Odorant Responses of Dual Polarity Are Mediated by cAMP in Mouse Olfactory Sensory Neurons

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Submitted 10 February 2004; accepted in final form 5 May 2004

Delay, Rona and Diego Restrepo. Odorant responses of dual polarity are mediated by cAMP in mouse olfactory sensory neurons. J Neurophysiol 92: 1312–1319, 2004; 10.1152/jn.00140.2004. Some olfactory sensory neurons (OSNs) respond to odors with hyperpolarization. Although transduction for excitatory responses is mediated by opening of a cyclic nucleotide-gated (CNG) channel, there is controversy on the mechanism underlying inhibitory responses. We find that mouse OSNs respond to odorants by either depolarizing or hyperpolarizing responses in loose-patch measurements. In the perforated-patch configuration, OSNs not only responded with a current consistent with CNG channel-mediated excitation but also displayed enhancement of outward currents, consistent with inhibitory responses. Increasing cAMP levels pharmacologically elicited excitatory or inhibitory responses in different OSNs. In addition, OSNs from mice defective for the CNGA2 subunit of the CNG channel displayed neither excitatory nor inhibitory responses. Thus CNG channels mediate inhibitory olfactory responses.

INTRODUCTION

The olfactory system is capable of detecting and discriminating a vast array of volatile molecules (Finger et al. 2000; Firestein 2001; Laurent 2002; Mori et al. 1999). The initial detection of odorants occurs at the apical endings of the olfactory sensory neurons in the cilia projecting to the lumen of the nasal cavity where odorants bind to olfactory receptor proteins. Once bound, odor receptors activate the G protein Gαqi (Jones and Reed 1989), which activates adenylyl cyclase type III (ACIII) (Bakalyar and Reed 1990). The subsequent increase in the concentration of adenosine 3′,5′-cyclic monophosphate (cAMP) elicits opening of a CNG channel made up of three subunits (CNGA2, CNGA4, and CNGB1) (Bonigk et al. 1999; Dhallan et al. 1990; Liman and Buck 1994). Opening of this channel allows Ca²⁺ to flow into the cilia where it binds to Ca²⁺-activated Cl⁻ channels causing cell depolarization (Kleene and Gesteland 1991; Kurashi and Yau 1993; Lowe and Gold 1993b). Genetic deletion of Gαqi, ACIII, or subunit CNGA2 of the CNG channel (which is required for channel function) abolishes odor-evoked field potential changes of the olfactory epithelium (Belluscio et al. 1998; Brunet et al. 1996; Wong et al. 2000). Thus it is clear that cAMP plays a mediatory role in odorant-excitatory OSNs.

While odor-induced excitation of olfactory sensory neurons (OSNs) is key to olfactory function, odor-induced suppression of voltage-gated conductances and odor-induced inhibition due to hyperpolarizing receptor potentials may also play a role in transmission of odor information to the olfactory bulb (Ache and Zhaninazarov 1995; Dionne 1992; Dionne and Dubin 1994; Kawai et al. 1997; Lischka et al. 1999; Pun and Kleene 2002; Sanhueza et al. 2000). In mammals little is known about inhibitory olfactory responses, but it is clear that some odors inhibit spontaneous action potential firing of OSNs in rats and mice (Duchamp-Viret et al. 1999; Maue and Dionne 1987; Sanhueza and Bacigalupo 1999; Sanhueza et al. 2000). If odors elicit a decrease in action potential firing in some OSNs, while increasing firing in other olfactory neurons, this would help “sharp” odor detection and discrimination, allowing a small response to stand out from the noise (Ache and Zhaninazarov 1995).

Although some odorants inhibit the basal rate of action potential firing in OSNs, in amphibians and mammals the mechanism is not well understood. In invertebrates, it is thought that the second-messenger, cAMP, mediates inhibitory odor responses by opening a CAMP-gated K⁺ channel (Ache and Zhaninazarov 1995). In contrast, in vertebrates the transduction mechanism mediating inhibitory odor responses is unclear (Morales et al. 1994, 1995; Pun and Kleene 2002; Sanhueza et al. 2000). In this study, we tested the involvement of CAMP and the CNG channel in inhibitory odor responses and odor-induced suppression of voltage-gated conductances with patch-clamp recording of odor responses in OSNs from wild-type and CNGA2-defective mice.

METHODS

Chemicals

3-Isobutyl-1-methylxanthine (IBMX), which inhibits the breakdown of cAMP by phosphodiesterase, and forskolin-7-deacetyl-7-[O-(N-methylpiperazino)-γ-butyryl]-dihydrochloride an agonist of adenylyl cyclase (fsk; Calbiochem No. 344273) were obtained from Calbiochem (La Jolla, CA). The CNG channel inhibitor, 1-cis-dililazem, was obtained from Sigma (St. Louis, MO). Odorants for mix A (100 μM of hedione, geraniol, citralva, citronellal, menthone, eugenol, and phenylethylalcohol) and mix B (100 μM each lilial, linalyl, ethylvanillin, triethylamine, isovaleric acid, and phenyl ethyl amine) were obtained from Firmenich (Switzerland) or Takasago (Japan). Odor mixtures were made in Ringer and required extensive mixing and/or sonicating to get in solution. Once in solution, the odor mixtures were stored in aliquots at −80°C until use.

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Solutions

Ringer solution contained (in mM) 145 NaCl, 5 KCl, 20 N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid; 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES-free acid), 1 MgCl₂, 1 CaCl₂, 1 Na-pyruvate, and 5 glucose pH adjusted to 7.2 with NaOH. Intracellular solution consisted of (in mM) 30 KCl, 110 Kgluconate, 10 NaCl, 20 HEPES-free acid, 1 EGTA, 0.023 CaCl₂, and 1 MgCl₂, pH adjusted to 7.2 with KOH. Gramicidin (2 mg/33 μl DMSO stock; 0.24 mg/ml intracellular solution) was made fresh for each experiment.

Animals

Adult C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Adult male CNGA2 knockout mice and their wild-type littermates were produced by backcrossing the heterozygous CNGA2 knockout female mice (Brunet et al. 1996) (provided kindly by Dr. John Ngai, University of California Berkeley) with wild-type C57BL/6 male mice. Their genotypes were determined by PCR or Southern blot analyses for the absence of the CNG channel subunit CNGA2 and presence of neomycin resistance genes, a reporter gene that replaced part of the CNG channel coding region to create the knockout [for details on the mice, see Brunet et al. (1996)].

The original publication by Brunet and co-workers reported a failure to thrive for male CNGA2 knockout hemizygous mice, presumably due to the lack of chemoosensory cues important for lactation (Brunet et al. 1996). Although initially it was difficult for us to raise CNGA2 knockout mice to adulthood, we found that judicious thinning of the litter, fostering of the pups, or use of wet solid food at weaning produced adult knockout mice (see also Baker et al. 1999). After weaning, the mice gained weight quickly and, by 2 mo of age, CNGA2 knockout mice could not be distinguished from their littersmates on the basis of weight. Mice used in this study were >2 mo. All experiments were performed with wild-type and hemizygous CNGA2 knockout male offspring of F1 crossings of CNGA2 heterozygous females and C57BL/6 males. All procedures were performed under approved protocols and followed guidelines from the Animal Care and Use Committee of the University of Colorado Health Sciences Center.

OSN isolation

Mice, from 3 wk to 1 yr of age, were killed by CO₂ inhalation followed by cervical dislocation. Cell isolation was as in Rawson et al. (1997). Briefly, olfactory epithelium was removed from the animal, cut into small pieces and placed in Ca-Mg-free mouse Ringer with 10–30 U/ml of papain and 2 mM cysteine for 15 min at room temperature. The cells were detached by gentle pipetting and filtered through nylon mesh to remove connective tissue. The cells were kept in saline until use, normally within 4 h of preparation. Only isolated OSN with cilia were used in these experiments.

Electrophysiology

All recordings were done with either an Axopatch 1D or an Axopatch 200 B amplifier (Axon Instruments, Union City CA) controlled by a PC computer using the software programs, Clampex 7 or 8 (Axon Instruments). All test solutions were applied using a Warner fast perfusion system (SF-77, Warner Instruments, Hamden, CT) or a puffer pipette controlled by a Picospritzer II (General Valve, Fairfield, NJ) in the presence of a bath perfusion flow.

Loose patch

Because whole cell measurements can result in the loss of or decreased response of olfactory neurons to certain odors, the loose-patch recording technique was used to undertake a survey of responses to applied odors or pharmacological agents. An electrode with a relatively large tip (1–4 μm, 2–3 MΩ resistance) filled with normal bath solution was pressed against the membrane of the cell and suction was applied until a seal of 20–80 MΩ was formed (Lowe and Gold 1993a; Reisert and Matthews 2001). Holding potential was 0 mV. Currents were low-pass filtered at 20 Hz.

Perforated patch

We used the perforated-patch technique to perform whole cell patch-clamp experiments while minimally disturbing the cytosolic contents of the cell (Lowe and Gold 1993b; Zhainazarov and Ache 1995). Gramicidin was chosen as the pore-forming agent because Na⁺ and K⁺ ions flow easily through the pore, whereas neutral metabolites, anions, and larger cationic molecules either do not permeate or permeate slowly. Pipettes with a resistance of 6–10 MΩ were filled at the tip with intracellular solution without gramicidin to form a tight seal (>1GΩ) with the membrane of the cell. Diffusion of gramicidin from the back of the pipette decreased the access resistance to 20–60 MΩ within 10–20 min of seal formation (measured by the kinetics of the capacitative current elicited by a 10-mV hyperpolarizing pulse). The decrease in access resistance was also evidenced by the increase in voltage-gated current elicited by a pulse from −80 to 0 mV. Currents were low-pass filtered at 1 or 5 kHz and sampled at 5 to 20 kHz to obey the Nyquist criterion. Unless noted otherwise, cells were held at −60 mV and pulsed in 20-mV steps from −80 to 100 mV.

Statistics

Significance for differences in responsiveness was tested using the Fisher exact probability test estimated using Statistica (Tulsa, OK). A level of P < 0.05 was considered significant.

Results

OSNs from wild-type mice respond to odors with either inward or outward currents under loose patch. To determine odor responsiveness using a minimally invasive technique, we recorded in the loose-patch configuration responses to two mixtures of odorants (mix A and mix B) that had displayed differential pharmacology in earlier studies in human OSNs (Rawson et al. 1997). With loose-patch recording, a large resistance electrode filled with normal bath solution forms a 40- to 80-MΩ seal with the membrane. This is similar to the cell-attached configuration of tight seal techniques. With zero holding potential applied to the pipette, the change in current is directly related to the membrane potential. If the cell depolarizes, the driving force on K⁺ is increased and generates an outward current (in the area of membrane under the electrode tip). If the neuron hyperpolarizes, the driving force decreases, and this is observed as an inward current. We recorded from a total of 54 mouse OSNs. Each cell was stimulated with Ringer solution, to control for mechanical artifacts due to a change in patch resistance, with a high K⁺ solution, to ensure that a sizable current could be measured on OSN depolarization, and with odors. Stimulation with Ringer produced small responses of either outward or inward polarity, presumably due to mechanical changes in seal resistance or drift (Fig. 1A). The mean amplitude of the responses to Ringer was 0.6 pA, and the SD was 2.1 pA (Fig. 1B). In contrast, stimulation with high K⁺ saline elicited a large outward current (Fig. 1C) that was variable from cell to cell (73 ± 264 pA, n = 38). The
variability in the $K^+$-induced current was due to differences in electrode diameter and the resistance of the seal. Under loose-patch recording conditions, a depolarization elicits an outward current across the cell body membrane (Lowe and Gold 1991; Reisert and Matthews 2001). Action potentials were not present in our records because of the use of a low-pass filter.

As expected, some OSNs responded to odorants with an outward current indicative of cell depolarization (Fig. 1D). However, the majority of the responses to odorants were inward currents indicative of cell hyperpolarization (Fig. 1E). Stimulation of the apical end of the OSN resulted in a larger and faster inward response consistent with a response mediated by receptors localized to the cilia (Fig. 1E). Figure 1F shows a histogram of the magnitude of the peak responses to odor mix A. Mixes A and B induced current changes that were significantly larger than the mechanical responses to Ringer (compare Fig. 1, F with A, not shown for mix B). Odor mixture A elicited responses that were of a magnitude larger than three times the SD for the response to Ringer in 14 of 55 OSNs (3 outward and 11 inward), and mix B elicited responses in 7 cells (3 inward and 4 outward, not shown). These experiments are in agreement with earlier on-cell patch measurements (Maue and Dionne 1987) that indicate that mouse OSNs respond to odors with inhibitory, hyperpolarizing, responses.

**Odors modulate conductances with distinct current-voltage dependencies**

We studied odor responsiveness under voltage clamp in mouse OSNs using the gramicidin perforated patch (Lischka et al. 1999; Zhainazarov and Ache 1995). Fifty-seven OSNs were tested. In two OSNs, mix B elicited a current with the current/voltage dependence typical of a CNG-mediated response: a current with a reversal between 0 and $-20 \text{ mV}$ and slight outward rectification (Fig. 2A). This current profile is similar to that observed in newt OSNs by Takeuchi and Kurahashi (2002) in response to release of caged cAMP. The only difference was they reported a reversal in the current response of about $-10 \text{ mV}$. Because they used the whole cell configuration to record current responses rather than the perforated patch used in this study, this difference can be assumed to be due a difference in the driving force on chloride, secondarily activated by the influx of $Ca^{2+}$ through the CNG channels. However, in addition to this type of response, we detected two additional distinct effects of odors. Mix A suppressed the voltage-dependent outward current in 17 OSNs (Fig. 2B), whereas mix B elicited suppression in only three cells (not shown). The odor suppression of the outward current was blocked by 20 mm TEA, a $K^+$ channel blocker (data not shown). We also observed suppression of the voltage-gated inward current (not
shown). Suppression of voltage-dependent currents has been described in amphibian and mammalian OSNs and is thought to be a direct effect of odors on voltage-gated conductances (Dubin and Dionne 1993; Kawai et al. 1997; Lischka et al. 1999; Sanhueza et al. 2000). However, in addition to the suppression of the outward current, odors elicited a marked increase in outward current with no increase in inward current at negative potentials in seven other OSNs (Fig. 2C). Interestingly, three cells responded to the two odor mixtures with different response profiles. The OSN that responded to mix B (Fig. 2A) with an outwardly rectifying current, reversal between 0 and −20 mV, responded to mixture A with an increase in the outward currents, a response similar to that shown in Fig. 2C. Odor mixtures repeatedly and reproducibly elicited the two types of responses in these dual responsive cells.

**IBMX and forskolin also elicit dual effects in mouse OSNs**

The experiments presented above indicate that in addition to the well characterized depolarizing effect of odorants on OSNs, odors elicit hyperpolarizing responses under loose-patch recording as well as an increase in outward current under voltage clamp in perforated patch. Hyperpolarizing responses and/or outward current enhancement by odorants has been reported in invertebrate and amphibian OSNs. To find out whether elevations in cyclic nucleotide concentration can elicit this type of response, we stimulated OSNs with 1 mM IBMX and 20 μM forskolin (IBMX/fsk). IBMX/fsk elicited two distinct types of responses in either loose- or perforated-patch recordings (Fig. 3). We tested 36 OSNs under loose-patch conditions. Among these, three OSNs responded to IBMX/fsk with an outward current, whereas three others responded with an inward current (Fig. 3, A and B). In a separate group of cells, we examined the involvement of cyclic nucleotide-gated channel by using the CNG channel inhibitor l-cis-diltiazem (LCD). We tested the effect of 20 μM LCD on inward current responses to odorants in the loose-patch configuration. L-cis-diltiazem decreased the inward current responses to 52 ± 12% of control, and the inhibition was fully reversible (n = 7, data not shown).
Experiments with IBMX/fsk in perforated-patch recordings yielded similar results. In these experiments, 5 of 19 OSNs responded to IBMX/fsk with an outwardly rectifying current reversing around 0 mV (Fig. 3, C and D), while 6 of 19 cells responded to IBMX/fsk with enhanced outward current (Fig. 3, E and F). IBMX/fsk did not suppress voltage-activated currents in any of the cells tested (n/H1100536). These experiments suggest that in addition to the conventional depolarizing cAMP-mediated response to odorants, cAMP elicits hyperpolarizing responses in mouse OSNs.

OSNs from CNGA2 knockout mice are unresponsive to IBMX/fsk and respond to odors only with suppressive responses

To test for the involvement of the CNG channel in the different types of responses to odors and IBMX/fsk, we tested OSNs isolated from CNGA2 knockout mice for their odor and IBMX/fsk responsiveness under voltage clamp in the perforated-patch configuration. None of 10 OSNs isolated from CNGA2 knockout mice responded to IBMX/fsk (Fig. 4A). The response frequency of CNGA2 knockout mice to IBMX/fsk is significantly different from the responsiveness of OSNs isolated from wild-type mice (11 of 19 cells) using a Fisher test. Odorants (mix A or B) did not elicit increases in inward or outward currents in any OSNs tested in CNGA2 knockouts (n = 20). When OSNs were stimulated with odorants, the only response type found in CNGA2 knockout OSNs was suppression of voltage-gated currents (detected in 7 of 20 cells) (Fig. 4, B–D). Thus suppressive responses are independent of the CNG channel.

DISCUSSION

CAMP-gated opening of the CNG channel mediates both depolarizing and hyperpolarizing odor responses

We find that mouse OSNs respond to odorant stimulation under loose patch with either depolarizing or hyperpolarizing responses. The dual polarity of odor responsiveness in the loose-patch configuration is mirrored by odor responses with distinct current-voltage dependencies in the perforated-patch configuration.
configuration. In this configuration, we detected odor-elicited responses that either displayed current-voltage relationship characteristics of CNG-mediated depolarizing currents (Fig. 2A) or odor-induced increases in outward current similar to those described in amphibian and rodent OSNs (Pun and Kleene 2002; Sanhueza et al. 2000) (Fig. 2C). We did not detect odor-stimulated outward currents at negative potentials in the perforated-patch configuration (Fig. 2C) although the loose-patch recordings clearly show that in some cells odors elicit hyperpolarizing currents at the resting potential (Fig. 1E). We do not know why there is a discrepancy here, but the difference may be due to a change in intracellular sodium or pH. Responses of both polarities were elicited by increases in cAMP elicited by stimulation with IBMX/fsk (Fig. 3). This evidence, together with the lack of responsiveness of OSNs isolated from CNGA2 knockout mice to either IBMX/fsk or odorants (Fig. 4), indicates that responses of both polarities are mediated by cAMP-mediated opening of CNG channels. Our experiments implicate the CNG channel in mediating hyperpolarizing responses in vertebrates.

The group of Bacigalupo has studied the mechanism underlying hyperpolarizing responses in toad and rat olfactory receptor neurons. These investigators do not make specific claims on the identity of second messenger that elicits the odor-induced elevation of ciliary Ca2+ that in turn, elicits opening of large conductance (BK type) Ca2+-activated K+ channels (Morales et al. 1995). Initially Bacigalupo and co-workers showed that the hyperpolarizing responses were elicited in toad only by odorants that had been shown to stimulate inositol-1,4,5-trisphosphate (InsP3) production in rat cilia homogenates (InsP3 odorants) (Breer and Boekhoff 1991; Morales et al. 1994). In those experiments, odorants that elicited cAMP production in biochemical studies in rat homogenates (cAMP odorants) only elicited depolarizing responses. On this basis, Bacigalupo and co-workers speculated that the hyperpolarizing responses could be mediated by InsP3 opening a plasma membrane InsP3-gated channel (Morales et al. 1994). However, studies with gene-targeted mice deficient for adenylyl cyclase III, the CNGA2 subunit of the CNG channel and Gαolf (Belluscio et al. 1998; Brunet et al. 1996; Wong et al. 2000) have called into question the validity of the results of the biochemical experiment within the context of intact OSNs. Indeed, subsequent publications from the group of Bacigalupo do not make specific claims on the identity of the second messenger involved in inhibitory odorant responses (Morales et al. 1997; Sanhueza et al. 2000).

Our studies provide evidence that InsP3 does not mediate the inhibitory (or excitatory) responses for the subset of odorants tested in this study and indicate that the CNG channel mediates both the excitatory and inhibitory responses. This conclusion is consistent with the finding by Bacigalupo and co-workers that nimodipine, which inhibits the CNG channel, inhibits odorant-induced hyperpolarizing responses in toad (Morales et al. 1997) as well as with experiments of Pun and Kleene implicating cAMP as the second messenger in inhibitory olfactory responses in grass frog (Pun and Kleene 2002). Further, the lack of responsiveness of OSNs from CNGA2 knockout mice implicates the CNG channel as a direct mediator of these responses. Taken together with the studies by the groups of Bacigalupo and Kleene, our study is consistent with mediation of odor-induced hyperpolarizing responses by CNG-mediated increases in intracellular Ca2+ leading to opening of BK type Ca2+-activated channels. An alternative explanation could be hyperpolarization elicited by opening of Ca2+-activated Cl− channels in cells (or local domains) where intracellular Cl− is at concentrations below electrochemical equilibrium (Delay and Verret 2003). Another possibility could be that a subset of CNG channels are inhibited due to localized higher intracellular concentrations of Ca2+ (microdomains). However, we should point out that we have not presented data probing the ionic nature of the odor-induced hyperpolarizing responses in mouse OSNs, and that any or all of the preceding options are possible.

Implications for odor detection and coding

The observation that wild-type mouse OSNs exhibit more inhibitory odor responses than excitatory was unexpected be-
cause the opposite was observed with rat olfactory neurons using the same odor stimuli (Lischka et al. 1999). Not only are inhibitory odor responses more prevalent in mouse OSNs, but a single mouse OSN can respond to one odor stimulus with a CNG-like response, while it responds to the other odor with an increase in outward current (Fig. 2, A and C). These findings are consistent with observations by others that show that single OSNs can respond to one odor with depolarization and to another with hyperpolarization (Ache and Zhainazarov 1995; Dionne 1992; Morales et al. 1994). Differential responses could be due to expression of two different receptors in one OSN (Rawson et al. 2000). Alternately, differential modification or protein-protein interaction of a single olfactory receptor could result in differential responsiveness of a single OSN to different odors. In either case, receptors responding to either odor should be localized in different microdomains to be able to elicit dual polarity responses in the same OSN.

Our observations raise interesting questions in terms of potential implications for odor detection and discrimination. Numerous electrophysiology studies suggest that olfactory neurons are broadly tuned, and most of these respond to a subset of odors rather than just a single odor (Restrepo et al. 1996). In addition, olfactory neurons are spontaneously active although the firing pattern can be quite variable. Thus the output from the olfactory epithelium is noisy and a small signal generated in response to an odor could be lost in the noise. One way to increase the signal to noise ratio would be to lower the surrounding noise while increasing the response to a specific odorant by inhibiting the spontaneous activity of olfactory neurons. This would happen when some OSNs are inhibited by an odorant, while others are stimulated by the same odorant. In addition, inhibitory responses could help increase contrast in odor quality detection between closely related odorants (Ache and Zhainazarov 1995; Dionne 1992).

**Suppression of voltage-dependent conductances is independent of the CNG pathway**

Suppression of voltage-dependent outward current occurs in ~30% of the OSNs tested with mix A in this study. While voltage-dependent current suppression also occurs in response to application of mix B, the frequency of current suppression was substantially less, only ~5%. The suppression of voltage-dependent currents in response to odors persisted in OSNs from CNGA2 knockout mice. This is consistent with a direct effect of odorants on voltage-gated conductances postulated by several groups (Kawai et al. 1997; Sanhueza et al. 2000). This decrease in outward current is blocked by the K channel blocker, TEA, which suggests that these odorants are suppressing a voltage-gated K current. This is similar to the extensive study by Lischka et al. (1999) on odor suppression of a voltage-gated K conductance observed in rat OSN. Similar odor suppression of a K conductance was observed in toad and rat by Sanhueza and Baccaglione (1999) and not studied further here. However, it still remains unclear how a nonspecific effect affects some OSNs but not others. Thus the nature of the mechanism mediating voltage-dependent current suppression, which could be relevant for odor detection and odor quality coding at high odorant concentrations, remains to be tested at the single-channel level, beyond the scope of this study.

In summary, our results are consistent with the results reported by Brunet et al. (1996) that olfactory neurons from CNGA2 knockout mice cannot generate excitatory odor responses in response to general odorants. In addition, we show that OSNs from CNGA2 knockout mice are deficient in inhibitory odor responses. Therefore the CNG channel is necessary for both excitatory and inhibitory odor responses but not for the odor-induced suppression of voltage-gated currents.

**Acknowledgments**

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