The long tale of the calcium activated Cl\(^{-}\) channels in olfactory transduction

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ABSTRACT

Ca\(^{2+}\)-activated Cl\(^{-}\) currents have been implicated in many cellular processes in different cells, but for many years, their molecular identity remained unknown. Particularly intriguing are Ca\(^{2+}\)-activated Cl\(^{-}\) currents in olfactory transduction, first described in the early 90s. Well characterized electrophysiologically, they carry most of the odorant-induced receptor current in the cilia of olfactory sensory neurons (OSNs). After many attempts to determine their molecular identity, TMEM16B was found to be abundantly expressed in the cilia of OSNs in 2009 and having biophysical properties like those of the native olfactory channel. A TMEM16B knockout mouse confirmed that TMEM16B was indeed the olfactory Cl\(^{-}\) channel but also suggested a limited role in olfactory physiology and behavior.

The question then arises of what the precise role of TMEM16b in olfaction is. Here we review the long story of this channel and its possible roles.

Introduction

In 1991, a revolution began in the olfactory field with the discovery of olfactory receptors (ORs) by Linda Buck and Richard Axel.\(^{1}\) This Nobel Prize discovery fueled rapid progress in our understanding how ORs are singly expressed in a given olfactory sensory neuron (OSN), how olfactory axons target to the brain and what the logic of olfactory coding might be.

Coincidentally, at the same time, Kleene and Gesteland discovered,\(^{2}\) in an effort to understand the role of Ca\(^{2+}\) in olfactory transduction, a Ca\(^{2+}\)-activated current in frog olfactory cilia that was almost entirely carried by Cl\(^{-}\). Later, Ca\(^{2+}\)-activated Cl\(^{-}\) currents were described in olfactory cilia of all vertebrates tested, including fish, amphibians, and rodents.\(^{3-6}\) It might seem much easier to determine the role of a single ion channel compared with 1000 ORs expressed in the mouse. But the quest to understand the role of the Ca\(^{2+}\)-activated Cl\(^{-}\) channel (CaCC) turned into a longer - at times convoluted - tale about olfactory signal transduction and the CaCC. This tale - that we will try to keep short - is still being, literally, written 25 y later.

By the mid 90s, the molecular identity of the olfactory transduction components needed to activate an odorant response had been completed, bar one. The identity of the CaCC participating in transduction remained elusive, hampering efforts to understand and characterize the role of this channel. Historically, the description, cloning, and characterization of Cl\(^{-}\) channels, in general, have always lagged behind that of Na\(^{+}\), Ca\(^{2+}\), and K\(^{+}\) channels. Although Cl\(^{-}\) is the most abundant anion in the extracellular fluids and plasma, Cl\(^{-}\) currents have long been considered a sort of background conductance with a lack of substantial interest by neuroscientists. Additionally, the lack of knowledge about natural toxins that target the Cl\(^{-}\) conductance certainly has not helped.

But – as it happens - Cl\(^{-}\) channels have received increasing attentions in the last few years. This interest has been partly driven by the appreciation that Cl\(^{-}\) can be seen as a “free agent:” it is unbound by, generally speaking, rather fixed ionic gradients across cell membranes like those for Na\(^{+}\) and K\(^{+}\), and thus can be used as an inhibitory or an excitatory ion. The increased
interest in Cl\(^{-}\) channels led to a rapid increase in our knowledge of their molecular identity and function. Indeed, in 2008 a new family of CaCCs was discovered\(^{7-9}\) and it was found that one member of this family, TMEM16B (also known as Anoctamin 2) is expressed in OSNs and is the long sought after CaCC, the main topic of our review.

We will guide the reader through the numerous twists of CaCCs, from Cl\(^{-}\) current characterization in native olfactory cilia, up to their cloning and possible role in olfaction.

**Odorant transduction and its components**

The very first step of odor detection takes place in the olfactory epithelium (OE) in which the bipolar OSNs are embedded. OSNs are the first functional unit of the olfactory system; their axons leave the epithelium to reach the olfactory bulb (OB) in the brain, and they extend their single dendrite to the apical surface of the OE ending with a knob which bears around 10–20 cilia. The components of the olfactory transduction machinery are expressed almost exclusively in the cilia (Fig. 1) and they convert the chemical information of the odorant-OR interaction into an electrical signal ultimately triggering action potentials (APs) that are sent to the brain.

ORs are 7 transmembrane G protein-coupled receptors and a single OR can respond to several different odorants and may be broadly tuned or more narrowly selective.\(^{10}\) When an odorant molecule binds to an OR, the ensuing conformational change triggers the activation of the G protein \(G_{olf}\),\(^{11}\) which in turn favors the enzymatic activity of the adenylyl cyclase 3 (AC3),\(^{12}\) thus leading to an increase in intra-ciliary levels of cAMP. cAMP opens cyclic nucleotide-gated (CNG) channels,\(^{13,14}\) allowing Na\(^{+}\) and Ca\(^{2+}\) to enter the ciliary lumen. This first series of events is necessary and sufficient to depolarize the OSN that will fire APs to be sent to the OB.

Ca\(^{2+}\) increase inside the cilia is responsible for various mechanisms: among those, the activation of CaCCs that leads to Cl\(^{-}\) exiting the cilia, further depolarizing the cell. The presence of a Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter, NKCC1, ensures that the Cl\(^{-}\) concentration inside the cilia is kept high, in the same range of that present in the mucus outside the cilia, supporting the depolarizing effect of CaCCs activation.\(^{15-17}\) The unusually high Cl\(^{-}\) concentration inside the cilia is the interesting feature that determines the depolarizing role of the Cl\(^{-}\) current. Although its exact value may be species-specific the measured intracellular Cl\(^{-}\) concentration varies from 55 mM up to 69 mM depending also on the techniques.
used to measure it;\textsuperscript{15,16} those values are close to the Cl\textsuperscript{−} concentration measured in the mucus surrounding the knob and cilia of the OSNs. Energy dispersive X-ray micro analysis on cryosections of rat OE estimated a Cl\textsuperscript{−} concentration of 69 mM inside the cells and 55 mM in the mucus, with a calculated equilibrium potential for Cl\textsuperscript{−} of \( 6 \text{ mV} \).\textsuperscript{16} In another study, Cl\textsuperscript{−} concentration was estimated from the intact OE of mice and rats using 2-photon fluorescence lifetime imaging of the Cl\textsuperscript{−}-sensitive dye MQAE (Fig. 2A & B). A maximal concentration reaching up to 70 mM was found within the dendritic knob, decreasing to 55 mM along the dendrite in the presence of a high extracellular Cl\textsuperscript{−} concentration.\textsuperscript{15} For a lower extracellular Cl\textsuperscript{−} concentration of 50 mM the gradient disappeared (Fig. 2B), suggesting the presence of an apical Cl\textsuperscript{−} uptake system.\textsuperscript{15} This data support the view that a Cl\textsuperscript{−} accumulation process charges the resting cilia prompting an excitatory Cl\textsuperscript{−} efflux in olfactory transduction.\textsuperscript{17–19}

Different mechanisms are responsible for the termination of the response. cAMP is hydrolyzed by 2 phosphodiesterases (PDEs), PDE1C in the cilia and PDE4A in the other part of OSNs. Although little is known about the fate of ciliary cAMP, recent

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**Figure 2.** Cl\textsuperscript{−} gradients in olfactory sensory neurons and activation of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents in olfactory cilia. The depolarizing action of the Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} is possible because the intraciliary Cl\textsuperscript{−} concentration is around 60 mM in the knob/cilia region of an OSN (A and B). Modified from Kaneko et al. 2004 with permission.\textsuperscript{15} A series of voltage steps in an excised frog cilium showed the existence of cAMP-activated current followed by a secondary current (C) that was absent when Cl\textsuperscript{−} was removed from the pseudointracellular bath (D) Modified from Kleene 1993 with permission.\textsuperscript{61} This secondary current was also present in mouse OSNs following cAMP uncaging (E and F). It constitutes up to the 90% of the transduction current and was blocked by the Cl\textsuperscript{−} channel blocker niflumic acid (gray traces). © American Physiological Society. Reproduced by permission of American Physiological Society. Permission to reuse must be obtained from the rightsholder.\textsuperscript{19}
knockout studies in mice revealed that hydrolysis of ciliary cAMP is not a rate-limiting factor for termination kinetics.\textsuperscript{20,21} Indeed, it is most likely that the diffusional escape of cAMP from the ciliary lumen is sufficient to allow a rapid response termination.\textsuperscript{22,23}

However, not only the CNG channels have to close, but also the CaCCs. Their closure kinetics are dictated by the rate of ciliary Ca\textsuperscript{2+} removal, which is mainly due to the activity of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. The role of a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in OSNs has been suggested since the mid-1990s. Preventing Ca\textsuperscript{2+} extrusion by reducing extracellular Na\textsuperscript{+}, and thus abolishing the driving gradient that fuels the exchange, prolongs the odorant-induced receptor current by seconds, generating a prolonged Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} current.\textsuperscript{24-26}

More recently it has been shown that the potassium-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger 4 (NCKX4) is expressed in the cilia of OSNs and plays the major role in Ca\textsuperscript{2+} extrusion and therefore, in controlling the termination of the odorant response.\textsuperscript{27-29} A further contribution to Ca\textsuperscript{2+} dynamics inside the cilia of OSNs is given by mitochondria in the dendritic knob. Not only do they contribute to the low resting Ca\textsuperscript{2+} but also they are responsible for its clearance during odorant stimulation. Noteworthy is that odorant responses are also affected since pharmacological alteration of the inner mitochondrial membrane potential shifts the dynamic range of the OSN stimulus-response function.\textsuperscript{30}

cAMP-activated currents

In OSN cilia, cAMP activates a cationic current through CNG channels. First described by Nakamura and Gold (1987),\textsuperscript{13} the cAMP-activated current has been extensively characterized and proven to be required for OSN response to most odorants.\textsuperscript{14} The native olfactory CNG channels are composed of 3 different subunits encoded by CNGA2, CNGA4 and CNGB1b forming a tetramer with the stoichiometry of 2 CNGA2, 1 CNGA4 and 1 CNGB1b.\textsuperscript{31-35} They are expressed in the cilia of all canonical OSNs.\textsuperscript{34,36,37}

The olfactory CNG channels are slightly more permeable to Na\textsuperscript{+} than to K\textsuperscript{+},\textsuperscript{38} and the current-voltage relation shows slight outward rectification in divalent free conditions.\textsuperscript{34,38,39} The relation between the concentration of cAMP and CNG current is well fitted by a Hill function with a Hill coefficient ranging from 1.3 to 2.3, indicating that the binding of at least 2 cAMP molecules is necessary to open the channel. It was shown that binding of the second cAMP molecule brings the channel almost to its maximum open probability,\textsuperscript{40,41} which is just 0.7–0.8.\textsuperscript{3,39,42,43} Once activated, the current does not spontaneously inactivate in the presence of cAMP.\textsuperscript{39,44,45} Ca\textsuperscript{2+} has a complex effect on CNG channel activity. Under physiologic conditions, much of the inward current is carried by Ca\textsuperscript{2+},\textsuperscript{46} but extracellular Ca\textsuperscript{2+} and Mg\textsuperscript{2+} greatly reduce inward CNG current by open-channel block. Moreover, elevated cytoplasmic Ca\textsuperscript{2+}, together with one or more Ca\textsuperscript{2+}-binding factors such as Ca\textsuperscript{2+}-calmodulin, reduces the sensitivity of the channels to cAMP, a mechanism relevant for adaptation.\textsuperscript{47-52}

cAMP activates cationic CNG channels in the ciliary membrane and knockout mice missing the CNG channel subunit CNGA2 lacked electro-olfactogram (EOG) responses (which represent the recorded summed generator potential from the surface of the OE)\textsuperscript{53,54} to most odorants tested.\textsuperscript{55} The absence of EOG responses indicated that CNGA2 KO mice are anosmic. CNGA2 KO mice struggle to survive because they are not even able to feed during nursing and weaning. Since that initial report, residual responses to some odorants have been demonstrated in mice lacking CNGA2.\textsuperscript{56,57}

Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} currents in olfactory cilia

The odorant-induced current is made of 2 components: a cationic one activated by cAMP and an anionic one activated by Ca\textsuperscript{2+}. This could be demonstrated by excising olfactory cilia and exposing them to cAMP (Fig. 2C). Only at negative holding potentials, which favors Ca\textsuperscript{2+} influx, was a bi-phasic current observed with the second component being absent in Cl\textsuperscript{-}-free solution (Fig. 2D). The latter current, a Cl\textsuperscript{-} current, is gated directly by the Ca\textsuperscript{2+} that entered through the CNG channels. Taking advantage of flash photolysis Boccaccio and Menini showed that upon cAMP uncaging in intact OSNs a clearly biphasic current developed at a holding potential of \(-50\) mV (Fig. 2E & F).\textsuperscript{19,58} The first and fastest step reached a plateau that in some cases lasted several ms and was then followed by a larger component that was absent at \(+50\) mV when, again only little Ca\textsuperscript{2+} entered through the CNG channel not sufficient to activate the Cl\textsuperscript{-} current. Moreover, the second component was strongly reduced by lowering the intracellular Cl\textsuperscript{-}.
induced current. While in excised patch experiments, some modulatory components of the channel may be when recorded from excised patches, indicating that the presence of a secondary Ca\(^{2+}\)-activated Cl\(^{-}\) current boosts the cooperativity of the odorant-induced current. While in excised patch experiments the apparent cooperativity of the cAMP-induced current is about 1.5, in intact OSNs, with the presence of Ca\(^{2+}\)-activated Cl\(^{-}\) current, the cooperativity reaches much higher values, between 3.5 and 5.4, clearly indicating that the presence of a secondary Ca\(^{2+}\)-activated Cl\(^{-}\) current increases the nonlinear amplification of the receptor current. Such nonlinear activation of the Cl\(^{-}\) current depends on the Ca\(^{2+}\) cooperativity of the conductance. Excised patch experiments from OSN cilia/knob show that the dose-response for Ca\(^{2+}\) was well fitted by the Hill equation with half-maximal activation between 2.2 and 4.7 \(\mu\)M and Hill coefficient between 2.0 and 2.8, sufficiently steep to support the high odorant-induced cooperativity observed in OSNs.

Moreover, Ca\(^{2+}\)-activated Cl\(^{-}\) currents show a Ca\(^{2+}\)-dependent inactivation, which is reversible after removal of Ca\(^{2+}\), but also an irreversible run-down when recorded from excised patches, indicating that some modulatory components of the channel may be lost after the excision of the membrane. The olfactory Ca\(^{2+}\)-activated Cl\(^{-}\) current is apparently not directly affected by Ca\(^{2+}\)–calmodulin and, at present, no modulators of channel activity are known. Another recently described property of the native channel is that upon flash photolysis of caged Ca\(^{2+}\), the reversal potential for some external large anions changes with time, a phenomenon which requires further mechanistic and physiologic studies.

Before the molecular identification of the Cl\(^{-}\) channel, the only genetic approach available to alter or investigate the excitatory role of the Ca\(^{2+}\)-activated Cl\(^{-}\) currents was by using a knockout mouse for the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter NKCC1. As mentioned earlier, NKCC1 provides for active accumulation of the intracellular ciliary Cl\(^{-}\) that reaches a concentration close to that of the embedding mucus. Thus, by knocking out NKCC1, Cl\(^{-}\) could not accumulate in the cilia, changing the Cl\(^{-}\) reversal potential, and the Ca\(^{2+}\)-activated Cl\(^{-}\) component of the odorant response was largely reduced, leaving only 20% of the total odorant response. The Cl\(^{-}\) channel, though, was still present when excised patch experiments were performed. In summary, although still elusive in its identity, the native CaCC was fully biophysically characterized and hypotheses about its functionality were possible.

**Hunting for the Ca\(^{2+}\)-activated Cl\(^{-}\) channel**

The elusive molecular nature of Ca\(^{2+}\)-activated Cl\(^{-}\) currents was an issue not only for olfaction but for other fields as well. Different Ca\(^{2+}\)-activated Cl\(^{-}\) currents have been characterized biophysically in various tissues and cell types without establishing their molecular identity.

In the OE, the first molecular candidate to be proposed was ClCa1 cloned from the rat olfactory mucosa in 2003, which was then followed by another candidate, Bestrophin2 (Best2). Best2, which belongs to the family of Bestrophin channels, was at the time a promising candidate because of several properties: it was cloned from mouse OE, was expressed in the ciliary layer of the OE and co-localized with the CNGA2 channel. Best2’s biophysical properties were very similar to those of the native channel (anion selectivity, unitary conductance, voltage dependences, sensitivity to blockers). But there was one obvious difference: Best2 is about 12 times more sensitive to Ca\(^{2+}\) than the native olfactory CaCC. Although such differences could simply be due to the absence of additional subunits or modulators in the expression system, it called for caution in concluding that Best2 was the native olfactory channel. Overall, it was reasonable to propose that Best2 might be the olfactory CaCC—or at least a part of it—but later it was shown that mice lacking Best2 still had the Ca\(^{2+}\)-activated Cl\(^{-}\) currents and displayed normal concentration and it was blocked by niflumic acid (Fig. 2E and F, gray traces), a commonly used Cl\(^{-}\) channel blocker. Single cell electrophysiology of OSNs indicated that the Ca\(^{2+}\)-activated Cl\(^{-}\) currents constitute up to 90% of the receptor current in rodents. The relative magnitude of the Cl\(^{-}\) compared with that of the CNG current is probably due to a higher density of CaCC in the olfactory cilia, even though this phenomenon and the estimated density of CaCC could be species-specific. Despite lacking its molecular counterpart at the time, the biophysical characteristics of the current have been extensively studied (see below).
olfactory behavior. In conclusion, these results eliminated a promising candidate, but sent the search for the molecular identity of the olfactory CaCC back to the beginning.

**TMEM16B is the cilial \(\text{Ca}^{2+}\)-activated \(\text{Cl}^-\) channel**

Help came from an unexpected side in 2008, when 3 groups using different approaches found that members of the TMEM16 family, a group of proteins with largely unclear functions, encoded for CaCCs. It was proposed to change the name of TMEM16 to Anoctamin because of the anion permeability and bioinformatics analysis predicted that they have 8 transmembrane domains (octamins). The appropriateness of the name has been actively discussed, in particular, because it is possible that only 2 members of this family are \(\text{Cl}^-\) channels and they might actually have 10, not 8, transmembrane domains.

But was one of the newly discovered TMEM16 family members the native olfactory channel? The answer came soon after when anocamin2/TMEM16B was biophysically characterized in axolotl oocytes and HEK 293 cells. Not only was it a CaCC but also most of its properties resembled those of the native olfactory channel. In addition, TMEM16B was found to be a prominent protein in the olfactory ciliary proteome with a relative abundance of TMEM16B to CNGA2, in agreement with electrophysiological data showing that CaCCs have a greater density than CNG channels. Anecdotally, in 2005, while trying to identify markers for different populations of cell types in the OE, it was shown that TMEM16B transcripts were abundantly localized in mature OSNs; unfortunately, at the time nobody could tell that TMEM16B was a CaCC. When, via adenoviral infections, GFP-tagged TMEM16B was expressed in OSNs, it trafficked to the ciliary membrane, and immunohistochemical experiments showed that it was localized in the ciliary layer of the OE (Fig. 3A).

Several other members of the TMEM16 family (16A, 16F, 16H, 16K) were found in the OE. Only—TMEM16A and TMEM16F—were further studied by immunofluorescence and only the latter was shown to be expressed in the cilia of the OSNs, while TMEM16A was expressed in some supporting cells of the OE. Whether TMEM16F is part of the native channel requires further studies.

Four different splice variants of TMEM16B are expressed in the OE (Fig. 3B). Two variants (isoform B, the most abundant, and isoform B\(\Delta4\)) have the transcription start site in a newly identified exon (exon 1b) therefore encoding for a shorter N-terminal region than TMEM16B in the retina, whereas the other 2, low-abundance variants (isoform A and A\(\Delta4\)) have the canonical start site. Interestingly, in 2 isoforms (A\(\Delta4\) and B\(\Delta4\)) exon 4 is spliced out and they are not able to form functional channels if expressed alone in a heterologous system.

A side-by-side comparison of the functional properties measured in excised inside-out patches from the native olfactory \(\text{Cl}^-\) channel and the TMEM16B-induced current in HEK 293 cells showed remarkable similarities (Fig. 3C - F). Both channels showed \(\text{Ca}^{2+}\)-activated currents (Fig. 3C) with dose–response relations having half-maximal concentration of \(\text{Ca}^{2+}\) of 4.9 \(\mu\)M for TMEM16B very similar to 4.7 \(\mu\)M for native channels at \(-50\) mV (Fig. 3D). Moreover, both channels have the same anion selectivity: they are more permeable to anions larger than \(\text{Cl}^-\) and have little permeability for methanesulfonate (Fig. 3E–F). In addition, both TMEM16B and native CaCCs are similarly blocked by niflumic acid (Fig. 3G–I). Moreover, TMEM16B displays a \(\text{Ca}^{2+}\)-dependent inactivation and an irreversible run-down similar to native olfactory CaCCs. Co-expression of the 2 splice variants B and B\(\Delta4\) gives rise to a channel that reproduces the native voltage-dependent inactivation at positive holding potentials better than isoform B alone. Thus, TMEM16B is an important and required component of the olfactory \(\text{Cl}^-\) channel that has been confirmed by several laboratories independently.

**Finally, the TMEM16B knockout**

Since the identification of TMEM16B expression in the OE, the knockout (KO) mouse model was in the air: it took 3 y to have the first mouse model where the TMEM1A\(\text{B}\) gene was knocked out. In 2011 Billig et al. published the TMEM16B KO mouse model in a paper with an unexpected title: “\(\text{Ca}^{2+}\)-activated \(\text{Cl}^-\) currents are dispensable for olfaction.” It was shown that the \(\text{Ca}^{2+}\)-activated \(\text{Cl}^-\) current was entirely absent in OSNs from TMEM16B KO mice, confirming that TMEM16B is a mandatory part of the \(\text{Cl}^-\) channel. However, KO mice performed indistinguishably from WT mice in an olfactory-guided Go/No Go task,
suggesting that Ca\(^{2+}\)–activated Cl\(^{-}\) currents are not relevant for near normal olfactory behavior. This result was truly puzzling, considering that the Cl\(^{-}\) channel is expressed at high levels at a significant metabolic cost and evolutionarily conserved across many species. Would this channel only have a merely minor accessory role or none at all?

Using the same animal model (kindly provided by Prof. Jentsch), Pietra et al. (2016)\(^{87}\) re-examined the question from a slightly different perspective, starting with a naïve behavioral task, the “finding the buried food test” which, unlike the Go/NoGo task, does not involve any animal training and pre-learning. Indeed, from the perspective of the mouse, the Go/NoGo task is an artificial operant conditioning experiment for which the mouse has to be extensively trained and be exposed to the same odorant repeatedly. Our approach, instead, involved odor-guided food-seeking where mice must use their olfactory ability to locate a food item buried under the bedding chips in the cage. This task did not require any learning sessions, making it a very simple and naïve task that lets the animals rely more on their innate olfactory abilities. TMEM16B KO mice were accomplishing the task significantly slower than wild type mice. Interestingly, when mice were tested once a day for several days, KO mice progressively caught up with WT mice and, after 2–3 d of testing, they performed

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**Figure 3.** TMEM16B is expressed in the OE and a side by side comparison with the olfactory native channel revealed surprisingly similar features. Immunofluorescence experiments revealed that TMEM16B is expressed in the cilia of OSNs (A). Modified from Maurya and Menini 2014 with permission.\(^{82}\) Its gene has 4 splice variants. (B) Diagram summarizes the 5' TMEM16B exon splicing structure. The green sections indicate the most 5' AUG translation start codons, and the subsequent black bars indicate predicted protein-coding sequence. The variants containing exons 1a and 2 are less abundant in the olfactory epithelium than the variants containing the previously unknown exon 1b. From Saidu et al. 2013 originally published in the Journal of General Physiology.\(^{85}\) (C) Currents activated by 100 \(\mu\)M Ca\(^{2+}\) at \(-50\) or \(+50\) mV. (D) Normalized currents measured at \(-50\) mV were plotted versus Ca\(^{2+}\) concentrations and fitted with the Hill equation. (E and F) Current–voltage relations from a ramp protocol activated by 100 \(\mu\)M Ca\(^{2+}\). Bath solutions contained 140 mM NaCl, or the sodium salt of methanesulfonate (MeS, green trace) or of the more permeable ion Iodide (I, cyan trace), as indicated, showing a similar shift in the reversal potential. Intracellular blockage by 300 \(\mu\)M niflumic acid (NFA, orange trace) of the current activated by 100 \(\mu\)M Ca\(^{2+}\) at \(-50\) mV. Modified from Pifferi et al. 2006\(^{b}\),\(^{59}\) copyright (2006) National Academy of Sciences, USA, and from Pifferi et al. 2009\(^{a}\).\(^{73}\)
equally well. This suggests that once KO mice “learned” the identity of the odor, they could perform equally well (Fig. 4A and B). If we challenged the KO with yet another novel odor, TMEM16B KO again required longer times to locate the food compared with WT mice, whereas for odors already known to the mice, e.g., retesting with the same food or using their standard chow, their performance was similar to WT (Fig. 4C). Thus our different behavioral tests gave different results compared with Billig et al., although their tests aimed at detecting different behavioral aspects. Given that we found that KO mice required longer times to identify new odorants, it would be interesting to investigate how, in a Go/No Go paradigm, mice trade off accuracy for odorant-sampling duration (meaning the KO mice might sample for a longer period to achieve the same accuracy), as WT mice do.

Clearly, though, in comparison to CNGA2 KO or AC3 KO mice, which are anosmic, TMEM16B KO are not anosmic. But CaCCs are not simply dispensable for all olfactory tasks, they may be dispensable for the kind of tasks which require extensive learning and training. Therefore, a broader battery of behavioral tests is required to delineate those differences. Also, as afore mentioned TMEM16B is expressed in other cell types and in particular in brain structures that may be linked to the olfactory systems.

Thus, a future approach to better dissect out the exact contribute of TMEM16B to the olfactory system may require conditional KO model where this channel is deleted in a population of mature OSNs.

**TMEM16B involvement in odorant-induced action potential firing**

The transduction current drives a generator potential that quickly reaches the threshold for AP firing to be conveyed to the OB. Since in OSNs, relatively small neurons with a high input resistance, CNG currents alone are sufficient to generate APs, what is the role of the CaCC? If in OSNs CNG currents alone are sufficient to generate APs, what is then the role of the Ca$^{2+}$-activated Cl$^{-}$ currents?
Usually, APs in OSNs are generated at the very onset of an odorant response and by the end of the rising phase of the transduction current, their spike amplitude has collapsed into the noise of the recording. This is most likely due to the progressive and ultimately complete inactivation of voltage-gated Na⁺ channels during strong and sustained depolarization. In addition, the presence of a rapidly inactivating K⁺ conductance that recovers only slowly from inactivation has been suggested to contribute to the generation of short bursts of spike firing.

While the transduction current saturates far above the odorant threshold, the number of APs fired increases monotonically with stimulus concentration only up to half maximal odorant concentrations, then showing a decreasing number of AP (typically 1–2) at higher concentrations.

In other words, the number of APs saturates well before that the transduction current saturates.

In response to IBMX (a phosphodiesterase blocker used as surrogate for odorant stimulation) or to an odorant, OSNs fire a train of few APs (Fig. 5A and C for WT) with a relatively short duration (Fig. 5F, black). However, when TMEM16B is absent the number of APs in the train increased and so did its duration (Fig. 5B and D for KO). The number of APs in the KO increased about 3-fold (Fig. 5E and F). In conclusion, the lack of TMEM16B, and thus of any Cl⁻ component, affects the firing behavior by prolonging the AP train duration and increasing the number of APs in response to a stimulus.

Therefore, the depolarizing action of CaCCs, although somewhat counterintuitive, is that of shortening AP firing by tuning it with the odorant strength, driving in this way the time course of the response that causes quick inactivation of voltage dependent Na⁺ channels. Both the number of APs and AP train duration might be required for the appropriate flow of information that ultimately is sent to the brain to correctly signal for the right odorant concentration and stimulus duration.

**TMEM16B involvement in spontaneous action potential firing**

Beginning with the early electrophysiological characterization of OSNs, spontaneous electrical activity in the form of basal spiking was described. OSNs showed basal membrane voltage fluctuations which are able to reach the threshold for spontaneous APs firing. This “noise” is caused by cAMP fluctuations generated by the spontaneous activity of an OR which in turn drives G protein and AC3 activation to open the CNG channel and then TMEM16B. Interestingly, different ORs have different constitutive activities that drive cAMP fluctuations, therefore spontaneous firing depends on the OR that an OSN expresses.

An interesting case is that of the I7 OR that has been extensively investigated for its pharmacological profile; it has a very high basal activity resulting in a higher spontaneous firing rate when compared with much quieter ORs (i.e. mOR-Eg). When we investigated the spontaneous firing rate of I7 OSNs lacking the TMEM16B channels, their spontaneous firing dramatically decreased (Fig. 6). These results along with the evidence that spontaneous APs firing is completely and reversibly blocked by applying the CaCC blocker niflumic acid demonstrate that even at very low levels of activity, the CaCC is what drives the AP output.

In conclusion, while for odorant responses TMEM16B is involved in controlling the spike train duration that may encode stimulus strength, for the spontaneous activity of an OSN expressing an OR with high basal activity, the CaCC provides the depolarizing current that amplifies the small CNG currents. OSNs expressing other ORs with different basal activity might be affected differently by the lack of TMEM16B, though. Forgotten for a long time, we think that spontaneous activity is an emerging and interesting aspect of the olfactory system and TMEM16B involvement.

**TMEM16B-mediated electrical activity has a role in glomerular targeting in the OB**

Basal activity of ORs and basal noise could be considered as an epiphenomenon of a system that expresses 1000 different G protein-coupled receptors, but recent evidence suggests that spontaneous activity could be more than just noise since, for example, spontaneously generated levels of cAMP could set the threshold above which a stimulus could give rise to a meaningful response. Additionally, basal activity has been implicated in targeting of OSN axons to the OB.

In sensory physiology, the notion that “neurons that fire together wire together” is used to describe that spiking can determine the fate of the projecting axons to
higher areas of the brain. In the olfactory system, this notion has been complicated by data showing that the “biochemical intrinsic spontaneous activity” (as opposed to electrical activity) is likely to be involved in the control of the glomerular formation and targeting.\textsuperscript{103,104}

The process of OR selection during OSN development is crucial and determines the fate of these neurons, not only for their response profile but also for the targeting of their axons to the bulb. Each OSN projects to a neuropil-like structure called glomerulus, which groups together axons from OSNs expressing the same OR. There are typically 2 glomeruli per OR, one on the lateral and one on the medial side of the OB. The OR identity regulates the expression levels of many molecules involved in glomerular positioning and targeting and the development of the olfactory map is largely target-independent. The guidance molecule implicated in anterior-posterior targeting was proposed to be Neuropilin1 (Nrp1) and recent evidence showed that OR spontaneous activity (and not ligand-induced activity), probably via the cAMP-PKA pathway,\textsuperscript{105-107} affects its expression level. Genetically swapping an OR of low basal activity with a high activity OR in a given OSN caused a posterior shift of

\textbf{Figure 5.} TMEM16 role in activity dependent firing. (A-B) IBMX responses recorded by using the suction electrode technique from isolated OSNs.\textsuperscript{98} OSNs from WT mice typically fired 2–3 APs in response to 1 mM IBMX (1 s stimulation) while the KO fired a more prolonged AP train. This finding was confirmed using a different approach: (C-D) loose patch recordings from the dendritic knob of OSNs in the intact epithelium (expressing the 17 OR stimulated with its ligand heptanal). OSNs from KO mice not only fired more APs (E) but also the AP train duration was prolonged (F). Modified from Pietra \textit{et al.} 2016 originally published in the Journal of General Physiology with permission.\textsuperscript{87}
the glomeruli. This model, though, has been questioned and requires further studies, but the idea of having an OR-dependent regulation of the expression of guidance molecules involved in OB targeting and glomerular formation seems to hold because of the mosaic pattern of expression of such molecules in the OB (i.e., Nrp1). Once the axons of OSNs expressing the same ORs converge in a general area, ligand-dependent activity determines the expression of molecules involved in axon sorting into individual glomeruli.

That the spontaneous AP firing could be important for glomerular formation is also supported by results from the TMEM16B KO mouse: additional, supernumerary glomeruli were observed for I7-expressing OSNs. The number of glomeruli in the OB onto which I7-expressing OSNs converge almost doubled in the TMEM16B KO mice. As the expression of I7 ORs confers to OSNs a high rate of spontaneous firing, I7-expressing OSNs are more sensitive to the reduction of the firing rate as is the case in the TMEM16B KO, while OSNs expressing other more silent ORs might not be affected. Indeed, this might be the case in the 2 other ORs, M72 and P2, for which glomerular targeting has also been studied in TMEM16B KO mice, which appeared to target normally to the OB. Thus, TMEM16B could play an important role to enable correct glomerular targeting, and the role of spontaneous firing in targeting needs to be carefully reconsidered. The TMEM16B KO mouse model could be a suitable tool to accomplish the task.

Conclusions and perspectives

The tale of the olfactory CaCC continues. It is like a journey, and the destination is to determine its role in OSNs and in the olfactory system. Since identifying its molecular identity, and hence being able to knock it out, TMEM16B has been shown to control the duration of OSN firing in response to a stimulus, with KO OSNs displaying a prolonged spike train and also axon mis-targeting to the OB. Alteration in AP firing alters the input to glomeruli in the OB where changes in temporal patterns could cause a change in how odor quality and intensity are encoded, hence leading to problems in performing naive behavioral tasks and recognizing novel odors. Changes in basal and evoked activity could also alter glomerular connectivity to second order neurons in the bulb, such as interneurons and mitral/tufted cells. Thus, the Cl− channel in OSNs might have multiple and complex effects on the physiology of OSNs themselves, their connectivity to higher order neurons and how they contribute to olfactory coding of odorants. This might be only the beginning of the journey to understand this complexity.

Disclosure of potential conflicts of interest

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Figure 6. The lack of TMEM16B reduces the spontaneous firing in I7-expressing OSNs. (A) Loose-patch recordings (60-s recordings) of the spontaneous firing activity from WT (top) and KO mice (bottom) OSNs expressing the I7 OR. (B) Interspike interval (ISI) distribution histograms (bin = 5 ms) normalized to the number of recorded neurons in control (black line) and KO mice (blue line) shows that brief ISIs were missing in TMEM16B KO OSNs compared with WT and that their distribution was shifted to longer ISIs. Modified from Pietra et al. 2016 originally published in the Journal of General Physiology with permission.
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