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Giovanni Neri
Nicola Donato
Arnaldo d'Amico
Corrado Di Natale
Editors

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In memory of Giuliano Martinelli.
A colleague, a scientist, a friend

Foreword

This book is the collection of most of the papers presented at the 15th Italian Conference on Sensors and Microsystems, promoted by the Italian Association on Sensors and Microsystems (AISEM). The book includes also three tutorial papers, which address basic concepts in the area of sensors and microsystems. This XV Conference edition, organized by the University of Messina, was held in the Faculty of Engineering, University of Messina, from 8 to 10th February 2010, in conjunction with the SIOF (Italian Society of Optics and Photonics) and GS-CSI (Sensor Group of the Italian Chemical Society).

Aim of AISEM 2010 Conference was to give an update overview of the different aspects (Materials, Processes, Devices, Systems and Applications) in the field of sensors and microsystems. At the Conference, organized in 5 topical sessions, attended about 100 participants, with 3 plenary lectures, 48 oral communications and 52 communications presented in the poster session. Plenary lectures were given by important researchers from University and industrial world. Luigi Campanella (University "La Sapienza", Rome) illustrated the multidisciplinary approach to sensors. Salvatore Coffa (STMicroelectronics, Catania) explained the strategy, both at technical and management level, in the sensors market. Anna Menini (SISSA, Trieste) presented the basic concepts of molecular sensing mechanisms in biological olfactory systems. Nicola Pinna (University of Aveiro) described the recent development in the synthesis of nanostructured metal oxides for sensing applications.

At the end of the Conference, three awards, sponsored by the Conference Organization, were assigned for the best posters. The Best Poster Award, was assigned ex-aequo to the papers "Nonlinear MEMS mechanism for energy harvesting from mechanical vibrations" by B. Andò, S. Baglio, C. Trigona, and "Ultrasensitive detection of non amplified genomic DNA" by L. M. Zanoli, R. D'Agata, G. Spoto, R. Corradini, R. Marchelli, C. Ferretti, M. Gatti and "A research study and development of a hydrogen sensor for fuel cells" by A. Bonavita, G. Micali, G. Neri, N. Donato, M. Latino, S. Licoccia.

This book represents then an exhaustive summary of the excellent scientific work presented at the Conference, with a deep discussion of the many subjects

under study. We hope that it may contribute to a further development of the field of sensors and microsystems in Italy and abroad. The appreciation by the readers will be the best awards for the efforts and time expended.

Special thanks are given to Dr. Mariangela Latino for his effort and dedication in the organization of the Conference and to the guys of LESST Lab. (Dr. A. Bramanti, F. Cincotta, S. Trocino, E. Cardillo, Dr. D. Aloisio, E. Fulco) for their support during the three days of the Conference. The Committee also thanks the sponsors BioAge, Libreria Bonanzinga and FINE Permeation Tubes for their support, and the artists Dr. G. Donato and Dr. V. Saija for their creations and the graphical arrangement of the Conference documents.

P.S. One of us, Prof. Giuliano Martinelli, died this year 24 May!

He was a very good friend, a kind person, an excellent researcher and an outstanding teacher. We will never forget the very sound contribution he gave to the AISEM scientific growth. In our mind we will keep alive also his unique polite and genteel behaviour and in our heart an immense pain.

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Part I
Tutorials

Chapter 1
Odorant Detection and Discrimination in the Olfactory System

Simone Pifferi and Anna Menini

Abstract The olfactory system excels in both discrimination and detection of odorants. In mammals, it reliably discriminates more than 3000 structurally diverse odorant molecules and has an amazingly high sensitivity that allows the detection of very low amounts of specific odorant molecules. In addition, the olfactory system has the capability to adapt to ambient odorants, allowing the recognition of a broad range of stimuli. The discrimination among different odorants is achieved by using hundreds of receptors, activated with a combinatorial code. Olfactory transduction uses a canonical second messenger system providing two critical attributes: amplification and high signal-to-noise characteristics, giving the system its remarkable detector capabilities. In this review, we present an introduction to the basic molecular mechanisms of olfactory transduction in olfactory sensory neurons.

1.1 Odorants

The process of chemosensation allows a living organism to detect and discriminate different chemical molecules in the external environment. This task is essential for survival of the individual and of the species, indeed it enables animals to locate nutritious food and suitable mating partners, as well as to smell the presence of predators and to avoid eating toxic substances [56].

The olfactory system is specialized in the detection of odorants, and many mammalian species recognize and discriminate among thousands of odorants with high specificity and sensitivity. For example, the threshold for human detection of

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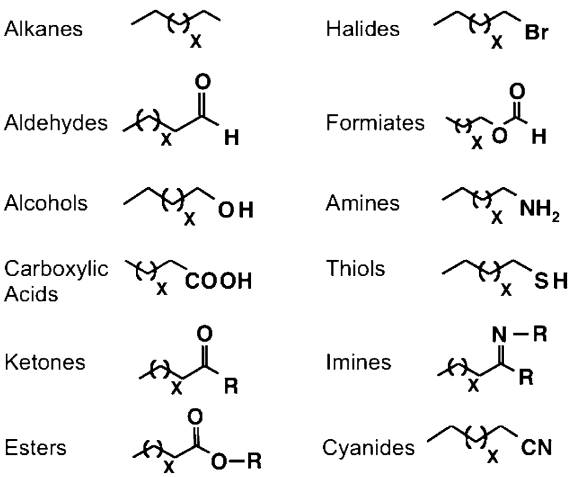

Fig. 1.1 Chemical structures of various functional groups in some odorant molecules

ethyl mercaptan (ethanethiol), commonly added to natural gas as an odorant, is as low as one part in 2.5 billion parts of air [58].

Odorants are mainly organic volatile compounds that bind to odorant receptors (see Sect. 1.6). A common odorant is usually made by a mixture of different types of volatile molecules and the relative concentrations of each component participate to determine the particular perception response. Odorant components include aliphatic and aromatic molecules with varied carbon backbones and diverse functional groups, such as alkanes, aldehydes, alcohols, carboxylic acids, ketones, esters, halides, formiates, amines, thiols, imines, cyanides, and others, as illustrated in Fig. 1.1 (for review see [38]).

1.2 The Olfactory Epithelium

Volatile molecules enter the nose during inspiration and contact the olfactory epithelium, located in the interior of the nasal cavity. The olfactory epithelium is made by three main types of cells: olfactory sensory neurons, which are devoted to the function of transducing chemical information into electrical signals, supporting cells and several types of basal cells (Fig. 1.2a). Olfactory sensory neurons are continuously regenerated by basal cells throughout the life span. Some olfactory glands, named glands of Bowman, produce most of the mucus that normally covers the epithelium surface. It is of interest to note that some odorant-binding proteins (OBPs) are found at high concentrations in the nasal mucus. Although their affinity for odorants suggests a role in olfactory perception, their physiological role in vertebrates is still unclear.

Olfactory sensory neurons have a bipolar morphology with a flask-like shape. Their apical part, located at the surface of the epithelium, is slightly swelled into

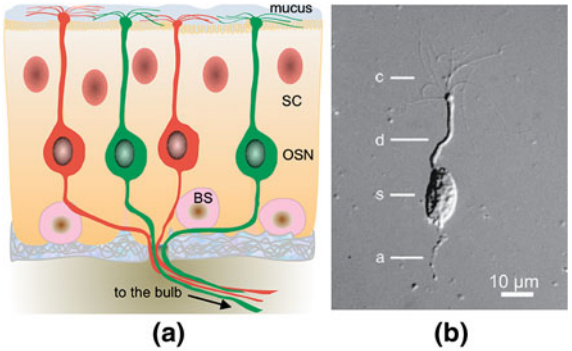

Fig. 1.2 The olfactory epithelium and an isolated olfactory sensory neuron. **a** Schematic diagram showing the various cell types composing the olfactory epithelium (*OSN* olfactory sensory neuron, *SC* supporting cell, *BC* basal cell). **b** Photograph of an isolated frog olfactory sensory neuron under differential interference optic, c cilia; d dendrite; s soma; a axon. Reprinted from Kleene and Gesteland [22], copyright 1981, with permission from Elsevier

an olfactory knob from which several very fine cilia depart (Fig. 1.2b). The cilia are embedded in the mucus covering the epithelium and expose their membrane to odorant molecules arriving from the external environment. Olfactory sensory neurons are primary sensory cells and their axons join in bundles to form the first cranial nerve that reach the olfactory bulbs in the brain.

The diameter of the cell body is about 5–8 μm, while that of the dendrite is about 1–2 μm. In mammals, the cilia are 15–50 μm long, while in some lower vertebrates they can be as long as 200 μm [35, 52]. The diameter of a mammalian cilium tapers from 0.28 μm near the base of the cilium to 0.19 μm in the distal portion [35]. The presence of numerous cilia greatly increases the surface membrane area that can interact with odorant molecules. Some estimations indicated that cilia may increase the cell bare surface some one thousand or more times [15].

The cilia play a fundamental role in olfaction, since they are the site of the sensory transduction apparatus (see Sect. 1.7). Indeed, olfactory sensory neurons deprived of cilia are no longer able to respond to odorants. Furthermore, the damage of the olfactory epithelium can produce a complete loss of the sense of smell, called anosmia.

1.3 Electrical Responses of Olfactory Sensory Neurons to Odorants

Even if olfactory sensory neurons are physiologically devoted to detect odorants, measuring an odorant response in one of these neurons is very challenging. The response to a brief pulse of odorant has been measured with several

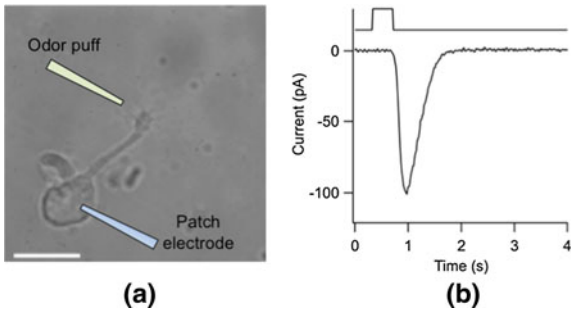

Fig. 1.3 Electrical response to odorants of an isolated olfactory sensory neuron. **a** Experimental method. An isolated olfactory sensory neuron was stimulated with odorants while the electrical response was measured with the patch-clamp technique in the voltage-clamp whole-cell configuration. **b** Current response evoked by the odorant amylacetate at the holding potential of −50 mV. The *top trace* indicates timing and duration of the odorant stimulus. Adapted by permission from Macmillan Publishers Ltd: (Nature) Kurahashi and Menini [25], copyright, 1997

electrophysiological techniques, including the patch-clamp technique (Fig. 1.3a). The percentages of responses to odorants range from 2 to 30%, depending on the choice of odorant. As shown in Fig. 1.3b, when the odorant stimulus causes the excitation of the neuron, a transient inward current is generated that will depolarize the neuron in situ. The response typically lasts 1 s or more. The latency between the arrival of the stimulus and the onset of the current ranges from 150 to 600 ms and, for a strong stimulus, the amplitude of the peak current can reach several hundred pA [21, 36, 52].

The basic electrical properties of olfactory sensory neurons, as well as the ion gradient across the ciliary membrane, play a fundamental role in shaping the properties of the odorant-induced response. In 1989, Lynch and Barry [33] reported that, in rat olfactory sensory neurons, the opening of a single ion channel was sufficient to induce the generation of action potentials. This is due to the very high input resistance, between 3 and 6 GΩ, typical of olfactory sensory neurons, producing a large depolarization also for very small odorant-induced currents [48]. Resting membrane potentials ranges between −90 and −45 mV, with a mean value of −55 mV [21, 28, 48, 52].

The electrical response to odorants is due to ion fluxes across the cell membrane, and therefore ion homeostasis is very important in signal transduction. Since the olfactory cilia are embedded in mucus covering the olfactory epithelium the relevant physiological ion concentrations are those in the mucus and inside the cilia. Data available about the intra- and extra-ciliary concentrations of major physiological ions are summarized in Table 1.1.

Table 1.1 Intracellular and extracellular ion concentrations at the apical side of olfactory sensory neurons

Ion	$[Ion]_{in}$ (mM)	$[Ion]_{out}$ (mM)	E_{Nernst} (mV)
Na^+	53 ± 31	55 ± 12	+1
K^+	172 ± 23	69 ± 10	−24
Free Ca^{2+}	40 ± 9 nM	4.8	+156
Cl^-	54 ± 4	55 ± 11	0

$[Ion]_{in}$ and $[Ion]_{out}$ are respectively the intracellular and extracellular (in the mucus) ion concentrations. E_{Nernst} is the calculated Nernst potential from the reported ion concentrations. Most of the ion concentrations were measured by energy-dispersive X-ray microanalysis in dendritic knobs of rat olfactory sensory neurons [51]. Intracellular Ca^{2+} was from [30]; extracellular Ca^{2+} was evaluated as the midpoint of the range (2.6–7.1 mM) measured by [11]; intracellular Cl^- from [16]

1.4 Dose–Response of Odorant-Induced Currents

Electrophysiological recordings from individual olfactory sensory neurons have shown that they respond to odorants in different ways: some neuron can detect several odorants, and a given odorant can activate neurons with various odorant specificity (Fig. 1.4a).

The relation between odorant dose and peak current is generally well fit by the Hill equation:

$$I = I_{max} \frac{C^n}{C^n + K_{1/2}^n}$$

where I_{max} is the maximal current, C is the concentration of odorant, $K_{1/2}$ is the odorant concentration producing 50% of the maximal current, and n is the Hill coefficient. In the neuron illustrated in Fig. 1.4b, c, $K_{1/2}$ was 53 μM. $K_{1/2}$ values for different odorants ranged between 3 and 100 μM. The Hill coefficient, n, describes the slope of the rising phase of the dose–response relation and its values ranges between 2.7 and 9.7. With such a non-linear amplification, only a slight change in the concentration of odorant molecules produces a large change in the response. As n decreases, the slope also decreases, causing an increase in the range of stimulus strengths over which the neuronal response varies (dynamic range) (for reviews see [21, 36, 52]).

1.5 Adaptation

It is common experience that olfactory sensation gradually decreases during continuous or repeated exposures to odorant stimuli. This phenomenon involves many processes along the entire olfactory pathway, but it begins in the cilia of olfactory sensory neurons. Indeed, during application of a prolonged odorant

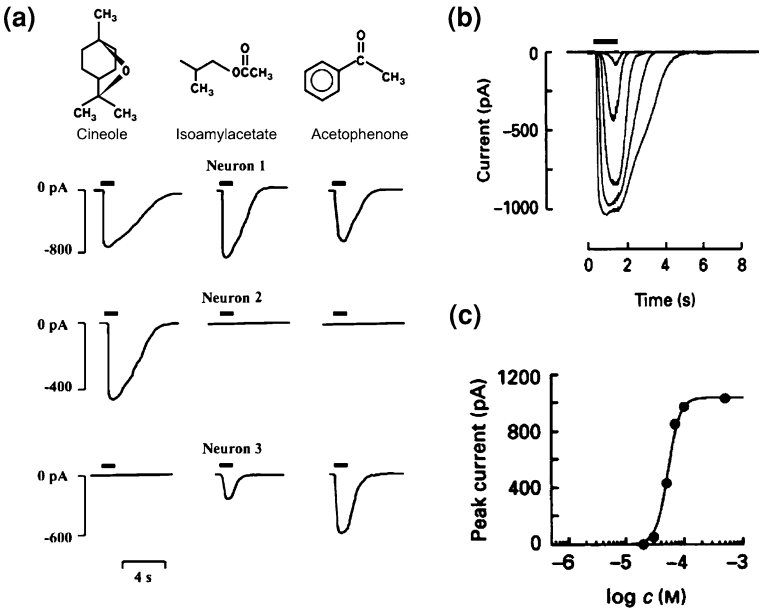

Fig. 1.4 Odorant responses of olfactory sensory neurons. **a** Responses of three different neurons to the odorants indicated at the top were measured with the voltage-clamp whole-cell configuration at −55 mV holding potential. Different neurons respond to a specific subset of odorants. Neuron 1 responded to all three odorants, while neuron 2 responded to only one odorant, and neuron 3 to two of the tested odorants. **b** Response of an olfactory sensory neuron to various concentrations of isoamylacetate. **c** Plot of the peak odorant-induced currents from (**b**) versus the odorant concentration. The *solid line* is the best fit of the Hill equation to the data with $K_{1/2} = 53$ μM and $n = 4.2$. Modified from Firestein et al. [13]

stimulus, the current amplitude decreases with time, despite the continued presence of the stimulus. Figure 1.5a shows that, when two brief odorant pulses are delivered within a short interval, the amplitude of the response to the second pulse is reduced. While the reduction is greater with shorter interstimulus intervals, the current amplitude gradually recovers to the initial value increasing the interval between odorant pulses (Fig. 1.5b).

It is important to note that sensory adaptation is not merely a reduction in response amplitude, but its physiological role involves the adjustment of the response to allow a cell to work over a broad range of stimuli (for review see [57]). Indeed, in odorant adaptation to repetitive stimuli there is a shift of the dynamic range (i.e. the range of stimulus concentrations over which the olfactory sensory neuron is able to respond) toward higher odorant concentrations compared with the control state.

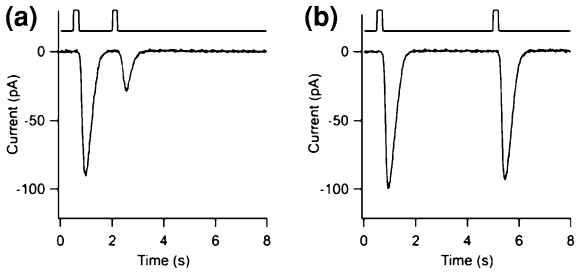

Fig. 1.5 Adaptation to repeated odorant stimuli. In each recording, the *top trace* indicates timing and duration of the odorant stimuli. Two identical pulses of the odorant amylacetate were delivered to an olfactory sensory neuron with different interstimulus intervals. The holding potential was −50 mV. **a** When the interstimulus interval was 1.5 s the response to the second pulse was reduced. **b** With a 4.5 s interval, the response to the second pulse was similar to the initial value. Adapted by permission from Macmillan Publishers Ltd: (Nature) Kurahashi and Menini [25], copyright, 1997

1.6 Odorant Receptors

The identification of the genes encoding for odorant receptors opened a new molecular era in olfactory research. The discovery of odorant receptor genes was first published in 1991 by Buck and Axel [7], who obtained the Nobel Prize in Physiology or Medicine in 2004 "for their discoveries of odorant receptors and the organization of the olfactory system". Odorant receptors belong to the superfamily of G-protein coupled receptors. The mouse repertoire contains about 1,000 potentially functional odorant receptor genes and is by far the largest gene superfamily in a mammalian genome [10, 38, 60]. In humans, about 350 odorant receptor sequences are potentially functional [37].

At the molecular level, odorant receptors share the same general structure of the other G-protein coupled receptors, with seven α-helical membrane-spanning domains connected by intracellular and extracellular loops of variable lengths, and numerous conserved short sequences. The most critical residues involved in odorant binding are hydrophopic and are located in the third, fifth and sixth transmembrane regions, which may form the ligand-binding pocket for odorant molecules [17]. The spatial localization of the binding pocket is similar to that for other members of the family, although the environment is quite different. For example catecholamines have been shown to form multiple electrostatic interactions through ionic bonds with adrenergic receptors. In contrast, in odorant receptors, the interaction of odorants with the binding pocket is based on hydrophobic and van der Waals interactions and therefore is rather weak, producing a low-affinity ligand-binding. Importantly, odorant receptors are still capable of selecting for shape, size and length of the ligand [17].

To understand how olfactory sensory neurons discriminate among odorants it is important to know how many types of odorant receptor genes are expressed in

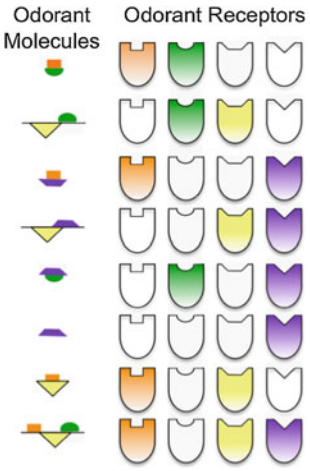

Fig. 1.6 Combinatorial code used by the olfactory system to discriminate between odorant molecules. *Figures on the right* represent odorant receptors activated by the odorants molecules on the *left*. Each type of odorant molecule activates a unique combination of receptors. Viceversa, each activated combination of receptors corresponds to one type of odorant molecule

each olfactory sensory neuron. It has been shown that every olfactory sensory neuron expresses a single odorant receptor gene. Moreover, the choice seems to be a stochastic process, which is likely to remain stable during the entire life of each olfactory sensory neuron, although not all odorant receptors are chosen with the same frequency [37, 38].

Another important information is the knowledge of how many and which odorants bind to each odorant receptor. It has been well established that each odorant receptor can be activated by several types of odorant molecules (Fig. 1.6). On the other hand, one single type of odorant can activate several types of odorant receptors. Thus, the odorant receptor family is used in a combinatorial manner to discriminate odorants and each odorant is recognized by a unique combination of receptors (Fig. 1.6) [34]. This scheme is consistent with previous observations that single olfactory sensory neurons can be stimulated by multiple odorants (Fig. 1.4a). Since each of these neurons expresses only one unknown odorant receptor type, a given neuron responds to a small and unpredictable subset among the many available odorants [13].

The combinatorial receptor coding scheme has the great advantage of allowing the olfactory system to recognize a large number of odorants and also to discriminate between odorants that have very similar but different structures, such as aliphatic odorants with different carbon chain lengths. Unfortunately, the identification of ligands for odorant receptors is still very limited, due to the difficulty to express odorant receptors in heterologous systems suitable for high-throughput screening [37, 38].

1.7 Olfactory Transduction

How is the binding of odorant molecules to odorant receptors converted into an electrical signal? When an odorant molecule binds to an odorant receptor, it

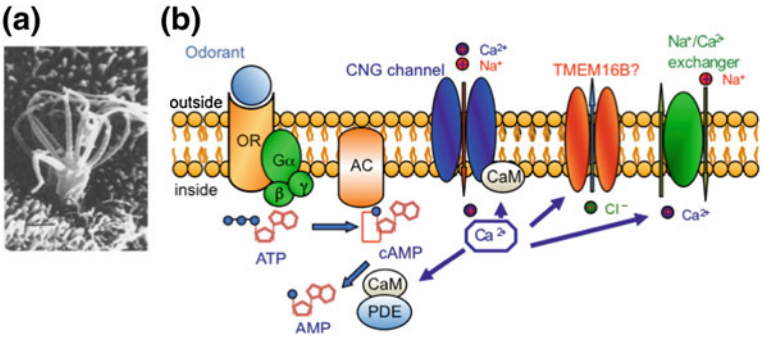

Fig. 1.7 Olfactory transduction in the cilia of olfactory sensory neurons. **a** A scanning electron micrograph of the knob of a human olfactory sensory neuron showing the protrusion of several cilia. Scale bar, 1 μm. Adapted from Morrison and Costanzo [39], with permission. **b** Schematic representation of the olfactory transduction taking place in the cilia. *OR* odorant receptor; *G*, G-protein, *AC* adenylyl cyclase, *CNG channel* cyclic nucleotide-gated channel, *CaM* calmodulin, *PDE* phosphodiesterase. *TMEM16B* indicates the candidate Ca^{2+}-activated Cl^- channel. Modified from Pifferi et al. [43], with permission

initiates a cascade of molecular events that transforms the chemical energy of binding into an electrical signal, as illustrated in Fig. 1.7.

The binding of an odorant molecule to an odorant receptor in the cilia induces a conformational change of the receptor causing the activation of an interacting G-protein. In turn, the G-protein stimulates the enzymatic activity of an adenylyl cyclase (ACIII) generating an increase in the concentration of cyclic AMP (cAMP). Cyclic nucleotide-gated (CNG) channels located in the ciliary membrane are directly activated by cAMP, causing a depolarizing influx of Na^+ and Ca^{2+} ions. The intracellular increase of Ca^{2+} concentration directly gates Ca^{2+}-activated Cl^- channels. As shown in Table 1.1, olfactory sensory neurons maintain an unusually high internal concentration of Cl^-, which is in the same range of the Cl^- concentration present in the mucus at the external side of the ciliary membrane. Therefore, in physiological conditions, the opening of Ca^{2+}-activated Cl^- channels causes an efflux of Cl^- ions from the cilia, corresponding to an inward current that further contributes to the depolarization of olfactory sensory neurons [16, 41, 42, 50–52]. The depolarization spreads passively to the dendrite and soma of the neuron, triggering action potentials that are conducted along the axon to the olfactory bulb.

Several mechanisms contribute to terminate the odorant response and to restore the initial conditions in olfactory sensory neurons. The cilia contain a phosphodiesterase that, after being activated by the complex Ca^{2+}-Calmodulin (CaM), hydrolyzes cAMP [4]; the G-protein is inactivated by its intrinsic GTPase function; the intracellular Ca^{2+} concentration is reduced by Ca^{2+}-extrusion through a Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase and, finally, the complex Ca^{2+}-CaM decreases the sensitivity of the CNG channel to cAMP, as further discussed in the following section (for reviews see [21, 36, 47, 52]).

1.8 Cyclic Nucleotide-Gated Channels

Cyclic nucleotide-gated (CNG) channels are the mediators of the chemo-electrical energy conversion in olfactory cilia. Indeed, information about odorant molecules is first transmitted as chemical information and then is converted into an electrical signal by ion fluxes through CNG channels activated by the increase in cAMP concentration [24, 31, 40].

The ciliary CNG channels are composed of three types of subunits with a stoichiometry of two CNGA2, one CNGA4, and one CNGB1b (Fig. 1.8a). Transgenic mice lacking CNGA2 are completely anosmic, demonstrating the importance of this channel in olfactory perception [3, 6, 60].

The topology of each CNG subunit consists of six transmembrane spanning domains, a pore region between the fifth and the sixth transmembrane domain, and intracellular N- and C-terminal regions. A cyclic nucleotide-binding site is located near the C-terminal at the cytoplasmic side in each subunit, for a total of four binding sites per each channel. Moreover, Ca^{2+}-calmodulin binding sites are also present at the cytoplasmic side (Fig. 1.8a) (for reviews see [18, 43]).

The relation between concentration of cAMP and CNG current is well fit with a Hill equation. Half-maximal activation ($K_{1/2}$) is in the micromolar range and varies in different species between 2 and 19 μM. The Hill coefficient ranges from 1.3 to 2.3 suggesting that at least two molecules of cAMP must bind before the channel gating (Fig. 1.8b, c).

The unitary conductance of single CNG channels in the presence of Ca^{2+} and Mg^{2+} is very small, with a value of about 1 pS. A small single channel conductance plays a relevant physiological role since, by using a large number of tiny events, the integrated current has a high signal-to-noise ratio.

It is important to note that, once open, CNG channels allow the flow along their electrochemical gradient, not only of monovalent ions, such as Na^+ and K^+, but also of Ca^{2+} [12]. The increase of Ca^{2+} increase in the intraciliary medium plays several important roles in olfactory transduction, mainly in the amplification of the odorant response (see Sect. 1.9) and in the adaptation process [26].

Kurahashi and Menini [25] investigated the localization of the principal molecular mechanism for adaptation in the olfactory transduction process. To determine whether the response reduction in the adapted state (Fig. 1.5a) was attributable to a reduction in the cAMP production or was instead due to other processes occurring after the production of cAMP, CNG channels in intact neurons were directly activated by flash photolysis of caged cAMP. The ciliary cytoplasm was loaded with caged cAMP through diffusion from a patch pipette and application of ultraviolet flashes to the cilia caused the photorelease of various cAMP concentrations. Therefore, cAMP-gated channels could be directly activated, bypassing the early stages of odorant transduction (i.e. receptor activation and G-protein and adenylate cyclase signalling). cAMP and odorant-induced responses were found to have similar adaptation properties, indicating that the entire adaptation process takes place after the production of cAMP. Furthermore, by using a

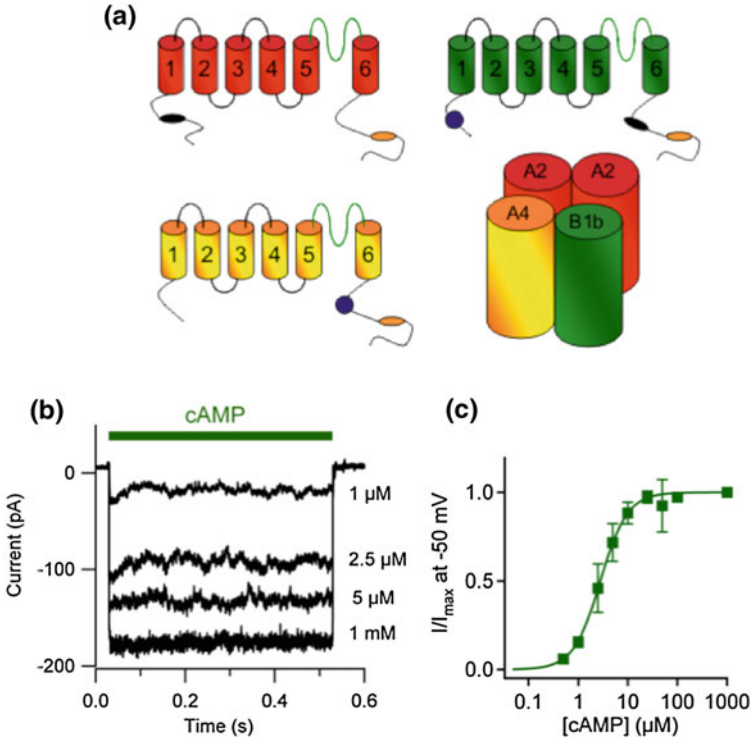

Fig. 1.8 Cyclic nucleotide-gated (CNG) channels. **a** Topological model and assembly of subunits of the olfactory CNG channel. Each transmembrane domain is indicated by a number, the pore loop is located between domains 5 and 6. The cyclic nucleotide-binding site is located in the C-terminal domain. Ca^{2+}-dependent calmodulin binding sites are shown in black. **b** A membrane patch was excised in the inside-out configuration from the knob or ciliary region of an olfactory sensory neuron. CNG channels were activated by the indicated concentrations of cAMP. The holding potential was −50 mV. **c** Normalized currents from experiments as in (**b**). The continuous lines is the best fit of the Hill equation to the data with $K_{1/2} = 2.7$ μM, $n = 1.5$. Modified from Pifferi et al. [43], with permission

hydrolysis resistant caged cAMP analogue, caged 8Br-cAMP, Boccaccio et al. [2] have shown that the hydrolysis of cAMP by PDE is not involved in adaptation. It has also been shown that Ca^{2+}-activated Cl^- channel are unrelated to olfactory adaptation.

All together, the previous experiments indicate that Ca^{2+} is likely to act through a negative feedback on the CNG channel. Indeed, micromolar concentrations of intracellular Ca^{2+} decrease the channel sensitivity to cAMP, probably by activating a Ca^{2+}-responsive endogenous factor already pre-associated with the channel [5, 9]. It has been proposed that the endogenous factor co-assembled with the CNG channel is Ca^{2+}-free calmodulin, called apocalmodulin, although a conclusive demonstration is still lacking [43].