from different sets of cells (control versus inhibitor), statistically comparing non pre-stimulated cells with each other.

Next, we plotted normalized receptor current amplitudes (I/I\(_{\text{max}}\)) from individual OSNs versus stimulus duration and fitted corresponding S-R curves using the Hill equation (Fig. 3.1 (C)). S-R curves of 15 cells under control conditions were super-imposed with 15 cells in presence of either FCCP, Ru360 or RuR, respectively. We observed a significantly (p\(_{3:3} < 0.0001\)) greater average slope for cells under drug treatment with Hill coefficients of 11.0 ± 1.2 for FCCP, 10.4 ± 0.8 for Ru360 and 13.4 ± 1.4 for RuR, compared to 3.3 ± 0.2 for controls (Fig. 3.1 (C), (D)). This increased slope is due to both a shifted odor detection threshold and a rapid approach to saturation. This results in a greatly reduced linear dynamic range when mitochondrial Ca\(^{2+}\) uptake was impaired.

In Fig. 3.2, S-R curves from Fig. 3.1 C were plotted separately for improved discrimination of individual cells. For each condition, 15 distinct cells are shown in a semi-logarithmic color-coded plot of receptor current amplitudes (I/I\(_{\text{max}}\)) as a function of stimulus duration. Sigmoid S-R curves of representative Olfr73-expressing neurons under control condition show broad dynamic ranges, but were shifted on the x-axis based on biological divergence. The same effect was observed for the three conditions when mitochondrial function was disrupted. Notably, those S-R curves displayed an increased slope and, consequently, a narrow dynamic range. Together these data indicate that mitochondrial Ca\(^{2+}\) recruitment in OSNs is essential.
to maintain a broad dynamic response range. When mitochondrial function is impaired, olfactory neurons function as simple stimulus detectors rather than intensity encoders.

Fig. 3.1: Mitochondrial Ca\textsuperscript{2+} regulation shapes the primary odor response in mouse OSNs. Olfr73-expressing neurons were stimulated with different odor concentrations under control conditions versus various drug treatments (FCCP, 1 µM; Ru360, 1 µM; RuR, 1 µM). (A) IR-DIC image showing the dorsal septum part of an acute MOE slice (thickness, 300 µm) of a mOR-EG mouse (left; scale bar, 50 μm); fluorescence image of the dashed box depicting a single eGFP-labeled Olfr73-positive neuron (middle; scale bar, 5 μm); merged IR-DIC and fluorescence image of the same area (right); d, dorsal; v, ventral. (B) Whole-cell recordings (V\text{hold} = −70 mV) of primary receptor currents of vanillin-induced responses from two representative OSNs under control conditions (top) and in the presence of Ru360 (1 µM, applied via the patch pipette; bottom). Vanillin (300 nM) was applied at increasing stimulation durations (horizontal bars) of 40 ms, 80 ms, 120 ms, 200 ms, 360 ms, 1 s and 3 s with inter-stimulus intervals of 30 s. Dashed lines indicate peak amplitudes; insets show corresponding S−R curves. (C) Super-imposed S-R curves (I/I\text{max}; Hill equation) of 15 randomly chosen OSNs under control (n = 29) and drug treatment conditions (FCCP, 1 µM; Ru360, 1 µM; RuR, 1 µM). (D) Hill coefficients (n) of vanillin-induced responses under control and drug treatment conditions (FCCP, 1 µM; Ru360, 1 µM; RuR, 1 µM).
µM, n = 44; Ru360, 1 µM, n = 49; RuR, 1 µM, n = 50). Semi-logarithmic stimulus duration is plotted against normalized response amplitudes. (D) Dot plot illustrating individual Hill coefficients (n) of single neuron S–R curves (horizontal bars, means ± SEM.: control, 3.3 ± 0.2; n = 29; FCCP, 11.0 ± 1.2; Ru360, 10.4 ± 0.8; RuR, 13.4 ± 1.4). Red dashed line indicated control mean. Significances compared to control, *p1, *p 2, *p 3 < 0.0001.

Fig. 3.2: S–R curves of individual OSNs show modified dynamic ranges of receptor responses upon drug treatment. Semi-logarithmic plot of receptor current amplitudes I/I_max (from representative individual Olfr73-expressing OSNs) as a function of stimulus duration (vanillin (300 nM); 40 ms, 80 ms, 120 ms, 200 ms, 360 ms, 1 s, 3 s). Sigmoid S–R curves are calculated using the Hill equation. Signals from individual neurons are color-coded; shown are the same 15 representative neurons for each condition as in Fig 1c. (A) Sigmoid S–R curves of 15 representative Olfr73-expressing neurons under control conditions. (B) Sigmoid S–R curves of 15 representative Olfr73-expressing neurons under FCCP treatment (1 µM). (C) Sigmoid S–R curves of 15 representative Olfr73-expressing neurons during Ru360 treatment (1 µM). (D) Sigmoid S–R curves of 15 representative Olfr73-expressing neurons under RuR treatment (1 µM). All drugs were administered via the patch pipette.

3.1.2 Basic response kinetics are not significantly changed in stimulated OSNs under control conditions versus mitochondrial Ca^{2+} uptake inhibition.

Next, we compared the odor response properties under control condition and during the three different pharmacological treatments. We asked whether mitochondrial Ca^{2+} buffering also affects sensory adaptation and analyzed response kinetics during prolonged stimulation (≥3 s). First, we examined current decay following bi-exponential kinetics, depicting an average fast decay time constant (τ_fast) of 0.15 s and average slow decay time constant (τ_slow) of 27.75 s under control conditions (Fig. 3.3 (A)). Neither FCCP nor Ru360 or RuR changed the average fast or the slow decay time constant significantly.

Second, we investigated the peak-plateau ratios (ΔI = I_max/I_plateau) during prolonged (≥3 s) stimulation (Fig. 3.3 (B)). Average peak-plateau ratios remained essentially unaffected. No statistical significance is observed between average ΔI values of 4.44 for control compared to 4.63, 4.62 and 3.36 for FCCP, Ru360 or RuR, respectively.
In addition, we tested the saturating current amplitudes for significant differences (Fig. 3.3 (C)). Average maximum current amplitudes were -153.99 ± 12.28 pA for control cells, -143.74 ± 15.04 pA for mitochondrial Ca\(^{2+}\) uptake inhibition by FCCP, -129.16 ± 13.48 pA for MCU-inhibited cells by Ru360 and -166.42 ± 15.89 pA for RuR treated cells. Consequently, maximum receptor current amplitudes were not significantly changed.

![Fig. 3.3: Analysis of response kinetics in stimulated OSNs under control conditions and mitochondrial Ca\(^{2+}\) uptake inhibition.](image)

**Fig. 3.3:** Analysis of response kinetics in stimulated OSNs under control conditions and mitochondrial Ca\(^{2+}\) uptake inhibition. Investigation of OSN sensory adaptation during prolonged vanillin exposure (300 nM; 3 to 5 s). (A) Analysis of time constants \(\tau\). Representative response trace. Fit is a bi-exponential decay (red dashed curve, top). Logarithmic bar diagram illustrating average fast and slow desensitization time constants under control (black; 0.15 ± 0.05 s (\(\tau_{fast}\)) / 27.75 ± 16.22 s (\(\tau_{slow}\))) versus \(\Delta \nu_m\) collapse (FCCP (blue); 0.15 ± 0.03 s (\(\tau_{fast}\)) / 19.78 ± 15.91 s (\(\tau_{slow}\))); MCU inhibition (Ru360 (green); 0.14 ± 0.05 s (\(\tau_{fast}\)) / 27.66 ± 10.58 s (\(\tau_{slow}\)); RuR (dark-red); 0.12 ± 0.02 s (\(\tau_{fast}\)) / 21.49 ± 13.83 s (\(\tau_{slow}\))). Values are means ± SEM; numbers of experiments are indicated above bars (bottom). (B) Analysis of peak-plateau current ratio \(\Delta I\). Representative response trace. Receptor current peak and plateau values are indicated by red dashed horizontal lines (top). Bar graph showing average peak-plateau ratios under control conditions (black; 4.44 ± 0.75) or after drug infusion (FCCP (blue), 4.63 ± 0.78; Ru360 (green), 4.62 ± 1.01; RuR (dark-red), 3.36 ± 0.97); means ± SEM; n as indicated (bottom). (C) Analysis of maximum current amplitudes. Representative response trace. Maximum receptor current amplitude and baseline are indicated by red dashed horizontal lines (top). Comparison of average saturating current amplitudes under control conditions (-153.99 ± 12.28 pA) and during inhibition of mitochondrial Ca\(^{2+}\) uptake (FCCP (-143.74 ± 15.04 pA); Ru360 (-129.16 ± 13.48 pA); RuR (-166.42 ± 15.89 pA)); means ± SEM; n as indicated (bottom). (D) Bar chart showing percentage of odor-sensitive OSNs at different odor pulse durations (vanillin (300 nM); 40 ms, 80 ms, 120 ms, 200 ms, 360 ms, 1 s; n = 37–77). Significances compared to control, *\(p\)12 ≤ 0.05; *\(p\)1, *\(p\)5, *\(p\)8 ≤ 0.01; *\(p\)2, *\(p\)3, *\(p\)4, *\(p\)6, *\(p\)10 ≤ 0.005; *\(p\)7, *\(p\)8, *\(p\)11 ≤ 0.001.

Furthermore, we compared the responsiveness of neurons under the four different treatment conditions towards the tested stimulus durations of 40 ms, 80 ms, 120 ms, 200 ms, 360 ms
and 1 s (Fig. 3.3 (D)). We recorded small, though well defined, receptor currents at 40 ms stimulus duration in 24.5% of vanillin-sensitive neurons under control conditions, but only few OSNs responded in presence of FCCP (7.4%), Ru360 (4.1%) or RuR (4.0%). Upon a 120 ms exposure to vanillin, almost all (92.5%) odor sensitive control neurons were recruited, whereas dissipation of ΔΨm or MCU inhibition significantly reduced this percentage to 50% with FCCP, 59.2% with Ru360 and 72% with RuR. These data indicate that mitochondrial Ca\(^{2+}\) mobilization in OSNs does not affect basic response kinetic properties but is required for odor-dependent OSN activation at low stimulus concentrations, thus, ensuring a broad dynamic detection range.

### 3.1.3 Investigation of passive membrane and basic response properties in OSNs

We next performed recordings in current-clamp mode to analyze membrane potential (V\(m\)) changes. Measured OSNs displayed a high input resistance (3.6 ± 0.3 GΩ; n = 23) and a resting membrane potential of −76 ± 1 mV (n = 30). To test for FCCP side effects, we recorded V\(m\) over extended periods of time (up to 180 s). Figure 3.4 (A) shows an original whole-cell current-clamp trace from an optically identified OSN (no current injection) in presence of different FCCP concentrations for prolonged periods. In (B), we quantified the data obtained from such experiments, comparing cells measured in presence or absence (control) of the protonophore and plotted the average maximum change in membrane potential ΔV\(m\) after 2 minutes. Both under control conditions and during FCCP incubation, V\(m\) was only slightly increased (+0.5 ± 0.4 mV control and +1.1 ± 0.4 mV FCCP) but remained essentially stable. ΔV\(m\) was not significantly different between control conditions and FCCP treatment.

Additionally, we investigated the spontaneous discharge frequency of OSNs in acute slice preparations from mOR-EG mice compared to C57BL/6 mice, as a wild-type control, in loose patch cell-attached configuration (Fig. 3.4 (C)). The spontaneous average spiking rates were 0.58 Hz in BL/6 control and 0.55 Hz in FCCP treated BL/6 mice, whereas spike frequencies of 0.58 Hz and 0.57 Hz were recorded from OSNs of mOR-EG mice under control conditions and in the presence of FCCP, respectively, showing no significant differences. These data indicate stable physiological OSN conditions during prolonged experiments as performed here.
Fig. 3.4: Basic passive membrane properties of OSNs are not affected by FCCP. (A) Original whole-cell current-clamp recording from an optically identified Olfr73-expressing OSN in an acute MOE slice from a mOR-EG mouse. OSNs display an average membrane potential of -76 ± 1 mV. The resting membrane potential $V_m$ is sampled over prolonged periods. Exposure to increasing FCCP concentrations (0.3 μM, 1.0 μM, 3.0 μM) mediates only marginal changes in $V_m$. (B) Quantitative analysis of OSN resting potential changes over prolonged time periods as a function of $\Delta \nu_m$ dissipation by FCCP compared to control. Average maximum change in membrane potential ($\Delta V_m$) during FCCP incubation (1 μM; 2 min) is $+1.1 \pm 0.4$ mV ($n = 8$). Under control conditions, $V_m$ shifts by $+0.5 \pm 0.4$ mV ($n = 10$); $\Delta V_m$ are plotted as means ± SEM; ns, not significantly different. (C) Quantified data of changes in spontaneous spiking under control conditions and after $\Delta \nu_m$ dissipation by FCCP. Spontaneous AP firing is sampled over a prolonged time. Bar graph depicting spontaneous discharge rates recorded in acute slices (loose-patch cell attached configuration) from randomly chosen OSNs of C57BL/6 mice (left) or Olfr73-expressing from OR-EG mice (right). Average frequencies (BL/6 control, 0.58 ± 0.26 Hz; BL/6 FCCP, 0.55 ± 0.23 Hz; mOR-EG control, 0.58 ± 0.19 Hz; mOR-EG FCCP, 0.57 ± 0.19 Hz; means ± SEM) are calculated from recordings in absence (black) and presence (blue) of FCCP (1 μM); numbers of experiments are indicated above bars.

Next, we focused on odor-evoked responses recorded in current-clamp mode. Identified Olfr73-expressing OSNs were again stimulated with vanillin and different response properties were analyzed. First, we considered the number of spikes evoked by a 1 s odor pulse (Fig. 3.5 (A)). APs were counted and plotted in a bar graph, depicting the results of control experiments versus FCCP treated cells. Though, on average, control cells showed less APs ($3.69 \pm 1$) than FCCP incubated OSNs ($8.09 \pm 3.04$), differences were not significant.

Second, we examined the maximum membrane depolarization $\Delta V_m$ upon odor stimulation (Fig. 3.5 (B)). $\Delta V_m$ remained essentially unaffected when cells were incubated with FCCP compared to untreated neurons.

As a third component of odor-triggered responses, we investigated the full duration at half maximum (FDHM) (Fig. 3.5 (C)). The presence of FCCP ($1,069 \pm 115$ ms) did not change average half-maximal response duration significantly when compared to OSNs measured under control conditions ($1,519 \pm 202$ ms).
Finally, the response rise time (10-90%) was calculated and quantified as indicated in Fig. 3.5 (D). The average rise time of 311 ± 41 ms in the control group was not significantly different from 247 ± 25 ms in FCCP treated cells.

![Fig. 3.5: Analysis of whole-cell current-clamp odor response properties.](image)

3.1.4 Mitochondrial Ca\(^{2+}\) mobilization regulates the action potential output in OSNs

Action potential firing mediates OSN information transfer to the brain. Therefore, we next performed extracellular loose-patch recordings of odor-stimulated responses in OSNs. This methodological approach allows discerning the OSN electrical output signal without disturbing the intracellular milieu and input resistance. Either FCCP or Ru360 were used as membrane permeant reagents to impair mitochondrial ability of Ca\(^{2+}\) regulation. In Olfr73-expressing neurons odor responses were triggered with vanillin at pulse durations of 40 ms, 200 ms, 350 ms, 500 ms, 1 s, 2 s, 3.5 s, 5 s and 10 s (Fig.3.6). A vanillin concentration of 10 nM was sufficient to trigger robust time-locked spike trains from a 200 ms stimulus duration in cells
under control conditions (Fig. 3.6 (A)). Therefore, we used a vanillin stimulus concentration of 10 nM for further experiments.

**Fig. 3.6: Mitochondrial Ca\textsuperscript{2+} mobilization regulates action potential output in OSNs.** Original representative extracellular recordings and corresponding average PSTHs illustrate stimulus-dependent AP discharge triggered by increasing vanillin (10 nM) exposure durations (horizontal bars (40 ms, 200 ms, 350 ms, 500 ms, 1 s, 2 s, 3.5 s, 5 s, 10 s) under control conditions (A, black) or either FCCP (1 µM, B, blue) or Ru360 (10 µM, C, green)
treatment. Traces in (A), (B) and (C) are from the same OSNs, respectively. In some recordings, stimulation-correlated baseline deflections are produced by local field potentials. Individual data points in a given PSTH (spike frequency versus time (1 s bin width)) depict means ± SEM. Numbers of experiments are indicated, black arrowheads mark stimulation onset. Asterisks indicate statistical significance ($p < 0.0001$).

Spike frequencies were averaged and plotted as peristimulus time histograms (PSTHs; 1 s bin width) to visualize spike rates and timing in relation to the external stimulus. Significant baseline versus peak differences were calculated using paired two-tailed t-tests. We observed significant time-locked odor responses to pulse durations ranging from 200 ms up to 10 s ($p^{*1} - p^{*8} \leq 0.0001$) and recorded odor-evoked mean firing rates up to $13.1 \pm 1.4$ Hz (2 s) in control cells. Sensory adaptation became evident in PSTHs as a sustained firing pattern at reduced frequencies upon prolonged odor exposure.

When mitochondrial Ca$^{2+}$ buffering was inhibited, significant increases in firing rate during short (0.2 - 0.35 s) stimulations were abolished (Fig.3.6 (B), (C)). In presence of either FCCP or Ru360, a significant increase in spike frequency was only detected in response to prolonged vanillin presentation (≥1 s with FCCP, $p^{*9} - p^{*13} < 0.0001$; ≥0.5 s with Ru360, $p^{*14} - p^{*19} < 0.0001$). These data demonstrate that inhibited mitochondrial Ca$^{2+}$ buffering leads to an impaired odor-encoded output, in particular during short stimulations.

To illustrate the highly time-locked vanillin triggered spike trains we plotted single trial firing patterns of individual neurons as raster plots (pulse durations of 0.5 s, 2 s and 10 s (Fig.3.7 (A))). Each row represents the firing pattern of a single Olfr73-expressing OSN. Vanillin-induced robust firing patterns occurred during application of all three stimulus durations, representing the odor-induced discharge output signal sent to the olfactory bulb and higher brain regions.

The drug-induced shift in output sensitivity reported in figure 3.6 was quantitatively compared to control experiments by plotting average spike response amplitudes ($\Delta f_{\text{peak}-\text{baseline}}$) versus pulse duration (Fig.3.7 (B)). Sigmoid S-R curves were fitted using the Hill equation. The fits revealed Hill coefficients of 3.3 for vanillin-triggered responses and 10.9 or 5.5 in the presence of FCCP or Ru360, respectively. The increased slope is caused by both, a shifted output signal and reduction in dynamic range when mitochondrial Ca$^{2+}$ uptake was impaired.

Next, we quantified spontaneous basal action potential discharge (Fig.3.7 (C)). The basal activity remained essentially unchanged by either FCCP or Ru360, indicating that biophysical properties remained stable during drug treatment. Together, our data suggest that mitochondria are critical determinants not only for the input, but also for the olfactory S−R output function.
Fig. 3.7: Action potential output in Olfr73-expressing OSNs is controlled by mitochondrial Ca\textsuperscript{2+} buffering. (A) Spike raster plots of vanillin-triggered (10 nM) responses of different OSNs measured under control conditions with increasing stimulus durations (0.5 s (left); 2 s (middle); 10 s (right)) depicted by black horizontal bars and gray shading; numbers of OSNs as indicated. (B) Response amplitudes Δf\textsubscript{peak} as a function of stimulus duration (vanillin 10 nM; 40 ms, 200 ms, 350 ms, 500 ms, 1 s, 2 s, 3 s, 5 s, 10 s). Sigmoid S-R curves are derived from significant vanillin-dependent changes (P ≤ 0.01) in average action potential discharge frequency (Δf = f\textsubscript{peak} − f\textsubscript{baseline}) and are calculated using the Hill equation (control, n = 36–78, η = 3.3; FCCP, n = 25–30, η = 10.9; Ru360, n = 36–41, η = 5.5). (C) Comparison of spontaneous basal AP frequency recorded from Olfr73-expressing neurons under control conditions (black; 4.19 ± 0.5 Hz; n = 54) and in presence of either FCCP (blue; 3.50 ± 0.9 Hz; n = 54) or Ru360 (green; 4.57 ± 0.7 Hz; n = 54); means ± SEM.

3.1.5 Odor-dependent mitochondrial distribution in OSN

Because mitochondria are highly dynamic organelles, we hypothesized that mitochondria might redistribute upon stimulus-induced activity. From previous experiments in our working group (done by Daniela Flügge), we learned that mitochondria travel from soma to knob regions upon odor stimulation (Fluegge et al., 2012). Ultrastructural analysis of OSN knobs showed that the content of predominantly spherical mitochondria (in unstimulated animals) shifted to an increased content of elongated morphologies as a result of stimulation (Fluegge et al., 2012). This indicates mitochondrial accumulation and fusion in areas of close proximity to where odor detection takes place.
We next asked whether stimulation-triggered mitochondrial accumulation resulted from organelle immobilization in knob regions or rather from augmented mitochondrial trafficking towards those regions. To address this question we employed the FRAP technique. Isolated neurons or MOE slices from mOR-EG mice were incubated with cell-permeant MitoTracker® Deep Red FM to stain the mitochondria. Then, circular regions spanning the entire knob of Olfr73-expressing cells were defined as photobleaching areas (Fig.3.8 (A)). MitoTracker fluorescence intensities were monitored before ((A), left), during ((A), center) and after ((A), right) bleaching under control conditions (extracellular solution) or during sustained vanillin exposure (60 s before and after bleaching) to allow mitochondrial accumulation in knob regions. Relative fluorescence intensities were normalized to average baseline intensity (pre-bleach = 1) and maximum photobleaching state, corresponding to the minimal fluorescence intensity (= 0). Recovery curves for isolated OSNs (Fig. 3.8 (B)) followed a single exponential time course and revealed an average maximum recovery to 73% under control conditions (Fig. 3.8 (C), left), whereas under vanillin exposure recovery was significantly reduced to 41% indicating a higher immobile fraction in stimulated neurons. In contrast, the mono-exponential recovery time constants were unaffected ((C), right). In OSNs from MOE-slices, normalized fluorescence recovery curves of control experiments were analyzed showing an average maximum recovery of 46% and a single exponential recovery time constant of 64 s indicating a larger less mobile and slower fraction of mitochondria than in isolated neurons (Fig. 3.8 (D), (E)). We suggest that this might be based on the increased physical stress caused by the procedure of cell dissociation during preparation. Together, these data suggest an odor-dependent mitochondrial accumulation in OSN dendritic knobs that seems to result from site-specific organelle arrest of a generally highly mobile mitochondrial population.
Fig. 3.8: Investigation of mitochondrial mobility by FRAP. For labeling of mitochondria, isolated or OSNs in MOE slices, respectively, are incubated with cell-permeant MitoTracker® Deep Red (500 nM; 20 min; RT). (A) Fluorescence micrographs of an Olfr73-expressing MitoTracker-stained isolated neuron before (left), during (middle) and 50 s after (right) knob (dashed-lined circle) photobleaching; scale bar, 2 μm. (B) Averaged recovery curves of dissociated OSNs under control conditions (n = 19) and during vanillin stimulation (100 μM; 60 s before and after bleaching; n = 19). Data (means ± SEM) are normalized to pre-bleaching values and the full extent of bleaching. (C) Bar graphs showing maximum recovery (FRAP max; left) under control (black; n = 19; 73.02 ± 7.93 %) versus stimulated (blue; n = 19; 41.07 ± 4.81 %) conditions and FRAP time constants τ (right) under control (black; n = 19; 31.77 ± 5.05 s) versus stimulated (blue; n = 19; 23.63 ± 3.13 s) conditions. Averaged τ values are calculated from single exponential fits of raw traces; means ± SEM; *p < 0.002; ns, not significant. (D) Averaged recovery curve of Olfr73-expressing OSNs in MOE slices under control conditions (n = 5). Data (means ± SEM) are normalized to pre-bleaching values and the full extent of bleaching. (E) Maximum recovery (FRAP max; left; 45.94 ± 10.98 %) and FRAP time constants τ (right; 64.19 ± 30.21 s) under control conditions (black; n = 5). Averaged τ values are determined from single exponential fits of raw traces; means ± SEM.
3.2 Electrophysiological characterization of sustentacular cells in the MOE of mice

The MOE consists of three major cell types: OSNs, basal progenitor cells and supporting cells (SCs), which are also called sustentacular cells. Each cell type is distinct in morphology and function. SCs usually have an oval shaped cell body with a length up to 20 µm and width up to 10 µm. They are situated along the apical epithelial surface, where they extend microvilli into the mucus. Most SCs possess a thick foot-like process towards the basal region of the OE (Vogalis et al., 2005a). SCs have been shown to be involved in the regulation of extracellular ionic gradients, i.e. K⁺ homeostasis in the MOE, and are able of secreting mucus (Okano & Takagi, 1974; Mellert et al., 1992). They are also thought to be responsible for the metabolism of noxious chemicals (Kulkarni et al., 1994) and they can function as phagocytes for dead cells, including OSNs (Suzuki et al., 1996). Additionally, SCs are supposed to be involved in intercellular communication via ATP (Czesnik et al., 2006; Dooley et al., 2011) and to be electrically coupled by gap junctional resistances (Vogalis et al., 2005b). Nevertheless, with one exception (Vogalis et al., 2005a) important basic electrophysiological characteristics have not been investigated. To address this, we used voltage-clamp and current-clamp whole-cell recordings of SCs in the MOE of C57BL/6 mice. Here, we analyzed different ionic conductances using different pulse protocols and pharmacological inhibitors.

3.2.1 Analysis of basic passive membrane properties of SCs in MOE slices

Passive membrane properties of cells are important because they provide crucial information how activation or inactivation of voltage-gated channels might affect the extent, time course and velocity of membrane depolarizations. Main parameters of passive membrane properties are the resting membrane potential (Vrest), the membrane input resistance (Rinput) and the membrane time constant (τmemb).

Figure 3.9 (A) shows an optically identified SC in the apical region of the MOE filled with Alexa 488 via the patch pipette. The relatively large soma and foot-like process towards the basal region could be clearly identified. This typical morphology of dye-filled cells confirmed that these cells were indeed SCs. We performed whole-cell current-clamp recordings to analyze the basic passive membrane properties. SCs had an average capacity of 14.63 ± 0.54 pF (mean ± SEM; n = 34). Next, we determined the resting membrane potential Vrest by recording the potential without current injection directly after break-through. Supporting cells showed a Vrest of -26 mV, when intra- and extracellular solutions had symmetric Cl⁻ concentrations, and -36 mV using a gluconate-based intracellular solution, containing only 15 mM Cl⁻ (Fig. 3.9 (D)). Employing a simple step protocol (Fig. 3.9 (B; top)), we obtained current-clamp traces such as the representative original trace shown in Fig. 3.9 (B) (bottom). We injected current (Imemb) to ‘clamp’ the cells at a potential of approximately -50 mV. Membrane time constant (τ) values
were calculated using a single exponential fit to voltage decays following a current step. \( \tau_{\text{memb}} \) describes how fast the voltage changes. The greater \( \tau \) is, the longer it will take to reach maximal voltage change and the slower is the decay of the voltage. We observed a relatively short average \( \tau_{\text{memb}} \) value of 4.13 ms for SCs (Fig. 3.9 (D)). Next, we defined the input resistance \( R_{\text{input}} \) of SCs (Fig. 3.9 (C), (D)) using the same step protocol as in (B). Applying the simple equation depicted in (C), we calculated an average input resistance of 255.5 MΩ for SCs, which is a relative low value compared to the much higher \( R_{\text{input}} \) of several gigaohms for OSNs.

### Table presenting basic passive membrane properties of SCs

<table>
<thead>
<tr>
<th>Passive membrane properties</th>
<th>Olfactory sensory neuron</th>
<th>Supporting cell</th>
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<tbody>
<tr>
<td>( V_{\text{rest}} )</td>
<td>- 76 mV</td>
<td>- 26 mV / - 36 mV</td>
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<tr>
<td>( \tau_{\text{memb}} )</td>
<td>n.d.</td>
<td>4.13 ms</td>
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<td>( R_{\text{input}} )</td>
<td>3.59 GΩ</td>
<td>255.5 MΩ</td>
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**Fig. 3.9: Analysis of basic passive membrane properties of SCs in the MOE.** Electrochemical properties of SCs were examined via whole-cell patch clamp experiments in acute MOE slices (thickness, 300 µm) from young (P0 – P5) C57BL/6 mice. To enable morphological identification of SCs the cells were filled with Alexa Fluor® 488 sodium salt (20 µM) via the patch pipette. (A) Fluorescence image of a representative Alexa 488-labeled SC in a part of the MOE (left) and merged with an IR-DIC image (right) of the same area showing the cell body lying adjacent to the apical surface and the process projecting towards the basal part of the OE. Dashed white lines indicate the apical surface of the OE or the patch pipette, respectively; ap, apical surface; pp, patch pipette; scale bar, 5 µm. **(B)** Original whole-cell current-clamp recording from an optically identified SC in an acute coronal MOE slice. Current is applied stepwise to the cell (+30 pA; -30 pA; 250 ms duration) as illustrated (top). Representative current trace (bottom) resulting from this current injection (above). \( \tau \) values were calculated from mono-exponential fits from raw traces (red curve). **(C)** \( R_{\text{input}} \) was determined by the displayed equation resulting from the indicated parameters (red color). **(D)** Table presenting basic passive membrane properties of SCs \((V_{\text{rest}} \) (sym. Cl⁻ intracellular solution) - 26.16 ± 1.17 mV \((n = 9\)); \( V_{\text{rest}} \) (gluconate intracellular solution) -35.72 ± 1.59 mV \((n = 7\)); \( \tau_{\text{memb}} \) 4.13 ± 0.46 ms \((n = 9\)); \( R_{\text{input}} \) 255.5 ± 41.7 MΩ \((n = 9\)) \) compared to OSNs \((V_{\text{rest}} \) (sym. Cl⁻ intracellular solution) -76 ± 1 mV \((n = 30\)); \( R_{\text{input}} \) 3.59 ± 0.31 GΩ \((n = 23\)); means ± SEM; n.d., not determined.
3.2.2 Investigation of voltage-gated Na\textsuperscript{+} channels

Voltage-gated channels mediate electrical signals that control essential functions, such as muscle contraction, central processing and perception of environmental stimuli, and are therefore typically expressed in neurons. One branch of members of this protein superfamily is the family of voltage-gated Na\textsuperscript{+} channels. Here, we investigated the role of voltage-gated Na\textsuperscript{+} channels in sustentacular cells, a cell type that shares common features with glial or epithelial cells.

We examined the physiological properties of Na\textsuperscript{+} currents from SCs located in MOE coronal slices from mice using an electrophysiological and pharmacological approach. First, we performed whole-cell voltage-clamp experiments, depolarizing the cells from negative to positive potentials by applying a voltage step protocol (Fig. 3.10 (A)). Membrane depolarization revealed prominent inward currents. We then analyzed the current-voltage relationship (IV) of those transient inward currents (Fig. 3.10 (B) and (Bi)) and observed a maximum $I_{\text{transient}}$ of -57.85 pA/pF at -8.8 mV. Next, we repeated those experiments under control conditions compared to treatment with TTX (1 µM), a specific inhibitor of voltage-dependent Na\textsuperscript{+} channels. Subsequently, TTX-insensitive currents were subtracted from control measurements uncovering the TTX-sensitive currents (Fig. 3.10 (C) and (D)). Plotting current density against membrane potential shows that $I_{\text{control}}$ is partly blocked by TTX, resulting in reduced maximum amplitudes of -23 pA/pF for TTX-insensitive and -25 pA/pF for TTX-sensitive currents. Next, we quantitatively described these data in a bar chart showing maximum inward currents normalized to the corresponding maximum current $I_{\text{max}}$ under control conditions (100 %; TTX-insensitive, 53.5 %; TTX-sensitive, 62.8 %) (Fig. 3.10 (E)).

Next, we checked for the existence of persistent Na\textsuperscript{+} currents, which would be indicated by an additional sustained inward current upon depolarization to voltages below the Na\textsuperscript{+} reversal potential. At the potential which evoked the maximum transient current, no lasting deviation from the baseline ($\Delta I_{\text{offset}}$), became evident (Fig. 3.10 (F)).

Next, we focused on $I_{\text{Na}}$ inactivation (Fig. 3.11). Using an inactivating pre-pulse protocol, we again performed recordings in voltage-clamp mode under control conditions and in presence of 1 µM TTX. As described, TTX-insensitive currents were subsequently subtracted to obtain the TTX-sensitive currents (Fig. 3.11 (A) and (B)). By plotting $I_{\text{Na}}$ current density amplitudes as a function of pre-pulse depolarization, we obtained steady-state inactivation curves. Inactivating Na\textsuperscript{+} currents were only partly blocked by TTX (Fig. 3.11 (C)). Data quantification shows that TTX-insensitive $I_{\text{max}}$ was 64% and TTX-sensitive $I_{\text{max}}$ was 59 % of maximum current of controls (Fig. 3.11 (D)). Maximum amplitudes of TTX-insensitive and TTX-sensitive currents exceeded 100 %, since $I_{\text{max}}$ of each condition (control, TTX-insensitive and TTX-sensitive) was measured at different step potentials (see Fig. 3.11 (C)).
Fig. 3.10: Supporting cells show voltage-dependent TTX-sensitive Na⁺ currents. (A) Schematic drawing of the depolarizing voltage step protocol used to activate Na⁺ channels in SCs. Cells are depolarized (5 mV intervals) from -123.8 mV up to +71.2 mV for 30 ms; LJP was corrected offline. (B) Current density plotted against voltage steps (shown in (A)) voltage-dependent inward currents as depicted in (Bi) (representative example traces); (I Na_max = -52.59 pA/pF at -8.8 mV; E_Na⁺ = +67.5 mV); means ± SEM; n as indicated. (C) Representative original traces revealing voltage-dependent inward and outward currents under control conditions (black; top) and in presence of TTX (1 µM; incubation, 30 s before and during measurement; dark blue; middle) and digitally subtracted traces uncovering the TTX-sensitive current (I_control - I_TTX = I_TTX insensitive) (light blue; bottom). (D) Graph illustrating current-voltage relationship of Na⁺ inward currents under control conditions (black; I Na_max = -45.27 pA/pF at -23.8 mV) with TTX treatment (dark blue; I Na_max = -23.49 pA/pF at -18.8 mV) and TTX-sensitive currents (light blue; I Na_max = -25.29 pA/pF at -38.8 mV); (E_Na⁺ = +67.5); means ± SEM; n as indicated. (E) Quantitative analysis of maximum Na⁺ inward currents normalized to the corresponding maximum current I Na_max under control: TTX insensitive, 53.5 ± 5.43 %; TTX sensitive, 62.8 ± 4.77 % (means ± SEM); n as indicated. (F) SCs do not show persistent Na⁺ currents. Depolarizing voltage step protocol used for activation of Na⁺ channels (top). Representative original current trace (red; bottom) generated by the depicted voltage step (red mark). Dotted black line indicates offset. No ΔI_offset after the transient current is observed during the prolonged voltage step.
Fig. 3.11: Investigation of voltage-dependent and TTX-sensitive Na⁺ current inactivation in SCs. (A) Schematic illustration of the voltage step protocol used for Na⁺ channel inactivation. Stepwise depolarization (5 mV intervals) from -123.8 mV up to +51.2 mV (10 ms) followed by a constant voltage step (16.2 mV; 30 ms duration); LJP was corrected offline. (B) Representative original traces produced by the voltage step protocol in (A). Transient voltage-dependent inward currents under control conditions (black; top) compared to TTX conditions (1 µM; incubation, 30 s before and during measurement; dark blue; middle) and subtracted traces (TTX-sensitive current; light blue; bottom). (C) Current-voltage relationship of control (I Naₘₐₓ = -49.06 pA/pF at -108.8 mV; I Naₐᵢₙₓ at -8.8 mV) compared to TTX-insensitive (I Naₘₐₓ = -28.35 pA/pF at -103.8 mV; I Naₐᵢₙₓ at 13.8 mV) and TTX-sensitive (I Naₘₐₓ = -23.19 pA/pF at -118.8 mV; I Naₐᵢₙₓ at -13.8 mV) current, current density amplitude plotted against voltage steps; means ± SEM; n as indicated. (D) Bar chart depicting normalized I Naₘₐₓ before (control, 100 ± 0 %) and after TTX treatment (TTX-insensitive, 64 ± 4.62 %; TTX-sensitive, 59.4 ± 6.61) (means ± SEM); n as indicated. Normalized I Na current densities recorded from SCs were fitted with a sigmoid function according to the Boltzmann equation and plotted as superimposed activation and inactivation curves (Fig. 3.12). Fig. 3.12 (A) describes the activation and inactivation under control conditions. Curves revealed relatively large slope values and accordingly a considerable window current. By contrast, the TTX-insensitive and TTX-sensitive population alone showed much steeper slope values and shifted maximal activation and complete inactivation, resulting in considerable smaller window currents meaning that less depolarization is required for maximum I Na activation. While full activation (100%) was achieved at +11.2 mV for combined populations (control), TTX-insensitive currents were fully activated at -8.8 mV. TTX-sensitive currents were fully activated at + 1.2 mV. This is also reflected in more negative potentials of half maximal activation (xᵢₕₐᵢₙₓ) of -41.3 and -42.8 mV for TTX-insensitive or TTX-sensitive channels, respectively, compared to control (xᵢₕₐᵢₙₓ = -35 mV). Similar effects were observed for inactivation curves. Approximate complete inactivation (≤ 5 %) occurred at -13.8 mV for mixed...
populations (control), while TTX-insensitive / TTX-sensitive fractions inactivated at -33.8 and -43.8 mV, which is also reflected in the more negative \( x_{\text{half}} \) values (see Fig. 3.12). Thus, our data show that there is a large transient inward conductance, which is in part inhibited by TTX, suggesting that there are at least two major voltage-dependent channel population in SCs contributing to the transient inward current: TTX-insensitive ion channels and TTX-sensitive Na\(^+\) channels. The TTX insensitive population might consist of another type of Na\(_v\) channel and / or Ca\(_v\) channel.

Fig. 3.12: Analysis of activation and inactivation of Na\(^+\) currents in SCs. (A)-(C) Normalized \( I_{\text{Na}} \) is plotted against injected voltage steps. All curves are fitted with Boltzmann sigmoid equation; \( x_{\text{half}} \) and slope for activation (black) and inactivation (red) and \( n \) as indicated; values were set to 0.0 after reaching 0 % \( I_{\text{Na}} \) (inactivation) or to 1.0 when reaching 100% \( I_{\text{Na}} \) (activation). (A) \( I_{\text{Na}} \) is fully activated (100 %) at +11.2 mV and completely inactivated (0 %) at -3.8 mV under control conditions. (B) TTX-insensitive current reaches total activation at -8.8 mV and absolute inactivation at -28.8 mV. (C) \( I_{\text{Na}} \) is extensively activated at 1.2 mV and inactivated overall at -23.8 mV for TTX-sensitive current.

3.2.3 Voltage-activated K\(^+\) currents

Voltage-gated K\(^+\) (K\(_V\)) channels are activated by depolarization, usually allowing an outward movement of K\(^+\) ions based on the electrochemical ion gradient. This ion channel family belongs to the six transmembrane domain (6TM) superfamily of 4 different classes of K\(^+\)
channels (Coetzee et al., 1999; Choe & Jolla, 2002). One important subfamily of the 6TM family is constituted by the Ca\(^{2+}\)-activated K\(^+\) (K\(_{\text{Ca}}\)) channels. This family can be classified into three major groups: big conductance Ca\(^{2+}\)-activated K\(^+\) channels (BK\(_{\text{Ca}}\)), small conductance channels (SK\(_{\text{Ca}}\)) and intermediate conductance channels (IK\(_{\text{Ca}}\)) (Stocker, 2004). Here, we focused on BK\(_{\text{Ca}}\) channels. These channels are synergistically activated by both depolarization and intracellular Ca\(^{2+}\) and can be blocked by relatively low TEA concentrations (~1 mM) (Berkefeld et al., 2010).

First, we used whole-cell voltage-clamp experiments to characterize TEA-sensitive currents employing the same voltage step protocol as for Na\(^+\) channel investigation (see Fig. 3.10 (A)). A large voltage-dependent K\(^+\) current occurred during depolarizing voltage steps (Fig. 3.13 (A), (D)). Currents sensitive to 1 mM or 30 mM TEA, respectively, were isolated by digital subtraction (Fig. 3.13 (B) - (E)). A substantial part of the maximum voltage-gated K\(^+\) current was blocked by a low TEA concentration (TEA 1 mM-insensitive, 46.9 %; TEA 1mM-sensitive, 55.21 %) and was completely abolished by high TEA concentrations (TEA 30 mM-insensitive, 2.3 %; TEA 30 mM-sensitive, 97.91 %) (Fig. 3.13 (G)). These data suggest that about half of the voltage-gated K\(^+\) current in SCs could be conducted by BK\(_{\text{Ca}}\) channels.

Because BK\(_{\text{Ca}}\) channels require Ca\(^{2+}\) for full activation (Berkefeld et al., 2010), we next examined the contribution of voltage-activated Ca\(^{2+}\) currents to I\(_{\text{BK}}\) using Cd\(^{2+}\) (200 µM) as an unspecific Ca\(_v\) channel blocker. This treatment prevents a Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\)-channels and thus an increase of intracellular Ca\(^{2+}\). By subtracting Cd\(^{2+}\)-insensitive currents from corresponding control traces, we isolated the Cd\(^{2+}\)-sensitive currents, corresponding to the fraction of I\(_{\text{BK}}\) activated by the Ca\(^{2+}\) current through Ca\(_v\) (Cd-sensitive, 57.52 %) (Fig. 3.13 (F), (G)). Together these data demonstrate expression of TEA- and Cd\(^{2+}\)-sensitive currents in SCs.

We next investigated the functional expression of SK\(_{\text{Ca}}\) channels in SCs (Fig. 3.13 (H)). To address this question, we used a voltage protocol that applied a brief hyperpolarizing voltage injection followed by a short depolarizing step and a sustained phase at repolarizing V\(_{\text{hold}}\) (top). The short hyperpolarization ensured the excitability while the depolarization ensured the complete activation of Ca\(_v\) channels. This is crucial because SK channels are solely activated by transient elevation of [Ca\(^{2+}\)]. For isolation of the SK channel-dependent K\(^+\) current, we also inhibited TTX-sensitive Na\(^+\) channels. Apamin, a specific SK channel blocker extracted from honey bee venom, was used to isolate SK\(_{\text{Ca}}\)-specific currents (Grunnet et al., 2001). We observed a transient current following depolarization, indicating that Ca\(^{2+}\) influx occurred as a pre-condition for SK\(_{\text{Ca}}\) currents. However, we found no SK\(_{\text{Ca}}\)-specific currents (control) and consequently no apamin-sensitive current in SCs.
Fig. 3.13: Supporting cells display voltage-dependent Ca$^{2+}$-activated TEA-sensitive K$^+$ currents. Experiments were performed using the depolarizing voltage step protocol as referred to in 3.10 (A). (A) –(C) Representative original traces show large voltage-dependent outward currents under control conditions (A); red box indicates
relevant data points) and during drug treatment (1 mM TEA) (B); incubation, 30 s before and during measurement; dark blue, TEA-insensitive; light blue, TEA-sensitive) or in the presence of 30 mM TEA (C); incubation, 30 s before and during measurement; dark green, TEA-insensitive; light green, TEA-sensitive. (D) Graph exemplifying current-voltage relationship of K+ outward currents under control conditions in comparison to TEA 1 mM-insensitive and TEA 1mM-sensitive current amplitudes; (Exk + = -84.7 mV) ; means ± SEM; n as indicated in (G). (E) Current density plotted against voltage steps showing K+ outward current amplitudes under control conditions versus TEA 30 mM treatment; means ± SEM; n as indicated in (G). (F) Current-voltage relationship of K+ outward currents under control conditions and incubation with Cd²⁺ (200 µM) and TTX (1 µM); means ± SEM; n as indicated in (G). (G) Bar diagram comparing normalized IKmax under control conditions (100 ± 0 %) and after drug treatment (TEA 1 mM-insensitive, 46.9 ± 4.56 %; TEA 1mM-sensitive, 55.21 ± 3.48 %; TEA 30 mM-insensitive, 2.3 ± 1.64 %; TEA 30 mM-sensitive, 97.91 ± 1.54 %; Cd-insensitive, 43.77 ± 4.03 %; Cd-sensitive, 57.52 ± 4.15 %) (means ± SEM); n as indicated. (H) Investigation of small-conductance K+ (SK) channels reveals no detectable SK in SCs. Short hyperpolarizing voltage injection is followed by a depolarizing step (100 ms) and a prolonged phase at Vhold (top). Representative original current traces, recorded from a SC in the presence of following blockers, used to isolate SK current: TEA, 1 mM; TTX, 1 µM; (control). Apamin, 100 nM was applied additionally and Apa-insensitive current was digital subtracted to gain the Apa-sensitive current (blue and light-blue)). We observe no Apa-sensitive SK in SCs; n as indicated.

3.2.4 Hyperpolarization-activated cyclic nucleotide-gated channels

Since we found hyperpolarizing K+ currents in SCs, we next checked for the existence of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. The slowly hyperpolarization-activated inward current produced by HCN channels is known as Ih and carried by Na+ and K+ ions, that depolarize the membrane potential (Kamondi & Rein, 1991; DiFrancesco, 1993). HCN channels were found in olfactory tissues, i.e. in both OSNs (Mobley et al., 2010) and VSNs (Dibattista et al., 2008). Here, we investigated their presence in SCs of the MOE.

We performed whole-cell current-clamp (Fig. 3.14 (A)) as well as voltage clamp (Fig. 3.14 (B)) experiments in visually identified SCs using hyperpolarizing current injections or voltage steps. Neither a voltage “sag” induced by hyperpolarizing current steps, a hallmark of Ih, nor the slow activation typically induced by hyperpolarizing voltage steps was observed. Therefore, we suggest that HCN channels are not functionally expressed in SCs of the mouse olfactory epithelium.

Fig. 3.14: Investigation of hyperpolarization-activated cyclic nucleotide-gated channels in SCs. (A) Representative original traces recorded in current-clamp mode. No typical sag potential was observed after hyperpolarizing current injection (stepwise; -20 pA intervals; 500 ms duration); n as indicated. (B) Original whole-cell voltage-clamp recording induced by the hyperpolarizing voltage step protocol shown above (step intervals, 10 mV; from -63.8 mV to -203.8 mV for 700 ms) n as indicated. No HCN channel activation can be observed.
3.2.5 Voltage-gated Ca\(^{2+}\) currents

Ca\(^{2+}\) ions orchestrate a number of intracellular events in different cell types, e.g. the activation of other ion channels such as K\(_{Ca}\) channels. The voltage-gated Ca\(^{2+}\) channel superfamily is divided into three subfamilies, Ca\(_{v}\)1 - 3. Ca\(_{v}\)1 and Ca\(_{v}\)2 families are high voltage-activated channels, Ca\(_{v}\)3 proteins are low voltage-activated channels. Here, we examined the contribution of persistent versus transient voltage-gated Ca\(^{2+}\) currents utilizing a depolarizing voltage step protocol (Fig. 3.15 (A)) and Cd\(^{2+}\) as a broad Ca\(_{v}\) channel blocker.

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**Fig. 3.15: Characterization of voltage-gated Ca\(^{2+}\) currents.** (A) Schematic illustration of the depolarizing voltage step protocol used to activate Ca\(^{2+}\) channels in SCs. Cells were depolarized (5 mV intervals) from -125.5 mV up to +69.5 mV for 200 ms; LJP was corrected offline. (B) Representative original traces recorded in voltage-clamp mode under control conditions (extracellular solution: TEA 25 mM, BaCl 5 mM, 4-AP, TTX 1µM; intracellular solution: K\(^{+}\) is substituted with Cs\(^{+}\); top) or after Cd\(^{2+}\) incubation (same conditions as control plus Cd\(^{2+}\) 200 µM; bottom). Colored boxes indicate relevant data points for I\(_{Ca}\)T-Type (blue box) or I\(_{Ca}\) persistent (green box) analysis, respectively. (C) Current-voltage relationship of Ca\(^{2+}\) T-Type inward currents under control conditions (I\(_{Ca}\)max = -22.92 pA/pF at -20.5 mV) compared to Cd\(^{2+}\)-insensitive (I\(_{Ca}\)max = -13.85 pA/pF at -20.5 mV) and Cd\(^{2+}\)-sensitive (I\(_{Ca}\)max = -11.79 pA/pF at -25.5 mV) current (E\(_{Ca2+}\) = 137.7 mV); means ± SEM; n as indicated. (D) Current density plotted against voltage steps comparing I\(_{Ca}\) persistent I-V relationships of control to Cd\(^{2+}\)-insensitive or Cd\(^{2+}\)-sensitive currents; means ± SEM; n as indicated; average curves were offset corrected (I\(_{Ca}\) T-Type: control, +2.75; Cd\(^{2+}\)-insensitive, +2.61; Cd\(^{2+}\)-sensitive, +3.56), (I\(_{Ca}\) persistent: control, +1.46; Cd\(^{2+}\)-insensitive, +1.5; Cd\(^{2+}\)-sensitive, +0.34).

To investigate I\(_{Ca}\) we pharmacologically isolated voltage-gated Ca\(^{2+}\) currents using Cs\(^{+}\)-based intracellular solution and E\(_{Ca}\) extracellular solution (see Materials and Methods) to which we added 4-AP (10 mM) and TTX (1 µM). Cd\(^{2+}\)-sensitive currents were obtained by subtracting
Cd\textsuperscript{2+}-insensitive from control currents and the transient and persistent component were quantitatively analyzed (Fig. 3.15 (B)). When we plotted maximal current amplitude against membrane voltage, we observed a transient Ca\textsuperscript{2+} current (I\textsubscript{CaT}) with threshold activation around -50 mV for Cd\textsuperscript{2+}-sensitive currents and between -65 and -60 mV for Cd\textsuperscript{2+}-insensitive currents (Fig. 3.15 (C)). These data revealed the existence of T-type Ca\textsubscript{v} channels with an activation near the SC resting membrane potential, a hallmark of LVA channels. The population of Cd\textsuperscript{2+}-insensitive currents may be attributed to the TTX-insensitive Na\textsuperscript{+} channels. By contrast, we found no persistent Cd\textsuperscript{2+}-sensitive Ca\textsuperscript{2+} current in SCs (Fig. 3.15 (D)), indicating a lack of Ca\textsubscript{v}1 and Ca\textsubscript{v}2 channels in SCs in the MOE of mice.

3.2.5 Sustentacular cells do not show odor-evoked activation

Because OSNs are encircled by supporting cells we hypothesized that SCs may also react somehow to odor stimulation, e.g. by regulation of the extracellular ionic milieu. To address this question I used mOR-EG mice as a tool to identify SCs in close proximity to GFP-labeled vanillin-responsive OSNs. For better discrimination of both cell types, we used Alexa 555 to stain SCs (Fig. 3.16 (A)). This way, we ensured the activation of a neuron in close contact to the investigated SC upon stimulation with vanillin. Additionally, we used an odor mixture of 10 components, 100 nM each, to activate distinct subsets of OSNs. Neither voltage-clamp, nor current-clamp recordings revealed odor-triggered responses in SCs (Fig. 3.16 (B) and (C)). Thus, our data suggest that there is no direct activation upon odor stimulation in SCs in close proximity to responding neurons under the employed conditions.

Fig. 3.16: Investigation of odor sensitivity in SCs. Either SCs of C57BL/6 mice or mOR-EG animals were stimulated with odorants (odor mix (10 components, 100 nM each; see Materials and Methods) or vanillin (100 nM), respectively). (A) Low-magnification IR-DIC image presenting the dorsal septum part of an acute MOE slice from a
mOR-EG mouse (top; scale bar, 20 μm). Dashed black box indicate the magnified area of the MOE; ap, apical surface; ba, basal layer; pp, patch pipette; se, septum. **Bottom:** Fluorescence image of a representative eGFP-labeled Olfr73-expressing OSN (left); scale bar, 10 μm. Micrographs of the same area showing an Alexa 555 (20 μM) filled SC (middle-left), an IR-DIC image (middle-right) and fluorescence images of both channels merged with the IR-DIC image (right). Cell bodies of both, OSN and SC, lying in close proximity to each other. **(B)** Original whole-cell voltage-clamp (top) or current-clamp (bottom) recording, respectively, from an SC in a proximate distance to an optically identified Olfr73-expressing OSN in an acute MOE slice from mOR-EG mouse, stimulated with vanillin (100 nM; 1 s duration); n as indicated. **(C)** Representative original voltage-clamp (top) or current-clamp (bottom) recording, from randomly chosen SCs in the MOE of C57BL/6 mice, stimulated with an odor mixture (10 components, 100 nM each; 1 s duration). No odorant-triggered responses occurred in SCs; n as indicated.
3.3 The juvenile mouse pheromone ESP22 activates vomeronasal sensory neurons

Pheromones are powerful regulators of mouse social behavior because they are mediating information about sex, age and physiological state of an individual. Several mouse behaviors require this information conveyed by an intact olfactory system. But they involve pheromones that remain unknown in most cases. Only a handful of pheromones have been identified until now. Starting this project, we collaborated with the laboratory of Prof. Dr. Stephen D. Liberles from Harvard Medical School. Dr. Liberles’ group identified several peptides with an age-dependent production in the extraorbital lacrimal gland (LG). This included exocrine gland secreting peptide 22 (ESP22) produced by juveniles. Since any juvenile pheromone was so far unknown, additional studies on ESP22 were performed. Here, we addressed the question whether ESP22 is detected by the mouse olfactory system. Because another pheromone of the ESP family, ESP1, activates basal VSNs (Kimoto et al., 2005) we examined electrophysiological responses in the VNO.

3.3.1 VSNs show dose-dependent activation upon ESP22 stimulation.

First, we used extracellular loose-seal recordings to examine ESP22 responses at single-cell units in acute coronal slices from C57BL/6 mice (Fig. 3.17 (A)). Therefore, recombinant ESP22 was prepared as a fusion protein with maltose binding protein (MBP) to enhance solubility (done in the group of Dr. Liberles). Different concentrations of ESP22, rMBP alone as a negative control (200 nM), and elevated $K^+$ (50 mM), as a positive control for excitability, were applied to the VNO sensory epithelium. In an exemplary dose-response measurement, threshold ESP22 responses occurred at concentrations of 20 pM (Fig. 3.17 (B)). Whereas ESP22 evoked highly repetitive responses at different concentrations, MBP alone did not trigger similar spike patterns. Next, we quantified the percentage of responsive cells relative to $K^+$ viability controls (= 100%) (Fig. 3.17 (C)). These data showed that the number of responsive cells was also dose-dependent. While 1.3% of cells responded to 20 pM ESP22, 2.3% were activated by a 100-fold increased concentration. This amount is consistent with the detection by one or a few VNO receptors.
Fig. 3.17: ESP22 activates VSNs in VNO slice preparations in a dose-dependent manner. Electrophysiological ESP22-triggered responses are examined using single-unit extracellular loose-seal recordings from distinct VSNs in VNO slices (200 µm thickness) from C57BL/6 mice. (A) Low-magnification IR-DIC image showing an acute VNO slice (left; scale bar, 50 µm) and a higher magnification (right; scale bar, 20 µm) with a patch pipette attached to the soma of a single neuron of the basal layer; bl, basal layer; bv, blood vessel; pp, perfusion pencil. (B) Representative original extracellular loose-seal recording from a single basal neuron, repetitively exposed to different stimuli and concentration of ESP22 (rMBP, 200 nM; ESP22, 2 pM, 20 pM, 200 pM, 2 nM; K⁺, 50 mM; 3 s duration; inter-stimulus intervals, 30 s; blue horizontal bars indicate stimulus application). (C) Bar chart depicting the percentages of basal VNO sensory neurons responsive to different concentrations of ESP22 in a dose-dependent way.

3.3.2 VSNs responding to ESP22 are also activated by the natural source of ESP22: juvenile tear fluid.

Because ESP22 was found in homogenates of extraorbital lacrimal glands (LG) that are responsible for tear fluid production, we tested if diluted juvenile tear fluid activates VSNs. Again, we performed single-unit extracellular loose-seal recording from single VNO sensory neurons repeatedly exposed to different stimuli. We observed that most neurons responsive to ESP22 were also activated by juvenile tears but not by MBP or adult tears, with neuron viability verified by K⁺-mediated depolarization, as indicated by a representative original trace of one example cell (Fig. 3.18 (A)). Recordings also revealed response reproducibility. Spike frequencies of repetitive responses from one cell, shown in (A), were averaged using PSTHs (1 s bin width). ESP22 responses (n = 7) were compared to discharge patterns upon juvenile (n = 6) or adult (n = 6) tear fluid exposure, respectively, to visualize the spike rates and timing in relation to the external stimulus (Fig. 3.18 (B)).
Fig. 3.18: ESP22-responding neurons can also be activated by pup tear fluid, the natural source of ESP22. (A) Original extracellular loose-seal recording from a basal VSN repeatedly exposed to different stimuli (ESP22, 2 nM, 200 nM; pup tear fluid, 1:2000; adult tear fluid, 1:2000; rMBP, 200 nM; K⁺, 50 mM; 3 s duration; inter-stimulus intervals, 30 s; blue horizontal bars indicate stimulus application) indicating reproducibility of responses. (B) PSTH comparing average stimulus-dependent AP discharge to ESP22, pup tear fluid or adult tear fluid from repetitive responses from one cell shown in (A). Spike frequency increase was judged as a stimulus-dependent response if the following criterion was fulfilled: peak frequency value exceeded a given threshold that was calculated as the average baseline frequency before stimulation plus twice the baseline frequency standard deviation ($f_{\text{peak}} > f_{\text{baseline}} + 2 \times \text{SD (f_{\text{baseline}})}$): ESP22 (n = 7); $p^1 = 0.001; \text{pup tear fluid (n = 6); } p^2 = 0.002; \text{adult tear fluid (n = 6); } \text{ns = not significant. Individual data points in a given PSTH (spike frequency versus time (1 s bin width)) depict means ± SEM; stimulus application starts at 0 s.} \text{(C) Table illustrating the percentages of basal VSNs responses to different stimuli, referring to different combination possibilities.}
PSTHs confirmed significant time-locked responses to ESP22 ($p^{*1} = 0.001$) and pup tear fluid ($p^{*2} = 0.002$) but not to adult tear fluid (ns, $p = 0.17$) when the following criterion was satisfied: peak frequency values exceeded a given threshold that was calculated as the average baseline frequency before stimulation plus twice the baseline frequency standard deviation. Extracellular loose-seal recordings were summarized as depicted in table Fig. 3.18 (C). The percentage of responsive basal VNO sensory neurons was correlated to combinatorial activations to different stimuli.

In summary, these findings indicate that ESP22 is a lacrimal peptide that is secreted into juvenile tears and serves as a chemo signal that activates the VNO response pathway.

### 3.3.3 ESP22-induced cytosolic Ca$^{2+}$ transients in VSNs of VNO slices

Next, we screened ESP22-triggered Ca$^{2+}$ transients performing confocal cytosolic Ca$^{2+}$ imaging. We measured the fluorescence intensities in user-defined ROIs visualized as a function of time. Therefore, acute VNO slice preparations loaded with the Ca$^{2+}$-sensitive reporter dye fluo-4/AM were challenged with different stimuli (Fig. 3.19 (A) – (D)). Ca$^{2+}$ signals in response to K$^+$ (50 mM)-mediated membrane depolarization, as a control for cell viability, were observed in 54.4 % of all counted cells (Fig. 3.19 (B)). Next, urine, a known potent activator of a large subset of VSNs, was used to test for ligand-triggered responses (Fig. 3.19 (C)). Urine elicited Ca$^{2+}$ responses in 20% ($n = 158$) of K$^+$-responsive cells ($n = 787$).

To investigate whether ESP22 was also detected by females, we next focused on ESP22-induced Ca$^{2+}$ transients in virgins versus sexually experienced females (Fig. 3.19 (D) – (F)). 200 nM ESP22 were applied onto the sensory epithelium of the C57BL/6 females. We challenged the cells with a single, repetitive or triple stimulus (60 s inter-stimulus intervals) and monitored the response probabilities of different activation patterns. Fig. 3.19 (D) shows an exemplary trace of a significant increase in fluorescence intensity upon a 10 s ESP22 stimulation in a VSN soma from a virgin female mouse.

Summarized in a bar diagram, our data show that ESP22-mediated Ca$^{2+}$ responses occurred with various probabilities for different response profiles (Fig. 3.19 (E)). Note, however, that theoretically possible response patterns which, according to our hypothesis make no biological sense, were not or only rarely (1.3 %; 2nd stimulus alone; virgin) represented. Table illustrates number of cells and experiments corresponding to the bar diagram in (E) (Fig. 3.19 (F)). Together, these results indicate a repetitive induction of ESP22-dependent Ca$^{2+}$ transients in basal sensory neurons in the VNO.
Fig. 3.19: Imaging of cytosolic Ca\(^{2+}\) dynamics in stimulated VSNs. (A) Confocal fluorescence image of a coronal VNO slice incubated with the Ca\(^{2+}\)-sensitive reporter dye fluo-4/AM (2 μM; for 30 min at RT), depicting a part of the sensory epithelium (left; scale bar, 50 μm). IR-DIC image (center) of the same region and merged image (right); bl, basal lamina. (B) Cell viability of visually identified fluo-4-labeled VSNs. 54.4 % (n = 285 / 524) of the total number of counted cells (100 %; n = 524) show [Ca\(^{2+}\)]\(_i\) transients in response to K\(^+\)-mediated membrane depolarization. (C) Stimulus-dependent [Ca\(^{2+}\)]\(_{cytosol}\) fluorescence signal. Original trace from representative single VSN illustrating [Ca\(^{2+}\)]\(_{cytosol}\) level (F/F\(_{versus}\) time) during resting conditions and stimulation (pooled mouse urine, 1:100; K\(^+\), 50 mM; 10 s duration). Arrowheads mark stimulation onsets (blue, urine; black, K\(^+\)). Bar diagram shows urine-mediated [Ca\(^{2+}\)]\(_{cytosol}\) response probability (20 %; n = 158 / 787) relative to K\(^+\) (50 mM; 100 %; n = 787) viability controls. (D) [Ca\(^{2+}\)]\(_{cytosol}\) transient recorded from the soma of single VSN challenged with ESP22 (200 nM) and an elevated external K\(^+\) concentration (50 mM); fluorescence intensities \(_{versus}\) time. (E) Bar chart describing different ESP22-mediated [Ca\(^{2+}\)]\(_{cytosol}\) response probabilities comparative to K\(^+\) (50 mM) viability controls (100 %). A single stimulus exposure triggers [Ca\(^{2+}\)]\(_{cytosol}\) transients in 2.8 % (virgin female C57BL/6 mice) and 1.3 % (sexually experienced female C57BL/6 mice) of trials. Repetitive ESP22-evoked responses are observed in 2.6 % (virgin) and 0.43 % (experienced) or in 0 % (virgin) and 0.5 % (experienced), respectively, upon triple stimulation. Response patterns that, biologically, make no sense do not or only rarely (1.3 %; 2\(^{nd}\) stimulus alone; virgin) occur (n as indicated in (F)). (F) Table depicting the number of cells and experiments corresponding to the bar diagram in (E).
3.4 Excursus: Single-cell electroporation as an imaging approach in VNO slices

Because conventional $[\text{Ca}^{2+}]_{\text{cytosol}}$ imaging in VNO sections was not free of slight tissue movements, it was sometimes difficult to track a single VSN soma over the entire measurement duration. Moreover, diffuse fluorescence signals made it hard to distinguish neighboring cells from each other. We therefore established a novel labeling and imaging approach. Instead of incubating slices with the membrane permeable $\text{Ca}^{2+}$-sensitive reporter dye fluo-4/AM, we here loaded single cells with the cell impermeable fluo-4 via electroporation. This technique allows transient exchange of charged material (here, fluorescence dyes) across the temporarily perturbed cell membrane (see Material and Methods).

Fig. 3.20 demonstrates that, in a single VNO slice, 20-30 cells were successfully loaded with fluorescence dye within 10 - 15 min using targeted electroporation. Upon applying the ejecting voltage pulse, dye was not only transferred into the cell, but also in small amounts to the surrounding from where it is quickly removed by the laminar flow of extracellular solution (Fig. 3.20 (A)). In cells, the dye diffused within seconds from the injection site at the basal soma to dendritic regions revealing the typical VSN bipolar morphology. Systematically beginning at one margin of the sensory epithelium, cells were successively electroporated along a virtual margin-to-margin axis (Fig. 3.20 (B) and (C)). Using this technique, enhanced contrast allowed the discrimination of single cells against the unstained epithelial background.

Next, we aimed to examine stimulus-evoked $[\text{Ca}^{2+}]_{\text{cytosol}}$ signals (Fig. 3.21) in VSNs that were dye-loaded by electroporation. Therefore, cells were stimulated with 50 mM and 100 mM K$^+$ solution (10 s duration), respectively, and $[\text{Ca}^{2+}]_{\text{cytosol}}$ levels were monitored as a function of time during resting conditions and stimulation. An original trace from a VSN located in the depicted VNO slice illustrates $\text{Ca}^{2+}$ transients in response to both K$^+$ concentrations (Fig. 3.21 (Ai)).

In another experiment, we recorded elevated $[\text{Ca}^{2+}]_{\text{cytosol}}$ during and after K$^+$ stimulus application in several cells, magnified in Fig. 3.21 (Bi) (top). Differently colored user-defined ROIs refer to the corresponding $\text{Ca}^{2+}$ signals below. In six selected regions (ROI1 – 6), robust K$^+$-dependent $\text{Ca}^{2+}$ transients were determined. Together, these results show that electroporation as a targeted dye loading method for $\text{Ca}^{2+}$ imaging is generally functional, but response rates as well as response strength remain relatively weak.
Fig. 3.20: Targeted single-cell electroporation in VNO slices. Pipettes (5-10 MΩ) containing external solution and cell impermeable fluo-4 (500 µM) are placed in close proximity to the somata of basal VSNs. Trains of positive voltage pulses are applied to electroporate the cells and allow dye transfer. Images in (A)–(C) were taken using a confocal microscope. (A) Fluorescence image of a single fluo-4-labeled neuron filled via the pipette in the sensory epithelium of the VNO (left) and corresponding IR-DIC (center) and merged (right) image (top). Scale bar, 100 µm. White dashed boxes indicate higher magnification (bottom) of the Fluo-4pp-labeled VSN. Soma or dendrite, respectively, are indicated by white arrowheads. Scale bar, 50 µm. (B) Electroporation of a group of neurons in the same VNO slice. bl, basal layer; bv, blood vessel; pp, perfusion pencil; scale bar, 100 µm. (C) Fluorescence image of the same VNO slice as in (A) and (B) showing sequentially electroporated cells, up to 20-30 cells with the same pipette within 10–15 min. Scale bar; 100 µm.
Fig. 3.21: Single-cell electroporation of VSNs as a Ca\textsuperscript{2+} imaging approach. (A) Electroporation of multiple cells with the Ca\textsuperscript{2+}-sensitive dye fluo-4pp (500 µM). Fluorescence image of a representative fluo-4pp-loaded VNO slice (left) and an IR-DIC (center) and merged (right) image of the same slice (top). bl, basal layer; bv, blood vessel; pp, perfusion pencil; scale bar, 100 µm. White dashed box indicates the region magnified (bottom). Knob, dendrite and soma of a representative fluo-4pp-labeled VSN are pointed out by white arrowheads. Basal layer (bl) and lumen as indicated. Scale bar, 50 µm. (i) Original trace from a single VSN located in the same depicted VNO slice, illustrating [Ca\textsuperscript{2+}]\textsubscript{cytosol} level (F/F versus time) during resting conditions and stimulation (K\textsuperscript{+}, 50 mM; K\textsuperscript{+} 100 mM; 10 s duration). [Ca\textsuperscript{2+}]\textsubscript{cytosol} is elevated during and after stimulus application (stimulus onset indicated by black arrowheads). (B) Low-magnification fluorescence image (left) of another typical electroporated VNO slice and
corresponding IR-DIC (middle) and fluorescence and IR-DIC image merged (right); scale bar 150 µm. Zooming into the white dashed box shows magnified representative region (i); top: scale bar, 30 µm. (i) Fluorescence intensity (ΔF/F) from user-defined ROIs in the sensory epithelium (ROI 1-6; colored circles) are plotted as a function of time resulting in [Ca²⁺]cytosol transients (bottom) recorded from single VSN stimulated with K⁺ (50 µM; 100 µM; duration, 10 s; black arrow heads, stimulation onset). Responses are triggered by both concentrations.
3.5 Cl⁻ imaging in the mouse VNO using 2P-FLIM technology

In mice, the vomeronasal organ (VNO) plays a significant role in social behavior. The detection of pheromones via vomeronasal sensory neurons (VSNs) takes place in the dendritic knob microvilli, where the binding of signal molecules to vomeronasal receptors leads to subsequent activation of a complex signal transduction cascade. Two different types of ion channels are supposed to be involved: TRPC2 and ANO2. The activation of TRPC2 (transient receptor potential canonical 2) channels leads to influx of Na⁺ and Ca²⁺ ions. These Ca²⁺ ions, in turn, induce opening of the Ca²⁺-dependent Cl⁻ channel ANO2 (Anoctamin 2). Though controversially discussed (Billig et al., 2011), it has been shown that the subsequent Ca²⁺-activated Cl⁻ efflux contributes up to 80% of the receptor current in response to urine in mouse VSNs (Yang & Delay, 2010). Moreover, the anoctamin subunits ANO1 and ANO2 are present in the apical layer of the vomeronasal epithelium, where they colocalize with the TRPC2 channel (Dibattista et al., 2012).

Based on these findings, some authors (Yang & Delay, 2010; Kim et al., 2011) have hypothesized an elevated Cl⁻ concentration in the vomeronasal neurons, similar to findings for OSNs in the MOE (Kaneko et al., 2004). This would provide an electrochemical gradient that is sufficient to generate an outward Cl⁻ current and would, thus, contribute to an excitatory depolarization (Yang & Delay, 2010; Kim et al., 2011). To investigate whether this is true, we used two-photon fluorescence lifetime imaging microscopy of the dendritic knob and sustentacular cell (SC) layer of the vomeronasal sensory epithelium in an en face preparation of the VNO (Riviere et al., 2009). These experiments were performed in collaboration with the group of Thomas Gensch (Institute of Complex Systems 4 (ICS-4, Cellular Biophysics); Forschungszentrum Jülich) who is an expert in 2P – FLIM.

3.5.1 The VNO en face preparation allows 2P-FLIM measurements of intracellular Cl⁻ concentrations in an intact epithelial environment

Here, we used an en face preparation to investigate Cl⁻ concentrations in cells of an intact VNO sensory epithelium in OMP-GFP gene-targeted mice. We performed a skull hemisection in adult mice (≥ 8 weeks). In Fig. 3.22 (A), the lateral blood vessel of the left VNO as well as the MOE endoturbinates and the MOB can be identified. When the VNO cartilaginous capsule and non-sensory epithelium (including the blood vessel) were carefully removed (see Material and Methods), the surface of the sensory epithelium became visible (Fig. 3.22 (B)) - a minimally invasive preparation that ensures epithelial integrity and intact axonal wiring. As every mature sensory neuron in the utilized mouse strain expresses eGFP under control of the OMP promoter, we could examine the sensory epithelial structure by exciting intrinsic GFP with a 488 nm argon laser. A cross section of the sensory epithelium allowed a transverse view, revealing layers of different fluorescence intensity (C) due to the predominant cell types located.
there. The strong GFP fluorescence in the apical region refers to knobs and dendrites of VSNs, whereas increased intensity at the basal area represents VSN somata. The non-fluorescent layer in between consists to a large extent of sustentacular cells.

The fluorescence confocal image in Fig. 3.22 (D) shows an en face perspective. A magnified image of a plane epithelial area is illustrated beneath (Di), uncovering single VSN knobs of 1-2 µm diameter. Together, these images demonstrate the largely intact structure of the vomeronasal sensory epithelium after performing an en face preparation.

Next, we used this en face preparation for the investigation of intracellular Cl⁻ concentrations ([Cl⁻]i). MQAE (6-methoxy-quinolyl acetoethyl ester) was used as a fluorescent indicator dye for intracellular Cl⁻ (Verkman, 1990). MQAE molecules are excited by absorption of a single ultraviolet photon (λ = 375 nm) or, alternatively, the absorption of two infrared photons (λ = 750 nm). Here, we used two-photon excitation (Denk et al., 1990; Denk & Svoboda, 1997) providing the advantage of low photo damage caused by the infrared light, even at relatively long observation times of 80 s per measurement. For the MQAE molecule, the fluorescence lifetime τ is modified by anions through collisional quenching. Accordingly, the higher the Cl⁻ concentration, the shorter the lifetime.

To discriminate the different cell types and cellular structures we used OMP-GFP mice for these experiments. In Fig. 3.23 (A) the fluorescence intensity images illustrate the eGFP fluorescence pattern obtained by two-photon excitation (λ = 910 nm)) in distinct focal planes of the sensory epithelium. In the knob layer (left column) a dense field of VSN knobs of 1-2 µm in diameter was visible. 5 µm below, small fluorescent spots (< 1 µm diameter) indicate VSN dendrites, forming a honey comb-like structure with the SCs designated by dark gaps here.
(middle left). In the layer 15 µm below the knob layer, still mainly dendrites and SC gaps but also a few VSN somata, specified by diffuse larger areas of fluorescence, were visible. At a depth of 30 µm, VSN somata filled most of the image.

MQAE fluorescence intensity images (Fig. 3.23. (B), top row) revealed a somewhat inverse illustration of VSN knobs and SCs: knobs appeared as darker ‘gaps’, whereas SCs showed stronger fluorescence intensity. False color lifetime images (bottom row) are demonstrated below with warm colors indicating VSN knobs and cooler colors referring to SCs. When comparing fluorescence lifetimes of knobs (left) and dendrites (middle left) a [Cl\textsuperscript{-}] gradient became visible with shorter lifetimes in the more apical knob region, indicating a higher [Cl\textsuperscript{-}] as in dendrites.

In summary, we showed that VSNs as well as SCs are capable of incorporating the MQAE molecules, resulting in a fluorescence signal. Hence, the en face preparation of VNO sensory epithelium is suitable for 2P-FLIM Cl\textsuperscript{-} imaging.

**Fig 3.23:** Analysis of cytosolic Cl\textsuperscript{-} concentrations by 2-photon fluorescence lifetime imaging microscopy in the VNO sensory epithelium. En face fluorescence intensity and lifetime images of GFP- or MQAE-labeled cells in different focal planes of the sensory epithelium. (A) OMP-GFP mice were used for better identification of cell types, i.e. VSNs and SCs. (A) shows GFP fluorescence intensity images of the apical layer (left panel), 5 µm (middle left), 15 (middle right), and 30 µm (right panel) below the apical layer. Knobs (left), dendrites (middle left) and somata (right) of sensory neurons can be clearly identified here. (B) For Cl\textsuperscript{-} lifetime imaging, sensory epithelium was loaded with the Cl\textsuperscript{-}-sensitive reporter dye MQAE. In intensity as well as in lifetime images the outlines of knobs, dendrites and SCs are visible (left and middle left). Knobs and dendrites in false-color 2P-FLIM representations show warmer colors compared to SCs displaying cooler colors under physiological conditions.
3.5.2 Quantitative calibration of Cl\(^{-}\) concentrations in 2P-FLIM measurements

We next attempted to obtain a quantitative calibration of intracellular Cl\(^{-}\) levels and MQAE fluorescence to gain absolute values for \([\text{Cl}^{-}]\). In this way, we aimed to prove our hypothesis of elevated \([\text{Cl}^{-}]\) in VSNs that would in turn favor an amplifying Cl\(^{-}\) efflux upon stimulus-induced activation as shown for OSNs in the MOE (Kaneko et al., 2004; Yang & Delay, 2010).

MQAE molecules are not only quenched by Cl\(^{-}\) but also by other ions, amino-acid residues and small cytosolic molecules. Therefore, MQAE properties depend on intracellular milieus and are consequently different for distinct cell types. For this reason, fluorescence lifetimes are not to be compared between different cell types. Cells require calibration to gain absolute \([\text{Cl}^{-}]\) to allow comparison of intracellular Cl\(^{-}\) levels. Here, we first used a high \([\text{Cl}^{-}]\) of 140 mM in our calibration solution and the ionophores nigericin and tributyltin (10 µM each) to equilibrate the \([\text{Cl}^{-}]\) and pH value across the plasma membrane.

![Fig. 3.24: Approach for quantitative calibration of the [Cl\(^{-}\)] in VSN knobs.](image)

MQAE fluorescence intensity and lifetime images depend on \([\text{Cl}^{-}]\) in knobs under physiological conditions (control) and incubated with 140 mM Cl\(^{-}\) and ionophores nigericin and tributyltin to dissipate Cl\(^{-}\) gradients across the plasma membrane and to set intracellular Cl\(^{-}\) to known values. *En face* intensity and lifetime decay images of VSN knobs as well as experiment condition are presented on the left side. Histograms were generated by plotting lifetime \(\tau\) of single knobs against number of cells per bin (bin width 20 ps). (A) Histogram of knob analysis under physiological conditions (control; black) displays the distribution of fluorescence lifetime \(\tau\) values in ps. The average \(\tau\) value of 197 analyzed knobs was 2245 ± 15.7 ps (mean ± s.e.m.) (B) Distribution of fluorescence lifetime \(\tau\) values under calibration conditions, i.e. incubation of 140 mM Cl\(^{-}\) solution in the presence of ionophores nigericin and tributyltin. Treatment with the ionophores did not produce uniform Cl\(^{-}\) levels after 7 min incubation, indicated by shift to wrong direction as expected for a high [Cl\(^{-}\)]. Average \(\tau\) value of 183 analyzed knobs was 2480 ± 17.8 ps. (C) Histogram plot of lifetime distribution after 19 min incubation with the same conditions as for (B), shows likewise no considerable changes in distribution. Average \(\tau\) value of 237 analyzed knobs was 2405 ± 13.6 ps. n as indicated.

Fig. 3.24 shows the results from the calibration approach of VSNs from an *en face* preparation recorded in the apical knob layer. To quantitatively determine the effect of calibration we plotted
the lifetime $\tau$ of single knobs against the number of cells per bin (bin width 20 ps). Under physiological conditions (control) $\tau$ values ranged from $\sim 1800 \pm 2800$ ps with an average lifetime of $2245 \pm 15.7$ ps (mean ± s.e.m.). To reveal the effect of calibration we compared the lifetime distribution under physiological conditions to 7 (average $\tau$ value $2480 \pm 17.8$ ps) and 19 min (average $\tau$ value $2405 \pm 13.6$ ps) incubation with 140 mM calibration solution and ionic ionophores nigericin and tributyltin. However, cell fluorescence lifetime did not change in a considerable extend, i.e. shifted to shorter lifetimes in response to the high external Cl$^-$ concentrations. Neither after 7 min, nor after 19 min of incubation, treatment with tributyltin produced a detectable decrease of fluorescence lifetime in cells indicating uniform Cl$^-$ levels. Thus, this technique did not lead to a satisfactory calibration providing quantitative information and therefore absolute values of [Cl$^-$] in VSNs. However, we also attempted to calibrate with 0 mM Cl$^-$ calibration solution, leading to slightly more promising results, which were though sometimes conflicting. Additionally, we tried another approach for quantitative calibration, in which we used VNO slices to achieve better access for ionophores to the cells, in case this might be the reason for our failed calibration. However, this technique did not lead to more satisfactory results either.

Our 2P-FLIM results are, therefore, instead qualitative. Accordingly, the 2P-FLIM measurements deliver information about relative signal differences instead of absolute values. They clearly demonstrate a [Cl$^-$] gradient indicating an increase of Cl$^-$ accumulation in VSN knobs compared to dendrites (see Fig. 2.23) but they do not give information about which level of [Cl$^-$] is reached during the experiments.

3.5.3 Cl$^-$ 2P-FLIM measurements revealed stimulus- and time-dependent [Cl$^-$]. changes in VSN knobs and SCs

One possibility to investigate and compare relative fluorescence lifetime signals is to analyze a [Cl$^-$] gradient within one cell, e.g. the [Cl$^-$] gradient indicated between VSN knobs and dendrites (see Fig. 3.23). Another method to use relative fluorescence signals is the analysis of stimulus-induced changes of [Cl$^-$] within one cell type. Here, we chose to next investigate stimulus-induced changes of [Cl$^-$] in VSN knobs and SCs. Mouse urine (1:100, diluted in extracellular solution) was used as a ‘broadband’ VSN stimulus because it is known to activate a large subset of vomeronasal neurons (Tirindelli et al., 2009). Lifetimes at the same region of sensory epithelium in one en face preparation were measured under physiological conditions (pre-stimulus; $E_{norm}$) and in the sustained presence of the stimulus (post-stimulus; urine, 1:100) (Fig. 3.25.). MQAE lifetime false color images implicate longer lifetimes in the stimulated compared to unstimulated knobs (Fig. 3.25 (A)). For quantification, we compared the lifetime $\tau$ before and after stimulus application in optically identified knobs. $\Delta \tau$ was determined as an index for change in lifetime. Therefore, each $\tau$ value of one cell at post-stimulus conditions was
subtracted from the pre-stimulus value of the same cell. Higher magnification images (bottom row; indicated by black boxes, top) show clearly identifiable knobs (dark gaps / warmer colors). ROIs (red circles) were set based on corresponding intensity images and average fluorescence lifetimes of these areas were used for further analysis. Solid red circles indicate one VSN knob, that shows an increased $\tau$ value upon stimulation ($\Delta \tau = -626$ ps), corresponding to a decrease in $[\text{Cl}^-]$. The knob lined by the red dashed loop exemplifies a minor decrease in $[\text{Cl}^-]$ ($\Delta \tau = -266$ ps).

**A**

pre-stimulus  
post-stimulus (urine 1:100)

**B**

pre-stimulus  
post-stimulus (urine 1:100)

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**Fig 3.25:** Stimulus-dependent changes in $[\text{Cl}^-]$ occurred in both VSN knobs and SCs. MQAE fluorescence intensity and lifetime images depend on $[\text{Cl}^-]$ in knobs and SCs under physiological conditions (pre-stimulus) and sustained exposure to mouse urine (1:100, pooled; post-stimulus). **(A)** Original intensity and lifetime decay images of VSN knobs are presented in the top row. Areas of higher magnification (bottom row) are indicated by black boxes. **Bottom row:** Solid red circles depict one VSN knob that reveals a decrease in $[\text{Cl}^-]$ upon urine stimulation ($\Delta \tau = -626$ ps), whereas the knob encircled by red dashed line shows essentially no change in lifetime ($\Delta \tau = -266$ ps). **(B)** Images depict the SC layer in the sensory epithelium 12 µm below the knob layer. Black boxes indicate higher magnifications below. The cell marked by a solid red sphere demonstrates a decrease in lifetime ($\Delta \tau = 204$ ps), compared to the SC lined by the red dashed loop, indicating nearly no change in lifetime ($\Delta \tau = 29$ ps). Scale bars as indicated.
As shown in Fig. 3.25 (B), the same analysis was performed for SCs within the same epithelial regions, but at a deeper focal plane around 12 µm under the knob layer. MQAE lifetime false color images (top row) reveal a shift to shorter lifetimes in the SCs during prolonged stimulation (post-stimulus), thus indicating an increase in [Cl\(^-\)]. Again, ROIs were drawn based on intensity images. SCs were larger and more irregular shaped than knobs and showed brighter fluorescence intensity and longer fluorescence lifetimes (cold colors). The cell marked by a solid red sphere demonstrates a decreased lifetime (Δ\(\tau\) = 204 ps), compared to the SC encircled by the red dashed line, indicating essentially no change in lifetime (Δ\(\tau\) = 29 ps). These results show that a subset of VSNs displayed a decrease of [Cl\(^-\)], in response to stimulation, whereas another subset seems to be unaffected by the presence of urine. In contrast, most SCs responded to urine stimulation with an increase of [Cl\(^-\)].

For quantification, we next determined Δ\(\tau\) values of each optically identified knob or SC and then plotting the number of cells against binned \(\tau\) values (Fig. 3.26). Pre- and post-stimulus measurements of knobs and SCs were generated from two different areas of one en face preparation each, either stimulated with urine (Fig. 3.26 (A) and (B)) or under control conditions (exchange of extracellular solution) (Fig. 3.26 (C) and (D)).

Histogram plots of Δ\(\tau\) analysis of urine stimulated VSN knobs (Fig. 3.26 (A) and (B)) (dark blue; bin width, 20 ps) revealed a shift to negative Δ\(\tau\) values and an indicated second peak. The main peak at around Δ\(\tau\) -200 ps (A) or around Δ\(\tau\) 0 ps (B) indicates a population of knobs showing no or only very slight change in lifetime upon stimulation, whereas the second peak around Δ\(\tau\) -380 ps (A) or around Δ\(\tau\) -200 ps (B) suggests a second VSN population that displays a more pronounced decrease of [Cl\(^-\)]. Thus, these data indicate a robust decrease of [Cl\(^-\)] in a knob population upon urine presentation.

By contrast, histograms of Δ\(\tau\) values in SCs (dark green; bin width, 10 ps) displayed a shift to positive values indicating an increase of [Cl\(^-\)], when stimulated with urine. Only one peak occurred for (A) close to a Δ\(\tau\) value of +130 ps with a maximum cell count per bin of 7 cells, and one main peak for (B) around Δ\(\tau\) +120 ps with a maximum number of 8 cells per bin. A minor peak was observed at a Δ\(\tau\) value of 0 ps indicating a small population of SCs that showed no change in lifetime upon urine stimulation. The majority of SCs though, was responding with an increase in [Cl\(^-\)] to the presence of urine.

Control experiments were performed under the same conditions, but here extracellular solution was exchanged with a blank control solution identical in composition (Fig. 3.26 (C) and (D)). For VSN knobs, histogram plots (light blue; bin width, 20 ps) still depict robust shifts to negative Δ\(\tau\) values. By contrast, SCs (light green; bin width, 10 ps) exhibited a peak around Δ\(\tau\) 0 ps (D) or a slight shift to negative values (C, peak around Δ\(\tau\) -100 ps).
Together, these data suggest a stimulus-induced increase of \([\text{Cl}]\) in SCs in response to urine stimulation, whereas the effect of decreased \([\text{Cl}]\) in knobs should rather be attributed to stimulus-independent effects.

**Fig 3.26: Histogram plots show different effects in \(\Delta\) lifetime \(\tau\) between urine stimulation and control experiments.** Histogram plots were generated by subtracting each lifetime \(\tau\) value of one cell at post-stimulus conditions from the pre-stimulus lifetime of the same cell, resulting in a negative or positive \(\Delta \tau\). (A), (B) Both knobs and SCs were measured under physiological (pre-stimulus) and stimulated (urine in extracellular solution, 1:100; post-stimulus) conditions at two different areas ((A) and (B)) of the sensory epithelium in one VNO whole-mount preparation. Histograms of knob analysis (dark blue; bin width, 20 ps) display a shift and a second peak at negative \(\Delta \tau\) values, suggesting a decrease of \([\text{Cl}]\) in the presence of urine. In contrast a shifted histogram for SCs (dark green; bin width, 10 ps) to positive \(\Delta \tau\) values indicates an increase of \([\text{Cl}]\) upon urine presentation. (C), (D) In these control experiments, physiological extracellular solution was just exchanged with identical extracellular solution devoid of a semiochemical stimulus (post-stimulus). While histogram plots of knob data (light blue; bin width, 20 ps) still demonstrate shifted \(\Delta \tau\) values to negative values, \(\Delta \tau\) data from SCs (light green; bin width, 10 ps) display a peak around a \(\Delta \tau\) of zero (D) or, compared to urine stimulation, a slight shift in opposite direction (C); \(n\) as indicated.
4. Discussion

In most mammals, the olfactory system plays a vital biological role. The detection and discrimination of thousands of different environmental cues is crucial for the individual and the species. Thus, a number of complex chemosensory detection systems have developed during evolution. However, many physiological mechanisms underlying mammalian chemodetection remain unclear. In this thesis, I therefore focused on basic physiological processes underlying olfactory signaling. In one line of research, I investigated the physiology of two different MOE cell types. Two other projects were directed to aspects of vomeronasal organ physiology. To address these issues, I used acute tissue preparations and employed electrophysiological as well as imaging techniques.

In the first project, my experiments revealed an essential role for mitochondria during odor-induced activation of OSNs. Using patch-clamp recordings, I showed that mitochondria are critical regulators of the odor response input-output function by orchestration of intracellular Ca\textsuperscript{2+} signaling. Furthermore, I showed mitochondrial arrest in dendritic knob regions upon odor stimulation using FRAP experiments.

In a second project, I characterized SCs in the MOE electrophysiologically. I found that these cells exhibit transient Na\textsuperscript{+} and Ca\textsuperscript{2+} inward as well as large K\textsuperscript{+} outward currents at negative membrane potentials. However, odor-dependent electrophysiological changes did not occur.

In collaboration with the laboratory of Prof. Dr. Stephen D. Liberles from Harvard Medical School, we identified an age-dependent pheromone. In my experiments, I showed that ESP22 activates basal VSNs in a dose-dependent and repetitive manner. Additionally, juvenile tear fluid, the natural source of ESP22, activates ESP22-responsive cells.

In addition, I established a new imaging approach in VNO slices. I used electroporation to transfer a Ca\textsuperscript{2+}-sensitive dye into VSNs. Subsequently, I measured Ca\textsuperscript{2+} responses upon K\textsuperscript{+}-dependent depolarization.

Last, I investigated the role of Cl\textsuperscript{-} in the VNO sensory epithelium. In collaboration with colleagues from the Forschungszentrum Jülich, we used 2-photon FLIM to examine physiological Cl\textsuperscript{-} concentrations in VSN knobs and vomeronasal sustentacular cells. I found that [Cl\textsuperscript{-}] in VSN knobs is elevated as compared to dendrites. Moreover, SCs show stimulus-induced changes in the fluorescence lifetime.

4.1 Mitochondrial Ca\textsuperscript{2+} sequestration plays a key role in olfactory signaling in mice

The role of Ca\textsuperscript{2+} within the odor-dependent signal transduction machinery in OSNs has been studied intensively. [Ca\textsuperscript{2+}]\textsubscript{i} is not only increased during odor-dependent activation, but is also involved in response termination (Kurahashi & Menini, 1997; Matthews & Reisert, 2003; Antolin
et al., 2010). For the activating function of Ca\(^{2+}\), the maintenance of a low resting cytosolic [Ca\(^{2+}\)] at nanomolar levels is required (Pietrobon et al., 1990). Therefore, the interaction of various channels, pumps and transporters is necessary. A recent study showed that Ca\(^{2+}\) extrusion in OSNs is mainly based on Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX) especially during response termination and adaptation (Stephan et al., 2012). In addition, PMCAs contribute to maintenance of low resting cytosolic [Ca\(^{2+}\)] (Carafoli, 1994; Antolin et al., 2010). Another mechanism to regulate low cytosolic [Ca\(^{2+}\)] is comprised by intracellular organelles. For a long period, the endoplasmic reticulum (ER) was thought to be the sole intracellular Ca\(^{2+}\) storage organelle relevant in olfactory signaling (Zufall et al., 2000; Park et al., 2000). Since a possible role of mitochondria in OSN Ca\(^{2+}\) homeostasis and olfactory signaling had not been investigated, we asked whether mitochondria contribute to olfactory Ca\(^{2+}\) signaling?

Mitochondria serve as Ca\(^{2+}\) storage organelles based on their negative membrane potential, which is the driving force for Ca\(^{2+}\) accumulation into the mitochondrial matrix (Gunter & Pfeiffer, 1990). The most studied Ca\(^{2+}\) influx mechanism is the mitochondrial Ca\(^{2+}\) uniporter, which transports Ca\(^{2+}\) down its electrochemical gradient without coupling the transport to that of any other ions (Kirichok et al., 2004; De Stefani et al., 2011). The main proposed physiological function of mitochondrial Ca\(^{2+}\) transport is to control the rate of oxidative phosphorylation (Gunter et al., 2000). Intramitochondrial Ca\(^{2+}\) has been shown to activate different dehydrogenases coupled to the Krebs cycle, suggesting that mitochondrial free Ca\(^{2+}\) concentration might act to modify the cellular metabolic rate (Denton et al., 1972; McCormack & Denton, 1979; Jouaville et al., 1999).

In previous experiments from our working group, done by Daniela Flügge, cytosolic and mitochondrial Ca\(^{2+}\) imaging experiments revealed that Ca\(^{2+}\) transients are elicited in both the cytosol and mitochondria upon odor stimulation in OSNs. Inhibition of the ACIII or removal of extracellular Ca\(^{2+}\) abolished Ca\(^{2+}\) influx into the cytoplasm and mitochondria, showing that mitochondrial Ca\(^{2+}\) entry is mediated by canonical OSN signaling pathways. Mitochondrial Ca\(^{2+}\) sequestration influences cellular Ca\(^{2+}\) homeostasis by shaping the kinetics of the cytosolic Ca\(^{2+}\) signal (Fluegge et al., 2012).

Based on these findings, in this follow-up work, I showed that mitochondrial Ca\(^{2+}\) uptake during odor responses shapes the input-output function of OSNs. Using the protonophore FCCP, RuR or its related compound Ru360 to cause either ΔΨ\(_m\) collapse or MCU inhibition (Rizzuto et al., 1994), we revealed direct and comparable effects on the dynamic range of primary receptor currents in OSNs (Fig. 3.1, Fig.3.2). Here, we showed that inhibition of mitochondrial Ca\(^{2+}\) uptake results in a narrowed activation range and increased slope of the stimulus-response (S-R) curve, which is also reflected by significantly increased Hill coefficients. Under FCCP, the lack of a proton gradient and, consequently, the collapsed membrane potential stops ATP production by the ATPase. The ATPase can instead turn into reverse mode, resulting in
degradation of ATP to ADP + P_i. To prevent such ATP hydrolysis, we co-applied oligomycin, an ATPase inhibitor (Schepina et al., 2002; Jastroch et al., 2010). Therefore, and because of additional diffusion loading of ATP via the patch pipette, insufficient energy supply is not considered as a reason for altered response amplitudes when mitochondrial function is inhibited. We hypothesize that the narrowed dynamic range of the S-R function is based on a complex signaling and feedback machinery, including components of the canonical transduction cascade, which is highly sensitive to even slight changes in baseline [Ca^{2+}]_c. We suggest that OSN mitochondria, in addition to NCX (Stephan et al., 2012) and PMCA (Saidu et al., 2009), ensure a relatively low resting [Ca^{2+}]_c of less than ~100 nM (Restrepo et al., 1993; Zufall et al., 2000). Especially in cilia, the maintenance of a reduced Ca^{2+} concentration at rest is important because at intraciliary [Ca^{2+}]_c >100 nM, CNG channel sensitivity is shifted by Ca^{2+} binding to the pre-associated apocalmodulin (Bradley et al., 2004, 2005). Given that we observed sustained [Ca^{2+}]_c elevation in the OSN knobs upon ΔΨ_m dissipation (done by Daniela Flügge), we suggest that intraciliary [Ca^{2+}]_c is increased accordingly. This [Ca^{2+}]_c elevation could, in turn, switch CNG channels to a pre-adapted state and induce the S-R curve shift. In parallel, raised ciliary [Ca^{2+}]_c would bring CaCC channels closer to activation threshold, thus rapidly amplifying (and saturating) the OSN odor response once CNG channels open. Together, both mechanisms would result in the narrowed S-R curve slope and shifted activation threshold, as observed in our experiments.

Next, we investigated basic primary response kinetics in stimulated OSNs under control conditions versus mitochondrial Ca^{2+} uptake inhibition (Fig. 3.3). We compared different features of sensory adaptation during prolonged stimulation (≥3 s). Neither bi-exponential decay time constants (τ_fast and τ_slow), nor peak-plateau ratios (ΔI = I_max/I_plateau) were significantly changed in the presence of either drug (FCCP, RuR or RuR360). Even the maximum current amplitudes remained unaffected by pharmacological treatment. These findings indicate, that elevated [Ca^{2+}]_c does not affect sensory adaptation or maximum currents, once a saturating response is elicited. By contrast, the percentage of responsive of neurons differed significantly between control conditions versus impaired mitochondrial Ca^{2+} uptake, particularly at short stimulus durations. This result reflects the shifted activation threshold described above (Fig.3.1 / 3.2) and, thus, we propose the same underlying molecular mechanisms.

Furthermore, we examined if passive membrane and basic output response properties in OSNs were altered by FCCP treatment compared to control experiments (Fig 3.4, Fig. 3.5). Current-clamp recordings revealed no substantial changes in resting membrane potential ΔV_m, nor in spontaneous AP discharge during sustained FCCP incubation (Fig 3.4). We observed average spontaneous AP frequencies ranging from 0.55 – 0.58 Hz. This fits literature values of individual OSNs typically showing low spontaneous AP firing rates (Frings et al., 1991; Reisert & Matthews, 2001b). However, in the absence of stimulation, the basal activity OSNs
from mOR-EG mice (same strain as used in our study) was reported to be only 0.06 ± 0.02 Hz (Reisert, 2010). This difference might be explained by different techniques used to obtain these data. While Dr. Reisert recorded from isolated OSNs using the suction pipette technique, we used whole-cell patch-clamp recordings from OSNs in acute slice preparations. This technique could provide superior cell viability as the surrounding tissue is intact.

In addition, we analyzed different response properties to saturating odor stimuli in current-clamp mode (Fig 3.5). No significant alterations occurred, regarding the quantity of APs, the maximum membrane depolarization, full duration at half maximum (FDHM) or the response rise time (10-90%). Our data indicate that FCCP-dependent alterations of primary odor responses do not rely on changes in basic physiological parameters, but that OSNs rather reveal stable physiological conditions during prolonged experiments.

Next, we focused on another critical function of mitochondrial Ca\(^{2+}\) buffering, which became evident from spike recordings in loose seal configuration (Fig 3.6, Fig. 3.7). The ‘loose-patch’ technique provides the advantage of preventing dialysis of intracellular components and allows simultaneous recording of the odor-induced spike firing and receptor current responses (Reisert & Matthews, 2001a). Using this method, spontaneous activity and resting \(V_m\) turned out to be largely unaffected by FCCP or Ru360 exposure (Fig 3.6). Depending on the recording mode, RuR could not be used as an inhibitor because of its membrane impermeability. Our experiments showed that the relation between stimulus intensity and action potential discharge was shifted to increased odor concentrations when mitochondrial Ca\(^{2+}\) sequestration was inhibited (Fig 3.6 and 3.7 (B)). This effect could, in part, result from changes in the primary receptor currents (see above). Though impaired mitochondrial Ca\(^{2+}\) uptake during action potential firing would also affect various Ca\(^{2+}\)-regulated components of the general spike generation machinery, such as big conductance 130 pS Ca\(^{2+}\)-activated K\(^+\) channels (Maue & Dionne, 1987). In other neurons, these channels are responsible for repolarization and after-hyperpolarization (Bean, 2007).

Given that neurons are highly polarized cells with several dendrites and a single long axon, dynamic ordering of mitochondria at areas with high-energy demands is critical for neuronal development and synaptic function (Sun et al., 2013; Maeder et al., 2014). Mitochondria are able of anterograde as well as retrograde transport along the cytoskeleton, that requires molecular kinesin or dynein motor proteins, respectively (Saxton & Hollenbeck, 2012). Ultrastructural findings and fluorescence-based mobility assays revealed that mitochondria are, for example, recruited to cellular compartments with increased requirement for ATP or to particular subcellular regions of high Ca\(^{2+}\) buffering demands. Here, recruitment is frequently based on activity-dependent Ca\(^{2+}\) signals (MacAskill & Kittler, 2010; McCarron et al., 2013). Mitochondrial arrest in those regions might be accomplished by increased [Ca \(^{2+}\)]\(_{\text{c}}\)-dependent inhibition of motor protein-driven movement (Yi et al., 2004; Goldstein et al., 2008). Recently,
several groups reported that $\text{Ca}^{2+}$-dependent regulation of mitochondrial dynamics is mediated by a Miro-Milton complex linking mitochondria to kinesin motors (Fransson et al., 2003, 2006; Liu & Hajnóczky, 2009; Wang & Schwarz, 2009).

Based on the above findings, we hypothesize that mitochondria might redistribute in OSNs upon similar activity-dependent immobilization mechanisms. Previous results from our working group (done by Daniela Flügge), showed indeed that mitochondria travel from soma to knob regions upon odor stimulation. Ultrastructural analysis of OSN knobs revealed that the content of predominantly spherical mitochondria (unstimulated animals) shifted to an increased content of elongated morphologies as a result of stimulation (Fluegge et al., 2012). This indicates mitochondrial accumulation and fusion in areas of close proximity to where odor detection takes place.

In FRAP experiments, I could show that this increased mitochondrial content in stimulated OSN knobs was rather based on organelle arrest than on increased mobility (Fig. 3.8). This was indicated by a higher immobile fraction in the dendritic knob of stimulated OSNs compared to unstimulated cells, which resulted in a significantly decreased maximal FRAP. Thus, our results indicate a local $\text{Ca}^{2+}$-dependent down regulation of mitochondrial mobility similar to activity-regulated immobilization pathway as proposed in various previous publications (see above).

Together, I showed that mitochondria function as key determinants of olfactory signaling in OSNs. Mitochondrial $\text{Ca}^{2+}$ mobilization during sensory stimulation ensures a broad S–R range and maintains sensitivity of the OSN spike generation machinery.

### 4.2 Electrophysiological characterization of sustentacular cells in the MOE of mice

Sustentacular cells (SCs) comprise one major population of MOE-resident cells, analogous to glial cells of the brain. However, much less is known about these cells and their functions. For example, studies showed that SCs are involved in the regulation of extracellular ionic gradients, i.e. $\text{K}^+$ homeostasis in the MOE, and are able of secreting mucus (Okano & Takagi, 1974; Mellert et al., 1992). They are also thought to be responsible for the metabolism of noxious chemicals (Kulkarni et al., 1994) and they can function as phagocytes for dead cells, including OSNs (Suzuki et al., 1996). Additionally, they are supposed to be involved in intercellular communication via ATP (Czesnik et al., 2006; Dooley et al., 2011).

Basic electrophysiological functions were investigated only by few groups, namely Thomas V. Getchell and co-workers, who studied SCs in the early 1970s, and the group of Mary T. Lucero, who published studies that are more recent. Getchell and colleagues revealed morphological relations between OSNs, sustentacular cells and Schwann cells in the olfactory epithelium of
the salamander (Rafols & Getchell, 1983). A few years later, they showed that SCs are able of odor-dependent mucus secretion. The protrusion of secretory material into the mucociliary matrix was time- and concentration-dependent with respect to odor stimulation (Getchell et al., 1987). They also showed that odor treatment caused ultrastructural changes in the apical and supranuclear regions of SCs that were associated with secretion (Zielinski et al., 1988). Using intracellular and electro-olfactogram (EOG) recordings, the authors postulated slow potential changes in SCs, which were not accompanied by action potentials in response to odor stimulation (Getchell, 1977). However, at the same time, Getchell's group acknowledged a caveat of their study. It remained unclear to which extent SC membrane de- and hyperpolarizations could be directly attributed to (a) a glial-type function, (b) an active secretory process, or (c) to stimulus removal mechanisms subsequent to OSN activation. Moreover, these experiments were performed in salamander, but not in mammals. Only recently, Mary T. Lucero and co-workers showed that SCs located in the MOE of mice are electrically coupled by gap junctions (Vogalis et al., 2005b). Furthermore, they showed that SCs generate oscillatory Ca\textsuperscript{2+} transients evoked by purinergic signals. These Ca\textsuperscript{2+} signals were dependent on Ca\textsuperscript{2+} release from intracellular stores, i.e. the ER (Hegg et al., 2009). A G-protein coupled PLC pathway contributes to purine-evoked increases in intracellular Ca\textsuperscript{2+} (Hegg et al., 2009). In electrophysiological experiments, SCs displayed voltage-gated Na\textsuperscript{+} and K\textsuperscript{+} currents that could support rapid electrical discharge, but the Na\textsuperscript{+} current was largely inactivated at the resting potential of SCs in slices due to an outwardly rectifying resting ‘leak’ conductance (Vogalis et al., 2005a).

Here, we aimed to provide an extended characterization of the basic electrophysiological properties of SCs in the mouse MOE. I showed that these cells exhibit transient Na\textsuperscript{+} and Ca\textsuperscript{2+} inward as well as large K\textsuperscript{+} currents. HCN currents or odor-dependent electrophysiological changes were not observed.

### 4.2.1 Basic passive membrane properties of SCs in MOE slices

First, I analyzed the basic passive membrane properties of SCs in MOE slices (Fig. 3.9). I found an average resting membrane potential $V_{\text{rest}}$ of -26 mV, when intra- and extracellular solutions had symmetric Cl\textsuperscript{-} concentrations, and -36 mV using a gluconate-based intracellular solution, containing only 15 mM Cl\textsuperscript{-}. These values are slightly more positive than expected from $V_{\text{rest}}$ determined by Getchell and Lucero, which was -49 mV (average) or ranged between -30 and -50 mV, respectively (Getchell, 1977; Vogalis et al., 2005a). Compared to Lucero’s data, this result might be based on differences between utilized intracellular solutions. Vogalis et al. (2005b) used potassium fluoride (KF) based intracellular solution containing only 15 mM Cl\textsuperscript{-}, when they detected $V_{\text{rest}}$ between -30 and -50 mV. This fitted quite well to a $V_{\text{rest}}$ of -36 mV that we determined when we also used an intracellular solution containing 15 mM Cl\textsuperscript{-} based
on potassium gluconate, but not on KF. By contrast we detected a $V_{rest}$ of -26 mV we applied KCl-based symmetric Cl$^-$ concentrations. This might indicate an important contribution of Cl$^-$ to the membrane potential and therefore a pronounced role for Cl$^-$ transporters / ion channels in SCs. In CNS glial cells such as astrocytes, microglia and oligodendrocytes the expression of cation-coupled Cl$^-$ cotransporters have been shown (Annunziato et al., 2013). In astrocytes for example, KCC 1-3 as well as NKCC 1 function in maintenance and regulation of cell volume or control of intracellular [K$^+$], respectively (Su et al., 2002; Ernest et al., 2005; Ringel & Plesnila, 2008). In oligodendrocytes and Schwann cells, KCC 2 and 3 may also participate in the regulation of cell volume (Pearson et al., 2001), whereas NKCC1 serves to maintain [Cl$^-$] above electrochemical equilibrium, thereby having a crucial role in GABAergic functions (Plotkin et al., 1997; Annunziato et al., 2013). Thus, it is tempting to speculate about a similar function in ion homeostasis in olfactory SCs, often described as glial-like cells. $E_{Cl^-}$-dependent changes in $V_{rest}$ indicate contribution of Cl$^-$ to the maintenance of $V_{rest}$.

The positive membrane potential might also point to the expression or non-expression of other ion channels, such as HCN channel. Such a positive membrane potential would not be favorable for HCN currents, based on the notably more negative threshold activation of these channels at -50 to -60 mV (Biel et al., 2009). It is questionable if such a hyperpolarization of the cells would occur under physiological conditions, and therefore HCN channel expression in SCs appears unlikely.

The average SC capacitance was 14.63 pF ($n = 34$), whereas OSN capacitance was ~4 pF, confirming previous findings of 18.6 pF and 4.4 pF, respectively (Vogalis et al., 2005a). By contrast, the SC input resistance ($R_{input}$) was much lower (255.5 MΩ) than in OSNs (3.59 GΩ).

The membrane time constant $\tau_{memb}$ is a measure of how fast the voltage changes upon current injection. The greater $\tau$ is, the longer it will take to reach maximal voltage change and the slower is the decay of the voltage. We observed a relatively short average $\tau_{memb}$ value of 4.13 ms for SCs, meaning that current flow affects membrane discharge relatively fast.

### 4.2.2 Voltage-dependent conductances

Next, we investigated different ionic conductances. Distinct voltage-gated ion channels are typically expressed in excitable / neuronal cells, although voltage-dependent conductances are also generated in a variety of nonneuronal cells (Verkhratsky & Steinhäuser, 2000; Black & Waxman, 2013). The expression of voltage-gated ion channels in SCs suggests electrically excitability, which is unusual for glial-like cells.

#### 4.2.2.1 Investigation of voltage-gated sodium channels

I first focused on voltage-gated sodium channels. Nine different channel subtypes can be distinguished by pharmacological properties of the $\alpha$-subunits $Na_1.1$-$Na_1.9$ (Catterall et al., 2003).
2005a). Here, I showed that transient inward currents recorded from SCs could be attributed to at least two different subpopulations that are distinguished by TTX-sensitivity. Under control conditions, we found a maximum transient inward current of -45.27 pA/pF at -23.8 mV, which could be blocked with TTX by 62.8% (Fig. 3.10). This percentage thus corresponds to a population of TTX-sensitive sodium channels. More specifically, these data indicate expression of one or more members of the following TTX-sensitive subgroup of Na\(_v\) channels: Na\(_v\)1.1, Na\(_v\)1.2, Na\(_v\)1.3, Na\(_v\)1.4, Na\(_v\)1.6 and Na\(_v\)1.7. These results largely confirm previously reported values of a maximum I\(_{\text{Na}}\) of -44 pA/pF at -38 mV and an incomplete block (48%) of the current by TTX (Vogalis et al., 2005a). Since protein expression can be strain-specific (Keane et al., 2011; Ferrero et al., 2013) the variation of TTX-sensitive proportion might be explained by different mouse strains utilized. While we used C57BL/6 mice, Lucero’s laboratory used Swiss Webster mice.

Under each condition (control, TTX-insensitive and TTX-sensitive), I\(_{\text{max}}\) was reached at distinct potentials. This confirms the recruitment of different ion channel populations. However, as I\(_{\text{transient}}\) fully inactivated over a 30 ms test pulse (Fig. 3.10 (F)), any substantial conductance of persistent Na\(^+\) channels could be excluded.

Next, we analyzed the steady-state inactivation of I\(_{\text{transient}}\). The current was completely inactivated at -8.8 mV indicating a leak conductance at resting potentials. This contradicts Lucero’s work in slices postulating a transient Na\(^+\) current largely inactivated at the SC resting potential (Vogalis et al., 2005a).

For comparison, we summarized these data by plotting normalized activation versus inactivation curves of I\(_{\text{transient}}\) for each condition (control, TTX-insensitive and TTX-sensitive) (Fig. 3.12). Superimposed activation and inactivation Boltzmann curves revealed a considerable ‘window current’ under control conditions between approximately -60 and -15 mV. The fitted Boltzmann functions for activation and inactivation yielded relatively large slope values and a voltage of half-maximal activation (x\(_{\text{half}}\)) of -35 mV or -58 mV, respectively. By contrast, the TTX-insensitive and TTX-sensitive population alone showed much steeper slope values and a shifted maximal activation and complete inactivation to more negative potentials, resulting in substantially smaller window currents. This further suggested that at least two subpopulations of voltage-activated channels contribute to the transient inward current in SCs. The TTX-insensitive current is likely based on TTX-insensitive Na\(^+\) channels, i.e. Na\(_v\)1.5, Na\(_v\)1.8, and Na\(_v\)1.9 and / or Ca\(_v\) channels, whereas the TTX-sensitive current represents conductance(s) of TTX-sensitive Na\(^+\) channels such as Na\(_v\)1.1 - 4, Na\(_v\)1.6 or Na\(_v\)1.7. Lucero and colleagues postulated that the inward current was carried exclusively by Na\(^+\) because it was abolished when extracellular Na\(^+\) was replaced by the large cation NMDG\(^+\).

In general, the expression of TTX-sensitive Na\(^+\) channels suggests an electrical excitability and possible generation of APs. Lucero and coworkers showed that SCs were indeed able of
generating a single AP when depolarized rapidly to about -60 mV from a holding potential negative of -90 mV (Vogalis et al., 2005a). Excitability and expression of TTX-sensitive Na\(^+\) channels was also shown in other nonneuronal cells. In rat hippocampal astrocytes, for example, TTX-sensitive Na\(^+\) conductances were observed. Here, the number of cells showing I\(_{\text{Na}}\) increased with age indicating a contribution in postnatal development processes (Bordey & Sontheimer, 1997). A similar role could be played by SCs in the mouse MOE, since we recorded I\(_{\text{Na}}\) from P0-5 but not from adult mice. In further experiments, it would be interesting to investigate Na\(^+\) conductances in SCs from adult mice and to compare response rates. Several roles for glial Na\(^+\) channels have been suggested (Sontheimer et al., 1996). Glial cells may serve as a donor of Na\(^+\) channels to be transferred to and inserted into axonal membranes (Shrager et al., 1985). Na\(^+\) channels may be activated by adjacent neuronal activity and allow glial cells to sense activity of adjacent neurons (Chao et al., 1994). This may also be feasible for SCs in the MOE, providing a mechanism in which SCs may regulate ionic homeostasis during OSN activity. Additional roles postulated for glial Na\(^+\) channels include Na\(^+\) leakage through at the resting potential which may drive Na\(^+\)/K\(^+\)-ATPase activity (Sontheimer H., 1994) or a role of Na\(^+\) influx in pH regulation via Na\(^+\)/H\(^+\) cotransport (Shrode & Putnam, 1994).

Furthermore, Na\(^+\) channel expression in nonneuronal cells was shown for odontoblasts, epithelial cells responsible for dentin formation. Here, Na\(^+\) channels are supposed to play a role as sensors that initiate tooth pain transmission (Allard et al., 2006). Based on TTX-sensitive Na\(^+\) channels these cells are even able to generate APs. An example for glial Na\(^+\) channel expression in another sensory tissue are the retinal Müller cells (Linnertz et al., 2011). In those cells, TTX-sensitive Na\(^+\) channels and T-type VGCCs are required for glutamate release from retinal glial cells implicated in cell volume regulation.

4.2.2.2 Voltage-activated potassium currents

We next aimed to characterize voltage-activated potassium currents, which we observed upon depolarization as large outward currents (Fig. 3.13). One of four main K\(^+\) channel classes is the family of 6TM predominantly voltage-gated (K\(_{\text{v}}\)) channels (Choe & Jolla, 2002). This family also comprises the subfamily of Ca\(^{2+}\)-activated K\(^+\) (K\(_{\text{Ca}}\)) channels (Coetzee et al., 1999; Swartz, 2004). K\(_{\text{v}}\) channels are activated by depolarization, usually allowing an outward movement of K\(^+\) ions, based on the electrochemical ion gradient. By contrast, K\(_{\text{Ca}}\) channels open upon increased intracellular [Ca\(^{2+}\)] and conduct an efflux of K\(^+\) at rest, which feeds back onto [Ca\(^{2+}\)] via re-/hyperpolarization of the membrane potential (Fakler & Adelman, 2008). Two important Ca\(^{2+}\)-activated K\(^+\) channel types are the big conductance K\(^+\) (BK) and the small conductance K\(^+\) (SK) channels. BK channels exhibit a large conductance and are gated cooperatively by both membrane depolarization and rising [Ca\(^{2+}\)], whereas SK channels are solely ligand-gated (Sah, 1996; Berkefeld et al., 2010). I showed here that SCs generate a large current via BK channels. By contrast, I found no evidence for solely Ca\(^{2+}\)-gated SK channels. Apamin, a
specific blocker of SK channels (Grunnet et al., 2001), and a dedicated pulse protocol were used to investigate SK channel conductance. However, we observed no detectable SK current in SCs. Since BK channels are blocked by low TEA concentrations (Coetzee et al., 1999) and do require Ca\(^{2+}\) for full activation, we used TEA (1 mM) and Cd\(^{2+}\) to block and identify BK currents. We observed that 55.21 % of the maximum voltage-gated K\(^{+}\) current was blocked by TEA (1 mM). Similarly, 57.52 % of the maximum K\(^{+}\) current was Cd\(^{2+}\)-sensitive and, thus, Ca\(^{2+}\)-dependent. We propose that both the TEA-sensitive and the Cd\(^{2+}\)-sensitive currents are largely conducted by the same channel type. The voltage-activated K\(^{+}\) current was completely abolished by a high TEA concentration of 30 mM, indicating the participation of other Kv channel family members.

Classically, BK channels contribute to repolarization in neuronal cells, thus terminating an AP and mediate the fast phase of the afterhyperpolarization following an AP (Storm, 1987; Bean, 2007). Since, the ability of AP generation by SCs was shown (Vogalis et al., 2005a), this may provide a possible function for BK current in olfactory SCs.

In the CNS, BK-Ca\(_v\) channel complexes assemble to mediate rapid and localized Ca\(^{2+}\)-activated K\(^{+}\) signaling (Berkefeld et al., 2006; Fakler & Adelman, 2008). Thus, Ca\(^{2+}\) influx through the Ca\(_v\) channel activates BK in the physiological voltage range in CNS neurons with submillisecond kinetics. Complex formation with distinct Ca\(_v\) channels enables BK-mediated membrane hyperpolarization that controls neuronal firing patterns and release of hormones and transmitters (Robitaille et al., 1993; Raffaelli et al., 2004; Berkefeld et al., 2006). Because BK activation is resulting in membrane repolarization and closing of Ca\(_v\) channels to reduce Ca\(^{2+}\) entry it provides a feedback regulation mechanism and allows integration of various cellular signals that are triggered by changes in [Ca\(^{2+}\)]\(_i\) homeostasis (Latorre & Brauchi, 2006; Fakler & Adelman, 2008).

BK-modulated Ca\(^{2+}\) signaling mechanisms that might involve BK-Ca\(_v\) channel complexes may also be present SCs in the MOE. For example, this mechanism may modulate the Ca\(^{2+}\) signal evoked by ATP-dependent activation of SCs in the MOE (Hegg et al., 2003), thus, possibly influencing the communication between SCs and OSNs, in which ATP signaling is suggested to play a role (Hegg et al., 2003).

**4.2.2.3 Hyperpolarization-activated cyclic nucleotide-gated channels**

Another class of voltage-gated ion channel that we investigated in SCs are HCN channels. The hyperpolarization-activated HCN channel current is known as I\(_h\), and carried by Na\(^{+}\) and K\(^{+}\) ions (Kamondi & Reiner, 1991; DiFrancesco, 1993). I\(_h\) is slowly activated by membrane hyperpolarization to potentials negative to -55 mV (Biel et al., 2009). Using hyperpolarizing current injections and voltage pulses, respectively, we recorded neither a typical voltage “sag”, nor a slow inward current (Fig.3.14). Based on the absence of these characteristics, I propose that HCN channels are not expressed in mouse SCs.
The absence of HCN currents may also indicate that APs are not generated during physiological conditions in SCs. This assumption is, on one hand, based on the finding of a relative positive $V_{\text{rest}}$ of -36 to -26 mV, which is far from the HCN activation threshold (Biel et al., 2009). On the other hand, this assumption relies on the lack of a mechanism to compensate the afterhyperpolarization of an AP, thus contributing significantly to the recovery of resting potential and the limitation of excessive hyperpolarization, which is one main function of HCN channels in excitable cells (Pape, 1996).

4.2.2.4 Voltage-gated calcium currents

$\text{Ca}_v$ channels are divided into three families, $\text{Ca}_v1$ - 3, based on their molecular diversity, their kinetics and pharmacological properties (Dolphin, 2009). $\text{Ca}_v1$ channels are comprised of L-type $\text{Ca}^{2+}$ channels, which typically require a strong depolarization for activation and show long-lasting currents (Tsien et al., 1986; Catterall, 2011). The $\text{Ca}_v2$ family contains P/Q-type, N-type and R-type channels, which show relatively slow and/or long-lasting activation kinetics (Catterall et al., 2005b). In contrast, members of the $\text{Ca}_v3$ family, also referred to as T-type channels, show smaller and more transient currents (Perez-Reyes et al., 1998) upon relatively modest depolarization. $\text{Ca}^{2+}$ ions orchestrate a number of intracellular events in different cell types, e.g. the activation of other ion channels such as BK channels that, as shown above, are also expressed in SCs. Here, I investigated the contribution of persistent versus transient voltage-gated $\text{Ca}^{2+}$ currents utilizing $\text{Cd}^{2+}$ as a broad $\text{Ca}_v$ channel blocker (Fig. 3.15). Therefore, we pharmacologically isolated voltage-gated $\text{Ca}^{2+}$ currents using $\text{Cs}^+$-based intracellular solution to abolish $\text{K}^+$ currents, and $\text{E}_{\text{Ca}}$ extracellular solution (see Materials and Methods) to which we added 4-AP (10 mM) and TTX (1 µM) to block TTX-sensitive $\text{Na}^+$ currents. We observed that the transient component of the pharmacologically isolated current was reduced ~50% by $\text{Cd}^{2+}$. Thus, our data suggest the existence of T-type $\text{Ca}_v$ channels with an activation near the SC resting membrane potential, a hallmark of LVA channels. The population of $\text{Cd}^{2+}$-insensitive current may be attributed to the TTX-insensitive component of a transient $\text{Na}_v$ current (see above). In future experiments, one could clarify this point by substituting $\text{Na}^+$ ions with NMDG. By contrast, we found no persistent $\text{Cd}^{2+}$-sensitive $\text{Ca}^{2+}$ current in SCs, indicating the lack of $\text{Ca}_v1$ and $\text{Ca}_v2$ channels in SCs of the mouse MOE.

Classical functional roles for T-type $\text{Ca}^{2+}$ currents in CNS neurons include generation of low-threshold $\text{Ca}^{2+}$spikes that lead to burst firing (Huguenard, 1996). However, this is unlikely here, because SCs were shown to be unable of generating more than one AP consecutively (Vogalis et al., 2005a). It is also doubtful that T-type $\text{Ca}^{2+}$ currents contribute to temporal patterns (oscillations) of $\text{Ca}^{2+}$ signaling upon purinergic stimulation (UTP and ATP) in SCs as shown by Lucero and coworkers (Hegg et al., 2009). They revealed that calcium-free extracellular solution or the non-specific $\text{Ca}^{2+}$ channel blocker nickel had no effect indicating no contribution
of external Ca\(^{2+}\) to Ca\(^{2+}\) waves found in SCs (Hegg \textit{et al.}, 2009). Thus, the exact function of T-type Ca\(^{2+}\) currents in SCs remains elusive.

4.2.3 Sustentacular cells do not show odor-evoked activation

In the MOE, OSNs are essentially enwrapped by supporting cells. Therefore, we hypothesized that SCs may also react to odor stimulation, e.g. by regulation of the extracellular ionic milieu. Previous findings on this issue by M.T. Lucero (Hegg & Lucero, 2006) and T.V. Getchell (Getchell, 1977) were controversial. While Getchell observed odor-stimulated responses, which consisted of slow potential changes in intracellular recordings (Getchell, 1977), Lucero and colleagues could not detect any Ca\(^{2+}\) response in imaging experiments (Hegg \textit{et al.}, 2003; Hegg & Lucero, 2006). Here, we aimed to test for either possibility.

To address this question I used mOR-EG mice as a tool to examine identified SCs in close proximity to GFP-labeled vanillin-responsive OSNs, employing whole-cell patch clamp recordings (Fig. 3.16). This way, we ensured the activation of a neuron in close proximity to the investigated SC. Additionally, we used an odor mixture of 10 components to activate distinct subsets of OSNs, hence increasing the possibility of responding OSNs in the SC proximity. Neither voltage-clamp, nor current-clamp recordings revealed odor-triggered responses in SCs. Thus, our data suggest that, at least on the electrophysiological level, there is no direct activation of SCs that reside in close proximity to activated OSNs. Nonetheless, SCs in general seem to be capable of odor-induced cellular responses such as heat shock protein 25 upregulation or purinergic signaling (Hegg \textit{et al.}, 2003; Hegg & Lucero, 2006).

It is also conceivable that odor-dependent metabolic clearance of ions from the mucus is a possible function for SCs as shown for other non-neuronal cells. In testes, for example, it was shown that Sertoli cells regulate ionic gradients (Veitinger \textit{et al.}, 2011). Local ATP release comprises a potential mechanism of Sertoli cell stimulation, since two different ATP receptor proteins are activated in Sertoli cells. Receptor activation, in turn, causes elevation of calcium ion levels inside the cells (Veitinger \textit{et al.}, 2011). A similar mechanism is also conceivable in other tissues, as already indicated by previous works on SCs in the MOE (Hegg \textit{et al.}, 2009).

4.3 The juvenile mouse pheromone ESP22 activates vomeronasal sensory neurons

Pheromones are powerful regulators of mouse social behavior because they provide information about sex, age, social hierarchy, as well as the health and endocrine state of an individual. Pheromone-mediated behaviors are highly stereotyped, but controlled by poorly understood mechanisms. Only few pheromones have been identified until now. One important pheromone class is the family of exocrine gland-secreting peptides (ESP), that are secreted
in mouse saliva and tears (Kimoto et al., 2005). In mice, the ESP family consists of 38 members, which were shown to be expressed in a sex- and strain-specific manner and to activate V2R-expressing sensory neurons in the VNO (Kimoto et al., 2005, 2007; Ferrero et al., 2013). Of particular interest is a male-specific ESP, secreted from the extraorbital lacrimal gland into tear fluid: ESP1 (Kimoto et al., 2005). ESP1 was among the first pheromones linked to a specific vomeronasal receptor and to a highly stereotyped behavior. ESP1 is detected by V2Rp5-expressing VSNs and enhances female sexual receptive behavior, i.e. lordosis, a posture to facilitate male sexual interaction (Haga et al., 2010). These findings indicate possible dedicated functions for other members of the ESP family. For example, only recently Prof. Dr. Stephen D. Liberles and colleagues from Harvard Medical School identified ESP22 as a juvenile-specific peptide secreted into tear fluid of prepubescent C57BL/6 mice (Ferrero et al., 2013). In a cooperation with Dr. Liberles and coworkers, we aimed to perform additional studies on ESP22 since a juvenile pheromone was so far unknown.

We addressed the question whether ESP22 is detected by the mouse olfactory system. Because another pheromone of the ESP family, ESP1, activates basal VSNs (Kimoto et al., 2005) we examined electrophysiological responses of basal neurons in the VNO. Electrovomeronasogram (EVG) recordings, performed in Dr. Kazushige Touhara’s group from the University of Tokyo, indicated that ESP22 evokes negative field potentials in the VNO. Moreover, ESP22 responses in the VNO required the ion channel TRPC2 as demonstrated in experiments with Trpc2 knock-out (Trpc2−/−) mice (Ferrero et al., 2013). Using extracellular loose-seal recordings, I showed that ESP22 activates basal VSNs in a dose-dependent and repetitive manner. Additionally, I revealed that juvenile tear fluid, the natural source of ESP22, activates ESP22-responsive cells. In my experiments, I detected threshold responses to ESP22 at concentrations of 20 pM, similar to threshold ESP1 responses previously measured using genetically encoded calcium indicators (He et al., 2010). At that concentration, ESP22 evoked robust and repetitive discharge patterns in 1.3% of basal VNO sensory neurons (5/383), consistent with detection by one or a few VNO receptors (Fig. 3.17). Most neurons responsive to ESP22 were also activated by juvenile tear fluid but not by maltose binding protein (MBP) or adult tears, with neuron viability verified by K+-mediated depolarization (Fig. 3.18). MBP served as a negative control here, because, we used recombinant ESP22, that was prepared as a fusion protein with MBP to enhance solubility.

High-affinity ESP22 responses were also detected in VNO sensory neurons using Ca2+ imaging (Fig. 3.19). ESP22 (200 nM) evoked repetitive Ca2+ transients in 0.5 – 2.6 % of the cells. These results indicate a repetitive induction of ESP22-dependent Ca2+ responses in VNO sensory neurons. However, because of slight tissue movements, it was sometimes difficult to track a single VSN soma over the entire measurement duration. Moreover, diffuse fluorescence signals made it hard to distinguish neighboring cells from each other.
Together, I have shown here that ESP22 activates basal VSNs in a dose-dependent and repetitive manner and that juvenile tear fluid, the natural source of ESP22, activates ESP22-responsive cells. Thus, ESP22 is a lacrimal peptide that is secreted into juvenile tears and serves as a chemosignal that activates the vomeronasal V2R-dependent response pathway. In further experiments, Dr. Liberles and colleagues attempted to link these results to an adult-juvenile social behavior. Using Trpc2\(^{-/-}\) mice, as a powerful tool for VNO loss-of-function studies (Liman et al., 1999), they could show that intact VNO signaling normally prevents mating advances towards prepubescent mice (Ferrero et al., 2013). Trpc2\(^{-/-}\) adult males displayed both highly enhanced mounting behavior towards juvenile C57BL/6 females and a significantly reduced latency to mount (Ferrero et al., 2013). Testing juveniles of the C3H strain, a strain that lack juvenile ESP22 expression (Ferrero et al., 2013), C57BL/6 males displayed a striking reduction of mounting attempts towards C3H juveniles when painted with ESP22. However, sexual behavior towards unpainted and MBP-painted juveniles was increased (Ferrero et al., 2013). In summary, these results showed that Trpc2\(^{-/-}\) mice exhibit increased sexual behavior towards juvenile mice, indicating an involvement of an intact VNO response pathway, and that ESP22 is indeed the relevant component inhibiting sexual behavior in wild-type C57BL/6 mice.

4.4 Excursus: Single-cell electroporation as an imaging approach in VNO slices
Because conventional \([\text{Ca}^{2+}]\) imaging in VNO sections posed technical challenges, I aimed to establish a novel labeling and imaging approach. Instead of bolus loading with the cell permeable \(\text{Ca}^{2+}\)-sensitive reporter dye fluo-4/AM, we loaded single cells with the cell impermeable fluo-4 via electroporation. Electroporation is a technique that allows transient exchange of charged material (here, fluorescence dye) across the temporary perturbed cell membrane. This technique was originally developed for targeting gene transfer to individual cells in intact tissue (Neumann & Rosenheck, 1972; Neumann et al., 1982; Haas et al., 2001). Using modified patch electrodes and standard electrophysiological equipment, this technique is applicable to various systems and tissues where cells can be targeted with a micropipette (Bestman et al., 2006). Before targeting single cells in slices, an important step was to control for dye leakage. Therefore, pipettes were dipped into bath solution (no specimen to avoid unspecific loading by dye exposure) and minimal positive pressure was applied to avoid clogging of the pipette. An offset potential of opposite charge compared to the fluorescence dye (fluor-4 is positively charged \(\rightarrow\) negative offset voltage) was applied until no dye leakage from the pipette tip occurred during pressure ejection of solution. Once the offset voltage was adjusted correctly (dye diffusion from the pipette tip is hardly detectable), the slice chamber was placed onto the microscope stage. In close proximity to individual neurons in the VNO basal layer a voltage pulse was set depending on dye charge so that the dye is ejected from
the pipette tip (fluo-4 → positive voltage pulse). The voltage pulse was repeated once or twice until a visible dye loading of the cell was observed.

Based on the observation that biomembranes show enhanced permeability upon short electric impulses without lasting damage to the membrane structure, the technique leads to a transient exchange of material across the perturbed membrane (Sale & Hamilton, 1968; Lindner et al., 1977). The increase in permeability was reported to be based on hydrophilic pore formation within a fraction of a microsecond (Kinosita & Tsong, 1977). Once the transmembrane potential induced by the applied electrical field reaches a critical value, a reversible pore is formed in the lipid part of the membrane (Benz & Zimmermann, 1981). After electroporation, the patch pipette was quickly removed without disrupting the cell membrane.

In this way, I successfully labeled 20-30 cells per acute vomeronasal slice within 10 - 15 min (Fig. 3.20). The dye diffused within seconds from the injection site at the basal soma to dendritic regions revealing the typical VSN bipolar morphology. Using this technique, enhanced contrast allowed the discrimination of single cells against the unstained epithelial background. Thus, problems of diffuse fluorescence signals were resolved. I next aimed to examine stimulus-evoked Ca$^{2+}$ signals in dye-loaded VSNs (Fig. 3.21). I was able to detect robust Ca$^{2+}$ transients to both a 50 mM and a 100 mM K$^+$ stimulus. Together I could show that electroporation, as a targeted dye loading method for single cell Ca$^{2+}$ imaging, is generally functional. However, response rates as well as response strength remained relatively weak.

Further steps or potential applications for this technique may be background-free investigations of labeled cells or immunohistological procedures. For example, single cells with an identified physiological phenotype could be labeled, allowing immunocytochemical post-hoc analysis to identify coexpression of proteins of interest.

4.5 Cl$^-$ imaging in the mouse VNO using 2P-FLIM

In mice, the detection of pheromones by VSNs requires the binding of signal molecules to vomeronasal receptors expressed in the dendritic knob microvillous membrane. An intracellular signaling pathway leads to subsequent activation of two different types of ion channels: TRPC2 and ANO2. The unspecific cation channel TRPC2 is highly expressed in VSN microvilli and leads to an influx of Na$^+$ and Ca$^{2+}$ (Liman et al., 1999). TRPC2-deficient mice have serious deficits in social or sexual behavior (Stowers et al., 2002; Leypold et al., 2002). Ca$^{2+}$ ions, in turn, open the calcium-dependent chloride channel ANO2. This is hypothesized to lead to an amplifying Cl$^-$ outward current during stimulus-induced responses (Yang & Delay, 2010; Kim et al., 2011). Though controversially discussed (Billig et al., 2011), it has been shown that Ca$^{2+}$-activated Cl$^-$ efflux in mouse VSNs contributes up to 80% of the receptor current in response to urine (Yang & Delay, 2010). Moreover, the anoctamin subunits
ANO1 and ANO2 have been identified to be highly expressed in the apical surface, i.e. microvillous layer of the VNO sensory epithelium where they colocalize with the TRPC2 channel (Dibattista et al., 2012).

Most mature neurons possess Cl$^-$ transporters that pump Cl$^-$ either into (Haas & Forbush, 2000; Schöbel et al., 2012) or out of the cells (Duran et al., 2010). Interestingly, PCR experiments on the rat MOE revealed expression of Na-K-Cl cotransporter 1 (NKCC1), Na-Cl cotransporter (NCC), and K-Cl cotransporter 1 (KCC1) (Kaneko et al., 2004). Especially NKCC1 is required for the excitatory amplifying Cl$^-$ outward current in OSNs (Reisert et al., 2005; Nickell et al., 2007). Notably, KCC2 is lacking in the rat MOE (Kaneko et al., 2004). This is plausible since this protein provides the main contribution to keeping [Cl$^-$]$_i$ low in CNS neurons (Rivera et al., 1999; Stein et al., 2004). Based on the above findings in the MOE, some authors (Yang & Delay, 2010; Kim et al., 2011) have proposed a similarly elevated Cl$^-$ concentration in VSNs too. To investigate if this is true, I used 2P-FLIM of the dendritic knob and SC layer of the vomeronasal sensory epithelium in a VNO en face preparation (Riviere et al., 2009). These experiments were performed in collaboration with the group of Dr. Thomas Gensch (Institute of Complex Systems 4 (ICS-4, Cellular Biophysics), Forschungszentrum Jülich), an expert in 2P-FLIM.

First, I confirmed that the VNO en face preparation provides experimental access to a largely intact VNO sensory epithelium (Fig. 3.22). Next, I revealed that this preparation was also suitable for 2P-FLIM Cl$^-$ imaging (Fig. 3.23). I used OMP-GFP mice to discriminate different cell types and structures. VSN knobs were identified in a dense apical field with diameters of 1-2 µm, whereas VSN dendrites were indicated by small fluorescent spots 5-15 µm below the knob layer. They formed a honey comb-like structure with SCs designated by dark gaps. Using MQAE as a fluorescent indicator of intracellular Cl$^-$ (Verkman, 1990), I revealed a somewhat inverse illustration of VSN knobs and SCs. Knobs appeared as dark ‘gaps’, whereas SCs showed strong fluorescence intensity. False color MQAE lifetime images revealed shorter lifetimes in the more apical knob region compared to dendrites indicating a higher [Cl$^-$]$_i$ in VSN knobs and a [Cl$^-$] gradient within single cells. A similar [Cl$^-$] gradient has been found in OSNs in 2P-FLIM measurements by Kaneko and coworkers (Kaneko et al, 2004). To summarize, I showed that VSNs as well as SCs take up MQAE, resulting in a fluorescence signal. Our results fit the hypothesis of elevated [Cl$^-$]$_i$ in VSNs (Yang & Delay, 2010; Kim et al., 2011) indicated by elevated [Cl$^-$] in knobs compared to dendrites.

To confirm the hypothesis of Cl$^-$ accumulation in VSNs (Yang & Delay, 2010; Kim et al., 2011) I next aimed to determine the exact [Cl$^-$]$_i$ under physiological conditions. I therefore attempted to obtain a quantitative calibration of intracellular Cl$^-$ levels and MQAE fluorescence to gain absolute values for [Cl$^-$]. (Fig 3.24). A quantitative calibration to determine Cl$^-$ concentrations is required because MQAE measurements display only relative changes of fluorescence.
intensity and lifetime. Not only Cl⁻ ions contribute to quenching of MQAE molecules but also other ions, amino-acid residues as well as small cytosolic molecules. Consequently, MQAE properties are different in distinct cell types since they depend on individual intracellular milieus. In contrast to fluorescence intensity, fluorescence lifetime is independent of dye concentration and cell volume and is therefore constant for one cell type. To gain absolute values of [Cl⁻] and to allow comparison of intracellular Cl⁻ levels each cell type requires its own calibration. I tried to calibrate the [Cl⁻] and the Cl⁻-sensor MQAE using the K⁺/H⁺ exchanger nigericin and the Cl⁻/OH⁻ antiporter tributyltin (Krapf et al., 1988; Chao et al., 1989; Markova et al., 2008). In these experiments, a K⁺ (instead of Na⁺)-based external solution was adjusted to specific predetermined Cl⁻ concentrations by substituting equimolar concentrations of K⁺-gluconate with KCl to a final concentration of 140 mM K⁺. Nigericin and tributyltin were freshly added to the external solution before bath solution was applied. However, this technique did not lead to a satisfactory calibration and quantitative information. Drug treatment did not produce equilibrated Cl⁻ levels in the VNO sensory epithelium. Instead, 2P-FLIM results in the VNO are, at this point, qualitative in nature, providing information about relative differences instead of absolute values. Because these first calibration experiments generated contradictory results, in future experiments we aim to establish a quantitative calibration.

One possibility to investigate and compare relative fluorescence lifetime signals is to analyze a [Cl⁻] gradient within one cell, e.g. the [Cl⁻] gradient indicated between VSN knobs and dendrites (see Fig. 3.23). Another method to use relative fluorescence signals is the analysis of stimulus-induced changes of [Cl⁻] within one cell type. Here, I chose to investigate stimulus-induced changes of [Cl⁻] in VSN knobs and SCs. In these experiments I showed that VSN knobs and SCs indeed exhibited stimulus-induced changes of [Cl⁻] (Fig. 3.25). Therefore, I used mouse urine as a ‘broadband’ VSN stimulus because it was known to activate a large subset of vomeronasal neurons (Tirindelli et al., 2009). To quantitatively analyze changes in [Cl⁻], I compared the lifetime τ before and after stimulus application in optically identified knobs and SCs. Δτ was determined as an index for change in lifetime. MQAE lifetime false color images implicated longer lifetimes in the stimulated knobs, hence suggesting a decrease in [Cl⁻]. MQAE lifetime measurements also revealed a shift to shorter lifetimes in SCs during prolonged stimulation, thus indicating an increased [Cl⁻]. These results showed that a subset of VSN knobs reacted with a decrease of [Cl⁻] to stimulation, whereas another subset seems to be unaffected by the presence of urine. This is in contrast to most SCs that responded with an increase of [Cl⁻] to urine stimulation. To quantify these data, I plotted the number of cells against the τ values summarized in bins of 20 ps (knobs) or 10 ps (SCs), respectively (Fig. 3.26). Pre- and post-stimulus measurements of knobs and SCs were generated from two different sets of experiments, under either urine
stimulation or control conditions (exchange of extracellular solution). Histogram plots of $\Delta \tau$ analysis of urine-stimulated VSN knobs revealed a shift to negative $\Delta \tau$ values and a second peak at 0 ps. I suggest that the main peak without any shift in $\Delta \tau$ indicated a population of knobs showing no or only a very slight decrease in $[\text{Cl}^-]_i$, whereas the shift to more negative $\Delta \tau$ values (second peak), suggested a second knob population reacting with a more pronounced decrease of $[\text{Cl}^-]_i$. In contrast, $\Delta \tau$ values of urine-stimulated SCs displayed a shift to positive values indicating that the majority of SCs responded with an increase of $[\text{Cl}^-]_i$. Based on these data I propose following hypothesis: Odor stimulation leads to a Cl$^-$ efflux from the VSN cytoplasm, based on their elevated $[\text{Cl}^-]_i$, and a simultaneous Cl$^-$ uptake by the SCs from the extracellular space to maintain the Cl$^-$ homeostasis in the sensory epithelium. Two populations of knobs observed in $\Delta \tau$ analysis would refer to a population of urine-activated VSN (~20%) and a larger fraction of not responding cells. In contrast, I observed only one population of SCs with an increased $[\text{Cl}^-]_i$, based on the uptake of Cl$^-$ from the extracellular space, where $[\text{Cl}^-]$ is elevated because of Cl$^-$ release from activated VSNs. However, control experiments revealed that this hypothesis is not completely valid. For VSN knobs, histogram plots from control experiments still depicted a robust shift to negative $\Delta \tau$ values. By contrast, SCs exhibited a peak around $\Delta \tau$ 0 ps or a slight shift to opposite negative values. Together, these data suggested a stimulus-induced increase of $[\text{Cl}^-]_i$ in SCs in response to urine stimulation, whereas the $[\text{Cl}^-]_i$ decrease in knobs should rather be attributed to stimulus-independent effects.

In summary, I established the VNO en face preparation as method suitable for 2P-FLIM Cl$^-$ imaging experiments. Because, so far, I was not able to obtain a quantitative calibration and thus absolute values for $[\text{Cl}^-]_i$, I used an experimental design suitable for qualitative comparison of 2P-FLIM results. My data suggested a $[\text{Cl}^-]$ gradient within VSNs and a stimulus-induced increase of $[\text{Cl}^-]_i$ in SCs in response to urine.
5. Summary

In most mammals, the detection of environmental cues is essential for survival of the individual and, accordingly, the whole species. For accurate detection and discrimination of a myriad of different environmental cues, the olfactory system has evolved several subsystems of high complexity. However, many basic molecular and cellular mechanisms of olfactory signaling are still unknown. In this thesis, I therefore aimed to gain more profound insight into distinct olfactory signaling mechanisms. Specifically, my research focused on four different physiological aspects of mouse peripheral olfaction in either the MOE or the VNO.

First, I aimed to characterize the functional role of mitochondrial Ca\textsuperscript{2+} mobilization in OSNs during odor activation. Using patch-clamp recordings, I showed that mitochondrial Ca\textsuperscript{2+} recruitment in OSNs is essential to maintain a broad dynamic response range. When mitochondrial function was impaired, OSNs function as simple stimulus detectors rather than intensity encoders. Moreover, extracellular loose-seal recordings of OSN output revealed impaired odor-encoded discharge, in particular during short stimulations, when mitochondrial Ca\textsuperscript{2+} buffering was inhibited. My findings suggest that mitochondria are critical determinants of the olfactory input - output function. Furthermore, FRAP experiments showed mitochondrial arrest in dendritic knob regions upon odor stimulation, indicating activity-dependent recruitment of mitochondria to areas of specific metabolic / biochemical demand.

Second, I aimed to provide an in-depth electrophysiologic characterization of SCs. I described a variety of voltage-dependent currents in SCs, including transient Na\textsuperscript{+} and Ca\textsuperscript{2+} as well as hardly inactivating K\textsuperscript{+} currents. However, I did not detect any odor-dependent electrophysiological changes in SCs.

Third, I aimed to analyze the physiological impact of ESP22, a previously undescribed strain- and age-specific mouse pheromone that is enriched in tears of prepubescent mice. Using extracellular recordings, I showed that ESP22 activates basal VSNs in a dose-dependent and repetitive manner. Additionally, I revealed that juvenile tear fluid activates ESP22-responsive cells. Thus, together with Dr. Liberles’ group, I showed that ESP22 is a lacrimal peptide that is secreted into juvenile tears and serves as a VNO chemosignal.

Fourth, I aimed to investigate the role of Cl\textsuperscript{-} in VNO signaling. Establishing FLIM in a dedicated en face preparation of the VNO sensory surface, I here showed an intracellular [Cl\textsuperscript{-}] gradient along the apical VSN dendrite. Moreover, my findings suggest a stimulus-induced [Cl\textsuperscript{-}] increase in SCs, whereas no stimulus-correlated effects on [Cl\textsuperscript{-}] in VSN knobs were observed. Together, the data I obtained in this thesis provide a deeper understanding of different physiological mechanisms in the peripheral olfactory system.
6. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A.dest</td>
<td>Aqua destillata</td>
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<tr>
<td>AC III</td>
<td>Adenylate cyclase III</td>
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<tr>
<td>ANO2</td>
<td>Anoctamin 2</td>
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<tr>
<td>AOB</td>
<td>Accessory olfactory bulb</td>
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<tr>
<td>AP</td>
<td>Action potential</td>
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<tr>
<td>ATP</td>
<td>Adenosine-triphosphate</td>
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<tr>
<td>BES</td>
<td>Bis(2-hydroxyethyl)-2 aminoethanesulfonic Acid</td>
</tr>
<tr>
<td>BK</td>
<td>Big-conductance Ca^{2+}-activated K^+ channels</td>
</tr>
<tr>
<td>Bv</td>
<td>Blood vessel</td>
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<tr>
<td>[Ca^{2+}]_e</td>
<td>Extracellular calcium</td>
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<tr>
<td>[Ca^{2+}]_i</td>
<td>Cytosolic / intracellular calcium</td>
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<tr>
<td>CaCC</td>
<td>Ca^{2+}-activated Cl^- channel</td>
</tr>
<tr>
<td>Ca_v</td>
<td>Voltage-gated calcium channels</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosinmonophosphat</td>
</tr>
<tr>
<td>CCC</td>
<td>Cation-coupled Cl^- cotransporter</td>
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<tr>
<td>CNBD</td>
<td>Cyclic-nucleotide binding domain</td>
</tr>
<tr>
<td>CNG</td>
<td>Cyclic nucleotide-gated ion channel</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerole</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
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<tr>
<td>Dr</td>
<td>Dorsal roof</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ESP</td>
<td>Exocrine gland-secreting peptide</td>
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<tr>
<td>Et</td>
<td>Endoturbinate</td>
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<tr>
<td>ETC</td>
<td>Electron transport chain</td>
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<tr>
<td>EVG</td>
<td>Electro-vomeronasogram</td>
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<tr>
<td>FADH2</td>
<td>Flavin-adenin-dinucleotid</td>
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<tr>
<td>FCCP</td>
<td>Carbonyl cyanid 4-(trifluoromethoxy) phenyl-hydrazone</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Fh</td>
<td>Frontal hemisphere</td>
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<tr>
<td>FLIM</td>
<td>Fluorescence lifetime imaging microscopy</td>
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<td>FPRs</td>
<td>Formyl peptide receptor like proteins</td>
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<tr>
<td>FRAP</td>
<td>Fluorescence recovery after Photobleaching</td>
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<td>GC-D</td>
<td>Guanylyl cyclase D</td>
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<td>GG</td>
<td>Grueneberg Ganglion</td>
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<td>G proteins</td>
<td>GTP binding proteins</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
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<tr>
<td>HCN</td>
<td>Hyperpolarization-activated cyclic nucleotide-gated channel</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid</td>
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<tr>
<td>HVA</td>
<td>High-voltage activated</td>
</tr>
<tr>
<td>I\textsubscript{Ca}</td>
<td>Ca\textsuperscript{2+} current</td>
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<tr>
<td>I\textsubscript{h}</td>
<td>Hyperpolarization-activated cyclic nucleotide-gated current</td>
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<tr>
<td>I\textsubscript{k}</td>
<td>Potassium current</td>
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<tr>
<td>I\textsubscript{Na}</td>
<td>Sodium current</td>
</tr>
<tr>
<td>IP\textsubscript{3}</td>
<td>Inositol 1,4,5-trisphosphate</td>
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<tr>
<td>KCC</td>
<td>K-Cl cotransporter</td>
</tr>
<tr>
<td>MCU</td>
<td>Mitochondrial Ca\textsuperscript{2+} uniporter</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MOB</td>
<td>Main olfactory bulb</td>
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<tr>
<td>MOE</td>
<td>Main olfactory epithelium</td>
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<td>MOS</td>
<td>Main olfactory system</td>
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<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MUP</td>
<td>Major urinary proteins</td>
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<tr>
<td>n</td>
<td>Number</td>
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<tr>
<td>NADH</td>
<td>Nicotinamid-adenin-dinucleotid</td>
</tr>
<tr>
<td>Nc</td>
<td>Nasal cavity</td>
</tr>
<tr>
<td>NCC</td>
<td>Na-Cl cotransporter</td>
</tr>
<tr>
<td>NCX</td>
<td>Na⁺/Ca²⁺ exchanger</td>
</tr>
<tr>
<td>NKCC</td>
<td>Na-K-Cl cotransporter</td>
</tr>
<tr>
<td>MQAE</td>
<td>N-(Ethoxycarbonylmethyl)-6 methoxyquinolinium Bromide</td>
</tr>
<tr>
<td>OMP</td>
<td>Olfactory marker protein</td>
</tr>
<tr>
<td>OR</td>
<td>Olfactory receptor</td>
</tr>
<tr>
<td>OSN</td>
<td>Olfactory sensory neuron</td>
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<tr>
<td>Pa</td>
<td>Palate</td>
</tr>
<tr>
<td>Ph</td>
<td>Parietal hemisphere</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5-biphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane Ca²⁺ ATPase</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>Rₗm</td>
<td>Membrane resistance</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Ru 360</td>
<td>Rutenium 360</td>
</tr>
<tr>
<td>RuR</td>
<td>Rutenium Red</td>
</tr>
<tr>
<td>SC</td>
<td>Sustentacular / supporting cell</td>
</tr>
<tr>
<td>Se</td>
<td>Septum</td>
</tr>
<tr>
<td>SK</td>
<td>Small conductance potassium channels</td>
</tr>
<tr>
<td>SO</td>
<td>Septal organ</td>
</tr>
<tr>
<td>SERCA</td>
<td>Smooth endoplasmic reticular Ca²⁺ ATPase</td>
</tr>
<tr>
<td>TAAR</td>
<td>Trace amine-associated receptors</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium chloride</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TMEM16B</td>
<td>Transmembrane protein 16, member B</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>TRPC2</td>
<td>Transient receptor potential cation channel, subfamily C, member 2</td>
</tr>
<tr>
<td>V1R</td>
<td>Vomeronasal receptor type 1</td>
</tr>
<tr>
<td>V2R</td>
<td>Vomeronasal receptor type 2</td>
</tr>
<tr>
<td>Vb</td>
<td>Vomerbone</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage gated Ca$^{2+}$ channels</td>
</tr>
<tr>
<td>VNO</td>
<td>Vomeronasal organ</td>
</tr>
<tr>
<td>VSN</td>
<td>Vomeronasal sensory neuron</td>
</tr>
</tbody>
</table>
7. Literature


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9. Curriculum vitae

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Education and academic degrees

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PhD thesis - RWTH Aachen University, Dept. of Biology II / Chemosensation “Physiological characterization of chemosensory mechanisms in mice.”

10 / 2008 – 09 / 2010
Master of Science (M.Sc.) in Biology - Justus-Liebig-University Gießen / RWTH Aachen University „Elektrophysiologische Untersuchungen der Mitochondrienfunktion in olfaktorischen sensorischen Neuronen der Maus“

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Bachelor of Science (B.Sc.) in Biology - Justus-Liebig-University Gießen / Hessen „Der globale Spleißfaktor hnRNP L - Konstruktion und Expression von epitopmarkierten Varianten des Spleißregulatorproteins hnRNP L“

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Article:


Meera P, Wallner M, Song M & Toro L (1997). Large conductance voltage- and calcium-dependent K+ channel, a distinct member of voltage-dependent ion channels with


Park MK, Petersen OH & Tepikin a V (2000). The endoplasmic reticulum as one continuous Ca(2+) pool: visualization of rapid Ca(2+) movements and equilibration. EMBO J 19, 5729–5739.


Article:

*Nature Neuroscience*, 2012, "Mitochondrial Ca²⁺ mobilization is a key element in olfactory signaling." Daniela Fluegge*, Lisa M. Moeller*, Annika Cichy, Monika Gorin, Agnes Weth, Sophie Veitinger, Silvia Cainarca, Stefan Lohmer, Sabrina Corazza, Eva M. Neuhaus, Werner Baumgartner, Jennifer Spehr and Marc Spehr, *These authors contributed equally to this work*

Awards

Travel Award: 300, - €
Awarded by: Neurowissenschaftlichen Gesellschaft e.V (NWG) Congress: 10th Göttingen meeting of the german neuroscience society 2013, Göttingen, Germany

Travel Award: 893, - €

Travel Award: 800, - €
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Congress participation / poster contribution

10th Göttingen Meeting of the German Neuroscience Society 2013, Göttingen, Germany
Lisa M Moeller, Daniela Fluegge, Annika Cichy, Monika Gorin, Agnes Weth, Sophie Veitinger, Silvia Cainarca, Stefan Lohmer, Sabrina Corazza, Eva M Neuhaus, Werner Baumgartner, Jennifer Spehr Marc Spehr
"Mitochondrial Ca²⁺ mobilization plays a key role in mouse olfactory signaling"

XVI International Symposium on Olfaction and Taste (ISOT) 2012, Stockholm, Sweden
Lisa M Moeller, Daniela Fluegge, Annika Cichy, Monika Gorin, Agnes Weth, Sophie Veitinger, Silvia Cainarca, Stefan Lohmer, Sabrina Corazza, Eva M Neuhaus, Werner Baumgartner, Jennifer Spehr Marc Spehr
"Mitochondrial Ca²⁺ mobilization is a key element in olfactory signaling"

Association for Chemoreception Sciences (AChemS) 33rd Annual Meeting 2011, Florida, USA
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"On the role of mitochondrial calcium in mouse olfactory sensory neurons"

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Daniela Fluegge, Lisa M Moeller, Jennifer Spehr, Sophie Veitinger, Silvia Cainarca, Stefan Lohmer, Sabrina Corazza, Eva M Neuhaus, Marc Spehr
"On the role of mitochondrial calcium in mouse olfactory sensory neurons"