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General Anosmia Caused by a Targeted Disruption of the Mouse Olfactory Cyclic Nucleotide–Gated Cation Channel

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Summary

Olfactory neurons transduce the binding of odorants into membrane depolarization. Two intracellular messengers, cyclic AMP (cAMP) and inositol trisphosphate (IP_3), are thought to mediate this process, with cAMP generating responses to some odorants and IP_3 mediating responses to others. cAMP causes membrane depolarization by activating a cation-selective cyclic nucleotide–gated (CNG) channel. We created a mutant "knockout" mouse lacking functional olfactory CNG channels to assess the roles of different second messenger pathways in olfactory transduction. Using an electrophysiological assay, we find that excitatory responses to both cAMP- and IP_3-producing odorants are undetectable in knockout mice. Our results provide direct evidence that the CNG channel subserves excitatory olfactory signal transduction, and further suggest that cAMP is the sole second messenger mediating this process.

Introduction

The vertebrate olfactory system is capable of recognizing and discriminating thousands of different odorant molecules. This complex process of sensory perception begins with the activation of primary sensory neurons, the olfactory neurons, within the olfactory epithelium of the nose (reviewed by Lancet, 1986). Odorants elicit an inward (depolarizing) current across the plasma membrane of the sensory cilia of these cells, which ultimately leads to the generation of action potentials (Firestein and Werblin, 1989; Kurahashi, 1989; Lowe and Gold, 1991). What are the molecular mechanisms underlying excitatory signaling in olfactory neurons? Odorants are thought to activate receptors encoded by a large multigene family intially identified in the rat (Buck and Axel, 1991). The predicted structure of these receptors exhibits a seven transmembrane domain topology characteristic of the superfamily of G protein–coupled receptors.

How, then, is the binding of an odorous ligand with its receptor transduced into an excitatory signal in the olfactory neuron? In vitro biochemical approaches have shown that some odorants elicit an increase in the intracellular second messenger cyclic AMP (cAMP) (Pace et al., 1985; Sklar et al., 1986; Breer et al., 1990), whereas other odorants cause an increase in the second messenger inositol trisphosphate (IP_3) (Huque and Bruch, 1986; Boekhoff et al., 1990; Breer and Boekhoff, 1991). These observations suggest that cAMP mediates excitatory responses to one subset of odorants, while IP_3 mediates responses to another subset of odorants. Consistent with the proposed role of cAMP in olfactory signaling, a specific $G_{s\alpha}$-like G protein isoform, termed G_{olf}, as well as type III adenylyl cyclase are highly enriched in olfactory sensory cilia (Jones and Reed, 1989; Bakalyar and Reed, 1990). Thus, odorant receptor activation may lead to an increase in adenylyl cyclase activity via an interaction with G_{olf}. Odorant-evoked elevations in cAMP are thought to activate a cation-selective cyclic nucleotide–gated (CNG) channel directly, resulting in membrane depolarization and the generation of action potentials (Nakamura and Gold, 1987; Kurahashi, 1989, 1990; Firestein et al., 1991; Frings and Lindemann, 1991; Lowe and Gold, 1993a). The olfactory CNG channel provides a means for rapid neuronal signaling in response to odorant receptor–mediated elevations in cAMP. In addition, calcium influx through the CNG channel activates a depolarizing chloride conductance, which further amplifies the cAMP-mediated response (Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993b; Zhainazarov and Ache, 1995).

The role of IP_3 in olfactory signaling, however, remains somewhat controversial. Whereas several studies have reported the presence of an IP_3-gated ion channel in the plasma membrane of olfactory cilia and dendrites (Restrepo et al., 1990; Kalinoski et al., 1992; Miyamoto et al., 1992; Cunningham et al., 1993; Okada et al., 1994; Schild et al., 1995), other attempts to demonstrate this conductance in olfactory neurons have failed (Firestein et al., 1991; Lowe and Gold, 1993a; Kleene et al., 1994; Nakamura et al., 1996; S. Frings, personal communication; R. Kramer and S. Siegelbaum, personal communication; T. Kurahashi, personal communication).

To clarify the contributions of different second messenger pathways in olfactory signal transduction, we created a "knockout" mouse in which the olfactory CNG channel has been disrupted by homologous recombination. Using extracellular field recording, we find that olfactory neurons in mice homozygous for this mutation fail to respond to a wide array of odorants, including those which have been reported to elicit elevations in IP_3 in vitro. These observations demonstrate that the olfactory CNG channel is required for olfactory signaling in response to many, if not all odorant stimuli. Moreover, our results suggest that cAMP serves as the sole second messenger mediating excitatory olfactory signal transduction.

Results

Disruption of the Mouse Olfactory CNG Channel by Homologous Recombination

CNG channels are heterotetramers (Liu et al., 1996) composed of ion-conducting α subunits (Kaupp et al., 1989; Dhallan et al., 1990; Goulding et al., 1992) and modulatory β subunits (Chen et al., 1993; Bradley et al., 1994;

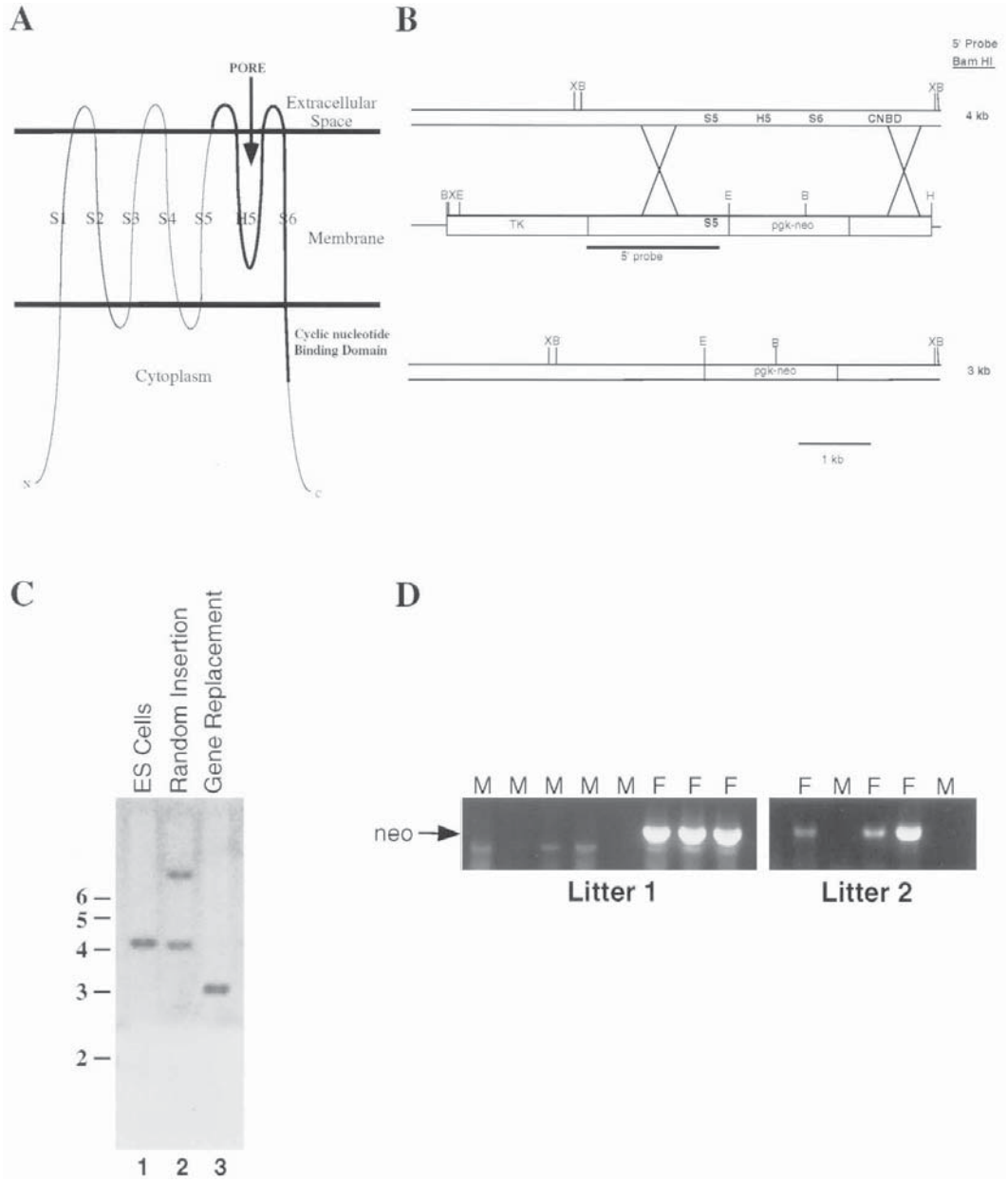

Figure 1. Targeted Disruption in the Olfactory CNG Channel α Subunit Gene

(A) Schematic of proposed transmembrane topology of the CNG channel α subunit. The portion of the channel subunit containing the pore-forming region (H5), the sixth membrane-spanning domain (S6), and part of the carboxyl terminus removed by homologous recombination is highlighted in bold.

(B) Homologous recombination of the wild-type olfactory CNG channel gene (top line) with the targeting vector (middle line) results in a disrupted CNG channel gene (bottom line).

(C) Southern blot analysis with the CNG channel probe shown in (B) indicates that the 4 kb wild-type BamHI fragment (lane 1) is present in a cell line in which there was a nonhomologous insertion (lane 2) and is replaced with the diagnostic 3 kb BamHI fragment (indicative of homologous recombination) in a bona fide knockout ES cell clone (lane 3).

(D) Genomic PCR analysis of offspring from one mouse chimera using primers for the neomycin resistance gene demonstrates that the targeted gene transmits through the germline to female (F), but not male (M) offspring.

Liman and Buck, 1994; Korschen et al., 1995). We therefore chose to mutate the olfactory CNG channel α subunit gene to ablate channel function. We designed a targeting vector that, upon homologous recombination within the olfactory CNG channel α subunit gene, would delete a portion of the channel that includes the extracellular loop following the fifth membrane-spanning region (S5), the putative pore-forming region (H5), the sixth membrane-spanning region (S6), and a portion of the intracellular carboxy-terminal tail (Figures 1A and 1B; see also Goulding et al., 1992). Thus, any protein product derived from this disrupted gene would not be expected to form a functional channel subunit.

Embryonic stem (ES) cells derived from the 129/Sv

strain of inbred mice were transfected with the targeting construct, and clones were isolated and screened for homologous recombination by Southern blot analysis (see Experimental Procedures). A blot containing DNA from two representative clones is shown in Figure 1C. The hybridization pattern indicates that the olfactory CNG channel gene underwent homologous recombination with the targeting vector in one of these cell lines (lane 3). Interestingly, no wild-type band is observed with the CNG channel probe in the homologous recombinant. Since the ES cell line we used has a normal XY karyotype (Szabo and Mann, 1994), this suggests that the olfactory CNG channel gene is located on the X chromosome.

ES cell lines containing the mutant olfactory CNG channel gene were expanded and used to generate chimeric mice. A total of seven chimeric mice were obtained using two independent cell lines. One male chimera transmitted the mutation through the germline when crossed with wild-type females, as judged by detection of the neomycin resistance gene in F1 offspring by polymerase chain reaction (PCR) analysis. An initial analysis of 37 F1 pups indicated that 17 out of 18 female offspring contained this gene, whereas all 19 males were negative. A representative gel showing this PCR analysis on 13 pups (derived from two litters) is shown in Figure 1D. The strict segregation of the targeted gene to female F1 offspring confirms that the olfactory CNG channel α subunit gene resides on the X chromosome.

Early Postnatal Lethality in Mice Hemizygous for the CNG Channel Mutation

Upon crossing F1 heterozygous mutant females with wild-type males, we found that an unusual number of F2 pups died within 1–2 days after birth. While these pups appeared healthy and pink immediately after birth, their stomachs remained devoid of milk and they quickly became dehydrated. These animals appeared unable to locate the mother's nipple and suckle, even when placed directly next to a nipple in the absence of any competing littermates. The dead pups were invariably males; genotypic analysis confirmed that they were also hemizygous for the targeted CNG channel gene (data not shown). Since olfactory cues are thought to play an important role in suckling behaviors (Teicher and Blass, 1977; Hudson and Distel, 1986; Risser and Slotnick, 1987), these observations gave an early hint that olfactory signaling was profoundly affected in the hemizygous mutant animals. Interestingly, a small percentage of mutant males ($\sim$1%–10%) learned to suckle effectively (presumably based on nonolfactory cues) and survived to adulthood. Owing to the difficulty in obtaining adult animals homozygous for the channel mutation, the studies described below were performed on 1-day-old pups.

Expression of Olfactory Neuron Markers Is Normal in Hemizygous Mutants

The goal of this study is to define the role of the CNG channel in olfactory signaling by studying the physiological effects of a targeted mutation in the olfactory CNG channel α subunit gene. This requires that we first establish that any effects of this mutation on signal transduction are due to an absence of functional CNG channels in mature olfactory neurons, rather than a nonspecific effect on olfactory neuron maturation or structure. We therefore performed RNA in situ hybridizations on olfactory epithelium preparations using molecular markers for mature olfactory neurons.

One such marker is the olfactory marker protein (OMP), an abundant cytoplasmic protein of unknown function that is expressed only in mature olfactory neurons (Margolis, 1985). In situ hybridizations with an OMP probe demonstrate the expression of OMP RNA in olfactory neurons throughout the sensory epithelium of the medial septum and complex nasal turbinates of both wild-type and mutant animals (Figures 2A and 2B). The density of olfactory neurons in mutants is indistinguishable from that in wild-type epithelium, as judged by the level of OMP expression (Figures 2C and 2D). In situ hybridizations using a probe complementary to the disrupted portion of the CNG channel gene confirm that intact CNG channel α subunit transcripts are not expressed in olfactory neurons of mutant animals (Figures 2E and 2F).

We next examined the expression patterns of odorant receptor genes in the olfactory epithelium. Odorant receptors are expressed in 3–4 distinct zones of the olfactory epithelium; each zone comprises a hemicylindrical ring along the anterior–posterior axis (Strotmann et al., 1992; Ressler et al., 1993; Vassar et al., 1993). Thus, in coronal sections, the expression zones appear as concentric dorsomedial to ventrolateral arcs. We find that odorant receptor expression is indistinguishable in mutant and wild-type pups. For example, in both wild-types and mutants, odorant receptor M50 is expressed appropriately in the ventrolateral-most zone (Figures 3A and 3B), whereas odorant receptor K4 is expressed in a medial zone (Figures 3C and 3D). From a survey of numerous tissue sections, the frequency of olfactory neurons expressing each of these receptors is similar in both cases (data not shown). Thus, odorant receptor expression appears to be unaffected by the targeted CNG channel mutation.

Olfactory Signal Transduction Components Are Expressed in Appropriate Subcellular Structures in Hemizygous Mutants

The cilia of olfactory neurons are specialized structures whose function is to receive odorant stimuli and transduce odorant receptor binding into changes in intracellular second messengers and membrane potential (Pace et al., 1985; Lowe and Gold, 1991; Kurahashi and Kaneko, 1993; Kleene et al., 1994). It is therefore important to show that olfactory cilia are intact in CNG channel mutants and that key signal transduction molecules are appropriately expressed in these structures. Toward this end, we performed immunohistochemistry on olfactory epithelium from wild-type and mutant animals, using an antibody directed against type III adenylyl cyclase and an antibody directed against a common epitope found in $G_{s\alpha}$ and G_{olf}. Both G_{olf} and type III adenylyl cyclase are highly enriched in olfactory cilia (Jones and Reed, 1989; Bakalyar and Reed, 1990). We find that type III adenylyl cyclase is localized to the olfactory cilia in mutant olfactory epithelium in a pattern indistinguishable from that found in wild-type animals (Figures 4A and 4B). Similarly, the anti-$G_{s\alpha}$/G_{olf} antibody shows strong immunoreactivity

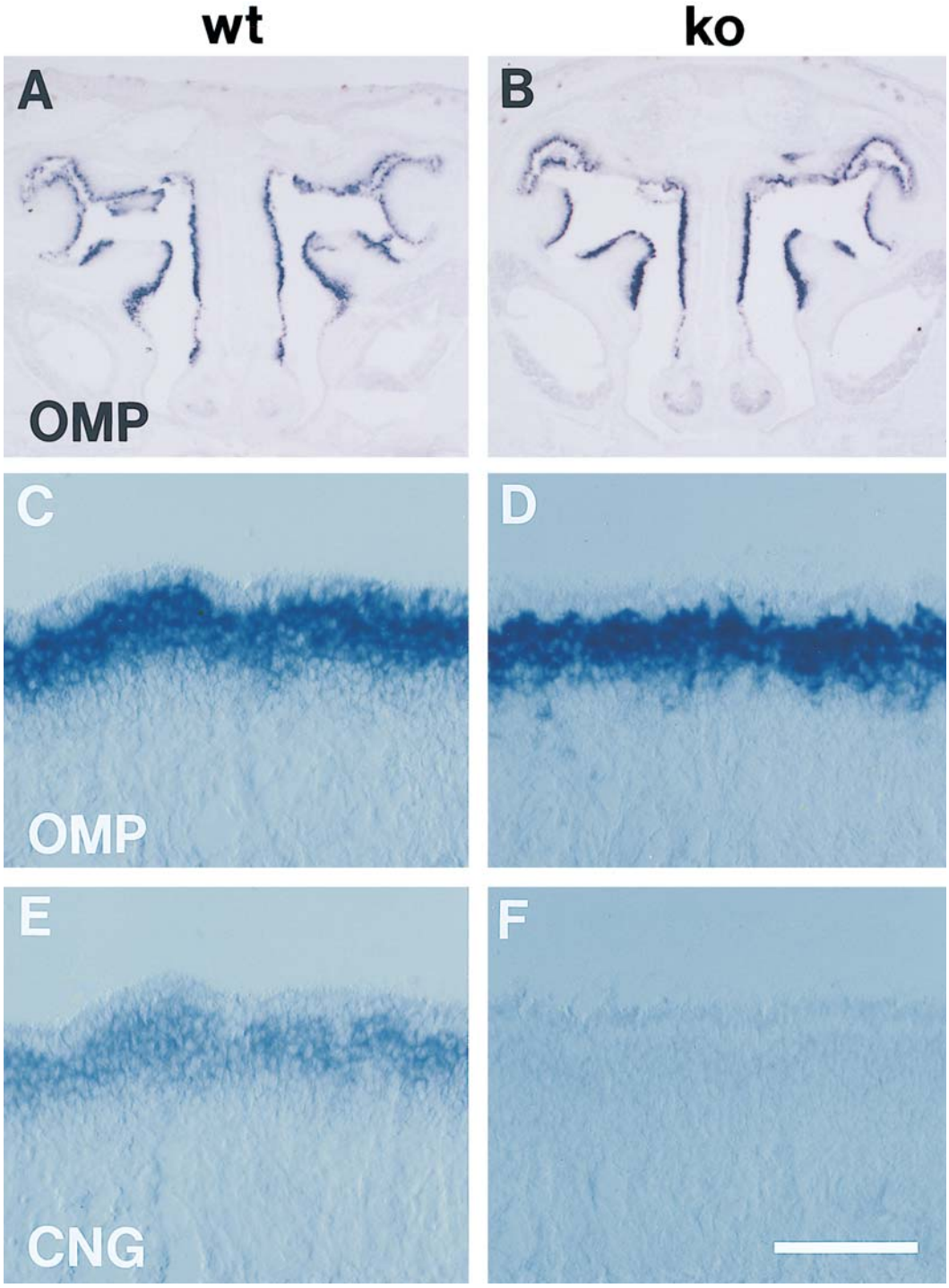

Figure 2. OMP and CNG Channel Expression in Wild-Type and Mutant Olfactory Epithelium

In situ hybridizations were performed on tissue sections from 1-day-old neonatal mice using digoxigenin-labeled antisense RNA probes. Digoxigenin-labeled probes were localized with an alkaline phosphatase–conjugated anti-digoxigenin antibody followed by a colorimetric reaction with BCIP/NBT. In situ hybridizations were carried out on noses from wild-type (+/o) males (wt) (A, C, and E) or hemizygous mutant (−/o) males (ko) (B, D, and F).

(A and B) Coronal sections hybridized with a digoxigenin-labeled OMP probe. Expression of OMP mRNA delineates the extent of the sensory neuroepithelium lining the medial septum, dorsal recesses, and lateral turbinates.

(C and D) High power differential interference contrast (DIC) views of septal olfactory epithelium from sections hybridized with the OMP probe. OMP reactivity is localized to the medially disposed olfactory neuron cell bodies in both the wild-type and mutant olfactory epithelium.

(E and F) High power DIC views of septal olfactory epithelium from sections hybridized with a digoxygenin-labeled CNG channel probe. (C) and (E) show adjacent sections from the same wild-type pup. Similarly, (D) and (F) show adjacent sections from the same hemizygous mutant pup. Reactivity is found in the olfactory neuron cell body layer in the wild-type, but not the mutant olfactory epithelium.

Scale bar in (F) indicates 1 mm for (A) and (B) and 100 μm for (C)–(F).

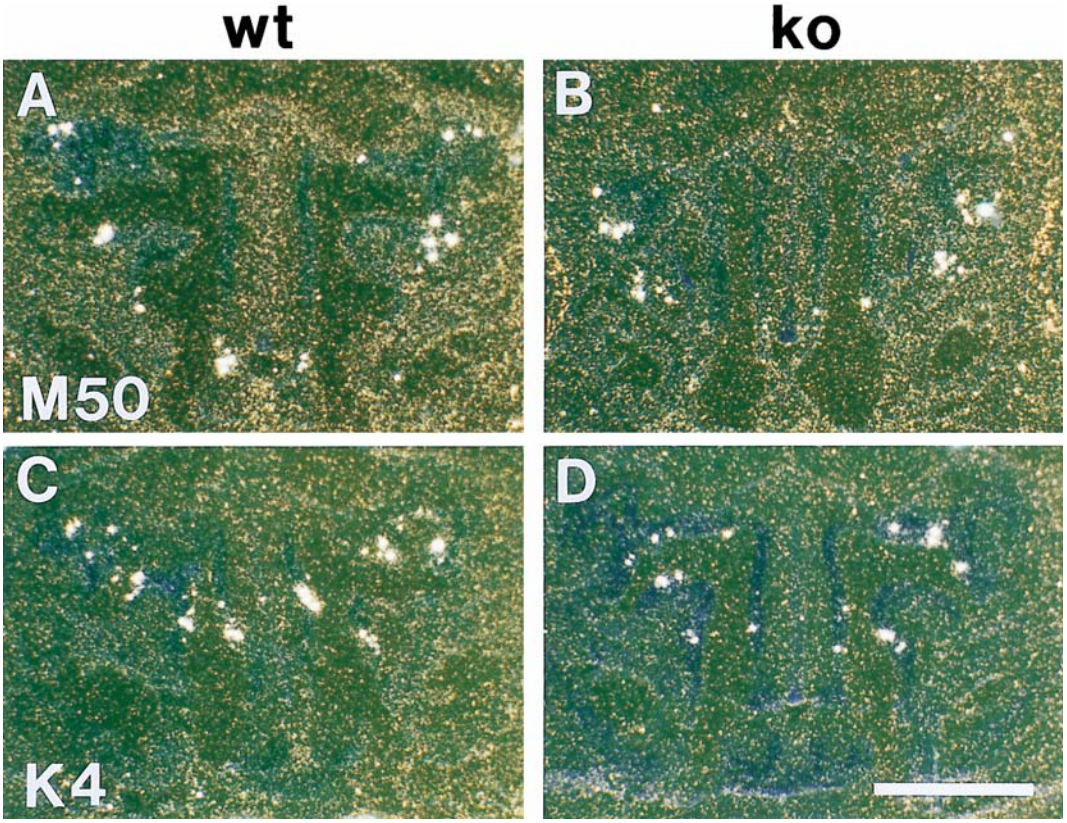

Figure 3. Localization of Odorant Receptor mRNAs in Wild-Type and Mutant Olfactory Epithelium

In situ hybridizations were performed on coronal sections from 1-day-old neonatal mice using ^{33}P-labeled antisense RNA probes complementary to odorant receptors M50 and K4. Hybridization signals were localized by dark-field microscopy following autoradiography. Tissue sections from wild-type males (wt) are shown in (A) and (C); tissue sections from hemizygous mutant males (ko) are shown in (B) and (D). In both wild-type and mutant preparations, odorant receptor M50 is expressed by cells within the ventrolateral-most zone of the olfactory epithelium (A and B), whereas odorant receptor K4 is expressed by cells within a medial zone (C and D).

Scale bar indicates 1 mm.

to the olfactory cilia in both wild-type and mutant preparations (Figures 4C and 4D). Thus, in mice hemizygous for the CNG channel mutation, expression of odorant receptors, G protein, and adenylyl cyclase is normal in olfactory neurons, and the olfactory cilia appear to be intact.

Previous studies have shown that G_{olf} is localized in olfactory neuron axons in addition to the sensory cilia (Jones and Reed, 1989). Immunohistochemistry with the anti-$G_{s\alpha}/G_{olf}$ antibody shows the expected localization of signal to the subepithelial axon fascicles in wild-type and mutant animals (Figures 4C and 4D). The population of olfactory neuron axons in mutants also appears to innervate the glomerular layer of the olfactory bulb (data not shown). These observations indicate that olfactory neuron structure is normal in mice hemizygous for the targeted CNG channel mutation.

Electrophysiological Properties Are Normal in Olfactory Neurons from Hemizygous Mutants

We wish to determine whether excitatory olfactory signaling is altered by the genetic removal of the olfactory CNG channel. It is therefore important to establish that any differences in odorant-evoked responses between wild-type and mutant mice are caused by the absence of the signal transducing CNG channel and not by abnormal electrophysiological properties of the mutant neurons. For example, if mutant neurons have an abnormally positive resting potential, this would decrease odorant responses mediated by all excitatory pathways. A normal resting potential in mutant mice can be inferred if the rate of spontaneous action potential generation is similar to the rate observed in wild-type mice. Since the threshold for spike generation lies close to the resting membrane potential (Lynch and Barry, 1989), a difference in resting potential between mutant and wild-type olfactory neurons would be manifested as a difference in the rate of spontaneous spike generation. We find that spontaneous action potentials occur at similarly low rates in cells from wild-type and mutant pups (Figure 5). The pattern of spontaneous action potential generation in mutant cells therefore indicates that the resting potential of olfactory neurons is very similar to that of wild-type cells. Moreover, the time course of individual action potentials is the same in cells from both backgrounds (Figure 5), further suggesting that the voltage-dependent properties of the mutant olfactory neuron membrane are normal. Thus, any observed differences in olfactory signaling between mutant and wild-type preparations (described below) can be ascribed to an

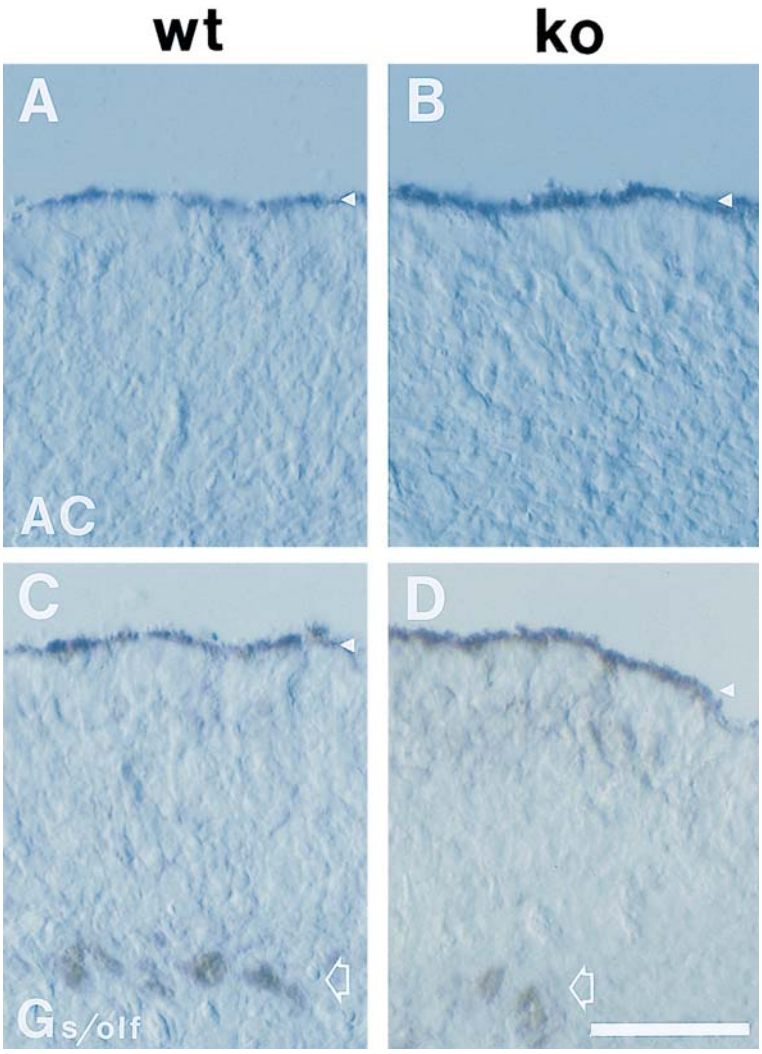

Figure 4. Immunolocalization of Signal Transduction Components in Wild-Type and Mutant Olfactory Epithelium

Tissue sections from olfactory epithelium of wild-type male (wt) (A and C) and hemizygous mutant male (ko) (B and D) were incubated with an antibody specific for type III adenylyl cyclase or an antibody directed against $G_{s\alpha}$ and G_{olf}. Specifically bound primary antibody was then localized with a horseradish peroxidase–conjugated secondary antibody, followed by chromogenic reaction with diaminobenzidine/H_2O_2. Sections were viewed using DIC optics. Type III adenylyl cyclase immunoreactivity is localized to the olfactory cilia (small arrowheads) in both wild-type and hemizygous mutant animals (A and B). $G_{s\alpha}/G_{olf}$ immunoreactivity is localized to the olfactory cilia (small arrowheads) as well as the subepithelial axon fascicles (open arrows) in both wild-type and mutant animals (C and D).

Scale bar indicates 50 μm.

absence of functional CNG channels rather than an alteration of resting membrane properties.

Absence of Odorant-Evoked Signaling in Olfactory Neurons of Hemizygous Mutant Animals

Having established that the expression of olfactory neuron–specific markers and the resting membrane properties of olfactory neurons are unaffected in mice hemizygous for the targeted CNG channel mutation, we next turned to an analysis of odorant-stimulated olfactory neuron function. Since the CNG channel α subunit is required for CNG channel function (Kaupp et al., 1989; Dhallan et al., 1990; Goulding et al., 1992; Chen et al., 1993; Liman and Buck, 1994), mice hemizygous for a mutation in this gene provide a means to assess the role of this channel in olfactory signaling. In one model, cAMP mediates excitatory olfactory responses to one subset of odorants, whereas IP_3 mediates excitatory signaling to another. In this case, we would expect the responses to some odorants to be reduced or eliminated and others to be intact in mice lacking a functional CNG channel α subunit gene. In an alternative model, cAMP serves as the sole second messenger mediating excitatory olfactory signaling. From this latter model, we would predict that mice hemizygous for the CNG channel mutation will exhibit a complete absence of neuronal responses to all odorants.

To assess olfactory neuron function, we recorded odorant-evoked changes in the voltage across the olfactory epithelium. This measurement, termed the electro-olfactogram (EOG; Ottoson, 1956), is an extracellular field potential that locally summates the activity of cells in the olfactory epithelium and thus provides a sensitive and facile assay for neuronal function. For most odorants, the EOG consists of a transient negative potential generated by the extracellular current flow resulting from an inward current across the olfactory cilia and an equivalent outward current across the dendritic and somatic membranes (Lowe and Gold, 1991). Some odorants evoke an initial positive transient, followed by the more common negative response. The positive component, but not the negative component of the EOG persists in epithelia whose receptor cells have been destroyed by denervation and is correlated with mucus secretion by non-neuronal supporting cells (Okano and Takagi, 1974). Thus, the positive component arises independently of the signal transduction mechanism within olfactory neurons.

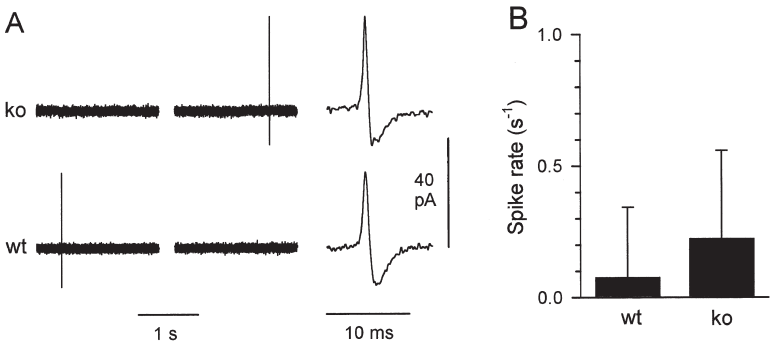

Figure 5. Single-Cell Recordings Demonstrate That Olfactory Neuron Membrane Properties Are Normal in CNG Channel Hemizygous Mutants

Dendritic cell–attached patch-clamp recordings were performed on individual olfactory neurons in tissue slices from olfactory epithelia from mutant and wild-type neonatal pups. (A) Representative traces from patch-clamp recordings (ko, hemizygous mutant; wt, wild type). The action potential currents are initially outward (see traces with expanded time scale at right), indicating that these are primarily capacitative currents, as shown previously for dendritic and ciliary recordings from the frog (Frings and Lindemann, 1990).

(B) Mean spike rates were compared between wild-type and mutant olfactory neurons (n = 3 cells from three wild-type animals; n = 4 cells from three mutant animals). Mean values are represented by solid bars, with the respective 95% confidence intervals indicated by error bars. Spike rates are low in cells from both backgrounds, and the difference between the means is not statistically significant (p = 0.25).

EOG recordings were performed on olfactory epithelium preparations from 1-day-old pups using the following compounds as stimulants: 2-hexylpyridine, isomenthone, citralva, and geraniol, which have been reported to elicit increases in cAMP (Sklar et al., 1986; Breer and Boekhoff, 1991); and pyrazine, lilial, ethylvanillin, isovaleric acid, and triethylamine, which have been reported to elicit increases in IP_3 (Boekhoff et al., 1990; Breer and Boekhoff, 1991). The concentrations of individual odorant stimuli were chosen to produce as large a response as possible in control preparations without causing an excessively long recovery time (see legend to Figure 6 and Experimental Procedures). To assess olfactory function in response to more complex or "natural" stimuli, EOG recordings were also carried out using three chemically undefined odorous substances: C57BL/6 mouse urine, coyote urine, and peanut butter. The urine samples were included in this analysis owing to their efficacies in eliciting olfactory-mediated behaviors. Mice can discriminate other mice of different genetic backgrounds based on odorants present in urine (Yamaguchi et al., 1981), and coyote urine contains aversive odorant cues that signal the presence of a predator (Nolte et al., 1994). Peanut butter was tested because of its potency as an attractive food source for mice (e.g., as bait in mouse traps; Davis, 1956).

Figure 6 shows representative EOG recordings from a wild-type female and a hemizygous mutant male. EOG recordings were typically performed by placing the electrode in the approximate middle of the first (anteriormost) turbinate, since responses to odorants in control preparations were largest at this location. For each odorant, the hemizygous mutant response (ko) is plotted directly above the wild-type (wt) response. A slow negative response is observed following exposure of the wild-type olfactory epithelium to each test odorant. Responses to odorants reported to elicit increases in cAMP are included in the top row of the figure, whereas responses to odorants reported to elicit increases in IP_3 are shown in the middle row. These response waveforms are typical of those observed in mice (e.g., see Wang et al., 1993). A small response is observed with mineral oil (the solvent carrier for all odorants except isovaleric acid and ethylvanillin, which were diluted in water) and is probably due to odorous hydrocarbons present in the mineral oil used for these experiments. In striking contrast with the responses found in wild-type epithelium, no detectable negative EOG is observed in the hemizygous mutant epithelium for any of the odorants tested. The absence of a detectable response is seen more clearly in the pyrazine knockout trace plotted at ten times higher gain (10× ko).

Considering that sensitivity to specific odorants varies across the surface of the olfactory epithelium (Thommesen and Doving, 1977; Ezeh et al., 1995), presumably due to the spatially restricted expression of individual olfactory receptor proteins to distinct longitudinal zones (Strotmann et al., 1992; Ressler et al., 1993; Vassar et al., 1993), it is formally possible that responses to some odorants might be detectable in knockout mice at other recording locations. To investigate this possibility, we carried out EOG recordings at two other locations in addition to the middle of the first turbinate: near the superior edge of the first turbinate and near the middle of the third turbinate. Activity from cells was therefore sampled across multiple odorant receptor expression zones. Identical results showing a complete absence of negative EOG responses were obtained at each of the three recording locations in six hemizygous mutant pups (data not shown).

The only response remaining in the mutant mouse was a rapidly rising positive potential upon stimulation with triethylamine, and is most obvious in the mutant owing to the absence of the negative EOG response. In normal olfactory epithelium, the summation of positive and negative components with slightly different latencies results in an intial positive transient followed by the more prolonged negative response (see inset below the triethylamine panel in Figure 6, which superimposes the wild-type and hemizygous mutant responses on an expanded time scale). The positive EOG reflects an odorant-evoked secretory mechanism in non-neuronal supporting cells (Okano and Takagi, 1974); its persistence in hemizygous mutant mice therefore is expected. Since the expression of the olfactory CNG channel is restricted to olfactory neurons (Dhallan et al., 1990; Goulding et al., 1992; see also Figure 2E), the absence of the negative EOG in knockout mice also confirms that the negative EOG indeed is of neuronal origin.

We quantitated EOG response amplitudes from six

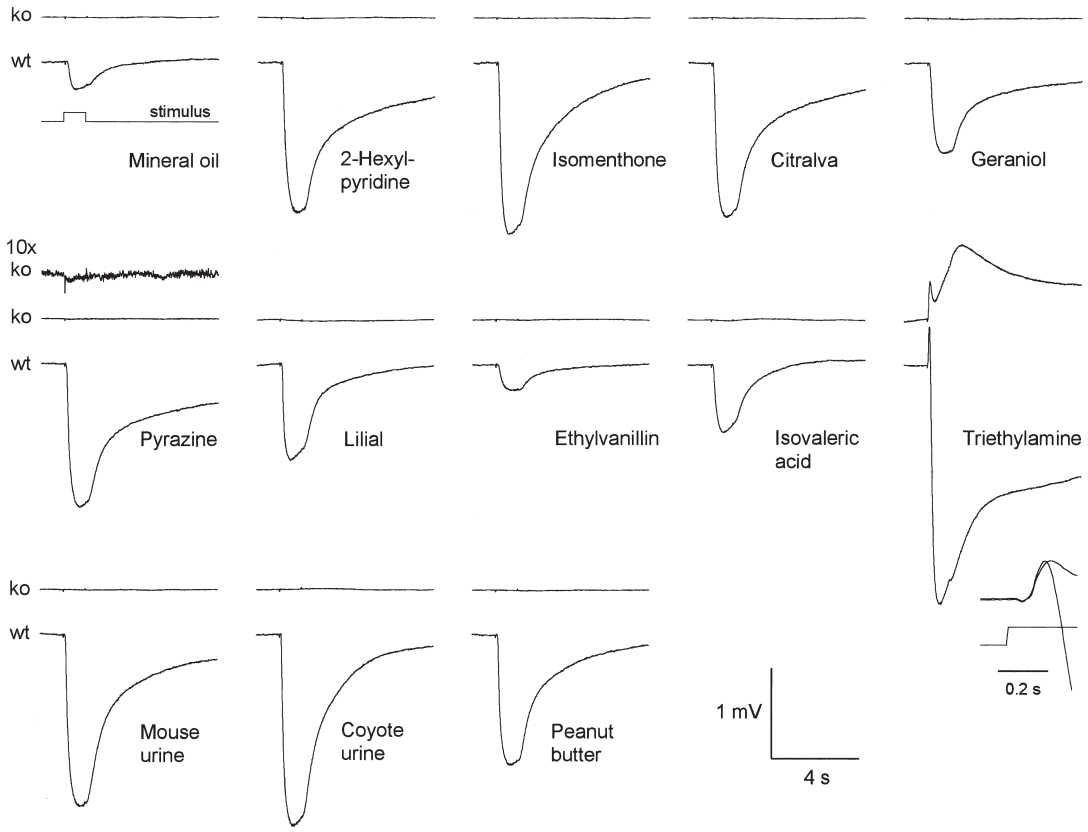

Figure 6. EOG Recordings from Olfactory Epithelium of Wild-Type and Hemizygous Mutant Pups

EOG responses to 13 odorants from a wild-type female and a hemizygous male, both from the same F2 litter which was derived on the FVB background. Each panel includes the wild-type (wt) and hemizygous (ko) responses to odorant, as indicated. Odorants were delivered in the vapor phase by passing an air stream over evaporation tubes containing odorant solutions (see Experimental Procedures). The following odorants were diluted in mineral oil (fold-dilutions from pure liquid stocks are indicated in parentheses): 2-hexylpyridine (10^{-3}), isomenthone (10^{-3}), citralva (10^{-3}), geraniol (10^{-3}), lilial (10^{-2}), triethylamine (10^{-3}). Pyrazine (a solid) was dissolved to a final concentration of 10^{-2} M in mineral oil. Isovaleric acid (final concentration: 0.02 M) and ethylvanillin (final concentration: 0.2 M) were each diluted in water. Urine from C57BL/6 male mice, coyote urine (from meat-fed animals), and peanut butter were not diluted. In all cases, odorants were applied 1 s after the beginning of each trace for a duration of 1 s. The stimulus trace beneath the mineral oil response shows an example of the time course of the computer-controlled stimulus pulse. The small initial negative deflection in each EOG trace (most easily visible in each of the knockout traces) is a mechanical artifact caused by actuation of the valve used to switch air streams; a similar positive artifact occurs when the stimulus is switched back to clean air. The knockout trace in response to pyrazine is also shown at 10-fold higher gain (10× ko) to demonstrate the absence of a detectable negative EOG. The positive EOG of the mutant in response to triehylamine is of non-neuronal origin (Okano and Takagi, 1974; see the text) and superimposes with the initial transient positive response of the wild-type trace (see inset with expanded time scale).

litters of inbred F2 pups derived from crosses between 129/Sv F1 heterozygous females (+/−) and 129/Sv wild-type males (+/o) (Figure 7). The negative EOG was always detected in wild-type and heterozygous mice (20 pups), but was never detected in mice bearing the (−/o) genotype (12 pups). Although a negative EOG was not observed in hemizygous mutant mice, we cannot rule out a response smaller than the noise of our recordings. Thus, in Figure 7, the values for hemizygous mutant responses are each expressed as an upper limit estimated as twice the magnitude of the peak-to-peak noise of the recording (peak-to-peak noise was typically <0.01 mV). For all odorants tested, mean EOG responses of wild-type and heterozygous mutant pups were highly significantly different from those of hemizygous mutant males (see legend to Figure 7). One might predict that heterozygous female mutants would exhibit EOG responses that are ~50% smaller than those found in wild-type animals due to random X inactivation. Response amplitudes from heterozygous mutant females, however, were not significantly different than those from wild-type preparations (e.g., $p = 0.28$ for pyrazine responses). Our inability to discern this difference may be due to the steps we employed to minimize recording noise; these methods introduced some variability in the EOG recordings, possibly obscuring any small quantitative differences (see Experimental Procedures).

To rule out the possibility that the severity of the knockout phenotype is influenced by genetic background or due to a second, unlinked mutation, we performed EOG recordings on F2 and F3 generation mice derived from successive back-crosses onto the FVB inbred background (see Experimental Procedures). From a total of 72 F2 generation mice, 14 displayed aberrant EOG responses typical of hemizygous mutants; all 14 of these animals were subsequently determined to have the (−/o) genotype (data not shown). Similarly, analysis of 25 F3 offspring revealed 4 pups that failed

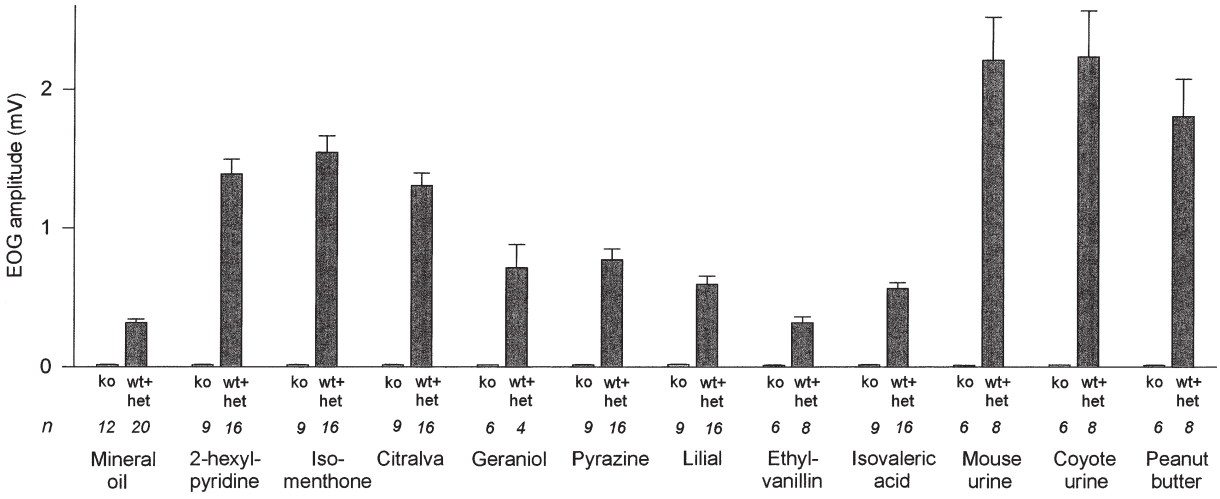

Figure 7. Quantitation of Negative EOG Response Amplitudes

Mean amplitudes of negative EOG responses were compared between hemizygous mutant mice (ko) and wild-type plus heterozygous mice (wt + het) derived on the inbred 129/Sv background. Each mean value is represented by a solid bar, with the standard error of the mean indicated by an error bar. Mineral oil response amplitudes were subtracted from responses in which odorants were diluted in mineral oil. No detectable negative EOG was observed in hemizygous mutant pups. The data for these animals are therefore expressed as an upper limit based on twice the mean peak-to-peak noise of the respective recordings (range: 0.012 to 0.018 mV). Since no significant difference in response magnitudes was observed between wild-type and heterozygous mutants (data not shown), these animals were considered as a single group. In all cases, the differences between hemizygous mutant responses and wild-type/heterozygote responses are statistically significant ($p \leq 0.0001$ for all odorants except geraniol, for which $p = 0.0008$). Sample sizes used to determine each value are indicated; a total of 32 mice were assayed, although not every odorant was tested on each mouse. Responses to triethylamine were excluded from this analysis owing to the presence of the non-neuronal positive EOG (see the text).

to show a negative EOG response to any of the odorants tested; all 4 were hemizygous (−/o) mutant males (data not shown). In summary, irrespective of genetic background, all 30 pups with the (−/o) genotype failed to exhibit a negative EOG, whereas each of the 99 wild-type or heterozygous mice always showed prominent odorant-evoked negative EOG responses. Taken together, our results demonstrate that the olfactory CNG channel is required for excitatory signaling in response to all of the odorants tested, including those which have been reported to elicit elevations in IP_3, but not cAMP (Boekhoff et al., 1990; Breer and Boekhoff, 1991).

Discussion

We have created a targeted mutation in the mouse olfactory CNG channel α subunit gene to assess the contributions of different second messenger pathways to olfactory signaling. Whereas the maturation, structure, and electrophysiological properties of olfactory neurons appear unaffected in mice lacking a wild-type CNG channel gene, profound deficits in olfactory function are observed. EOG recording, a method that measures the locally summated activity of neurons in the olfactory epithelium, reveals an absence of excitatory olfactory signaling in response to nine odorant compounds of diverse chemical structure and perceived odor quality. Of these nine compounds, previous in vitro studies have reported that four elicit increases only in cAMP in olfactory cilia (Sklar et al., 1986; Breer and Boekhoff, 1991), whereas five cause an increase only in IP_3 (Boekhoff et al., 1990; Breer and Boekhoff, 1991). We have also tested four complex mixtures as olfactory stimulants: mineral oil, which contains odorous hydrocarbons; mouse urine, with which mice can discriminate other mice from different genetic backgrounds (Yamaguchi et al., 1981); coyote urine, a potent aversive odorant cue signifying the presence of a predator (Nolte et al., 1994); and peanut butter, an effective bait for mouse traps (Davis, 1956). Mice hemizygous for the targeted mutation in the CNG channel gene fail to exhibit a detectable negative EOG in response to any of these complex stimuli. In addition, the mutant mice show extreme deficits in suckling, a behavior critically dependent on olfactory function (Teicher and Blass, 1977; Hudson and Distel, 1986; Risser and Slotnick, 1987). Considering the number and diversity of odorants tested, as well as the diversity of behaviors normally associated with the complex odorant cues, our results strongly suggest that the olfactory CNG channel is required for the transduction of most, if not all odorant stimuli. While future studies employing additional odorants as test stimuli will be required to extend and confirm this conclusion, it appears that mice lacking intact olfactory CNG channels are completely anosmic (i.e., they are unable to detect any odorant stimuli).

The Role of cAMP in Excitatory Olfactory Signal Transduction

Several independent approaches support the hypothesis that cAMP mediates odorant-evoked excitatory signaling in olfactory neurons. First, in vitro biochemical studies have demonstrated that many odorants elicit elevations in cAMP in isolated olfactory cilia preparations (Pace et al., 1985; Sklar et al., 1986; Breer et al., 1990). Second, adenylyl cyclase, G_{olf} (a $G_{s\alpha}$-like G protein

isoform), and the CNG channel are concentrated in olfactory cilia, the site at which odorant binding and receptor potential changes are thought to occur (Pace et al., 1985; Sklar et al., 1986; Jones and Reed, 1989; Kurahashi, 1989; Bakalyar and Reed, 1990; Lowe and Gold, 1991, 1993a; Kurahashi and Kaneko, 1993; Kleene et al., 1994). Third, a positive correlation is observed between the magnitude of adenylyl cyclase stimulation and the amplitude of the negative EOG response evoked by individual odorants (Lowe et al., 1989). The observation that some odorants do not elicit detectable elevations in cAMP in vitro (Sklar et al., 1986) probably reflects the low abundance of receptor cells that are activated by those odorants, rather than the involvement of other signaling pathways (see Lowe et al., 1989). Fourth, electrophysiological assays using isolated olfactory neurons have shown that the properties of odorant-induced ionic currents are remarkably similar to those of the olfactory cyclic nucleotide–gated conductance, suggesting that odorants cause changes in membrane potential through activation of CNG channels (Nakamura and Gold, 1987; Kurahashi, 1989, 1990; Firestein et al., 1991; Frings and Lindemann, 1991; Lowe and Gold, 1993a). Finally, in the present study, we demonstrate that mice lacking functional olfactory CNG channels fail to exhibit detectable excitatory responses to a wide variety of odorous stimuli, using EOG recording as our assay. These latter observations provide direct evidence that the CNG channel is required for excitatory olfactory signal transduction, and further suggest that cAMP is the sole second messenger mediating this process.

Do Multiple Second Messenger Pathways Mediate Olfactory Signaling?

IP_3 has been widely implicated as an intracellular second messenger subserving excitatory olfactory signal transduction. For example, some odorants have been reported to stimulate phospholipase C activity, but not adenylyl cyclase activity in isolated olfactory cilia (Huque and Bruch, 1986; Boekhoff et al., 1990; Breer and Boekhoff, 1991; Restrepo et al., 1993). Elevations in IP_3 have been proposed to generate a depolarizing current by activating a plasma membrane–associated IP_3-gated conductance (Restrepo et al., 1990; Kalinoski et al., 1992; Miyamoto et al., 1992; Cunningham et al., 1993; Okada et al., 1994; Schild et al., 1995). The proposed influx of calcium through this channel would also be expected to activate a calcium-dependent chloride current (Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993b; Zhainazarov and Ache, 1995), resulting in further membrane depolarization. However, numerous attempts have failed to identify an IP_3-gated conductance in olfactory neurons of sufficient magnitude to account for its contribution to olfactory signaling. These latter studies utilized intracellular dialysis of IP_3 in isolated olfactory neurons (Firestein et al., 1991; Lowe and Gold, 1993a; T. Kurahashi, personal communication) and application of IP_3 to inside-out patches of ciliary and dendritic membranes (Kleene et al., 1994; Nakamura et al., 1996; R. Kramer and S. Siegelbaum, personal communication; S. Frings, personal communication). Moreover, the absence of a negative EOG response in mice lacking functional olfactory CNG channels is inconsistent with a role for IP_3 as a mediator of excitatory olfactory signal transduction. While our data cannot rule out an IP_3-mediated response smaller than the baseline noise in our recordings, the magnitude of this noise (<0.01 mV) places an upper limit of $\sim$2% on the fraction of the EOG response that could be mediated by second messengers other than cyclic nucleotides. This upper limit is based on the ratio of twice the mean peak-to-peak noise in knockout traces to the mean amplitude of the wild-type responses to pyrazine. Responses to pyrazine were used for this calculation because pyrazine evokes the largest stimulation of phospholipase C of all odorants tested and would therefore be most likely to evoke an IP_3-mediated response (Boekhoff et al., 1990; Breer and Boekhoff, 1991). Such a small fraction is incompatible with the high frequency of cells reported to express the IP_3-gated channel (Kalinoski et al., 1992; Miyamoto et al., 1992; Cunningham et al., 1993; Okada et al., 1994).

Can the anosmic phenotype of the olfactory CNG channel knockout be reconciled with those studies implicating IP_3 as a second messenger in excitatory olfactory signaling? The observation that some odorants stimulate phospholipase C (Huque and Bruch, 1986; Boekhoff et al., 1990; Breer and Boekhoff, 1991; Restrepo et al., 1993) is consistent with, but does not prove the hypothesis that this enzyme pathway mediates excitatory signaling in olfactory neurons. Considering that our results argue against its direct role in excitatory transduction, perhaps phospholipase C serves instead to modulate the cAMP-mediated response of the olfactory neuron to odorant stimulation. For example, diacylglycerol, the other product of phospholipase C activity, may sensitize adenylyl cyclase via activation of protein kinase C (Frings, 1993) or desensitize the olfactory CNG channel directly, as described for the CNG channel expressed in rod photoreceptor cells (Gordon et al., 1995). It might also be proposed that IP_3-gated channels mediate a calcium influx that is too small to be detected as a current, but nevertheless can influence cAMP metabolism or the CNG channel due to their sensitivities to calcium (Anholt and Rivers, 1990; Borisy et al., 1992; Kramer and Siegelbaum, 1992; Chen and Yau, 1994). We find this possibility unlikely, however, since an influx of calcium through IP_3-gated channels in ciliary membranes would be expected to generate a depolarizing current by activating the calcium-dependent chloride conductance (Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993b; Zhainazarov and Ache, 1995); this scenario is inconsistent with our observation that olfactory CNG channel knockout mice fail to exhibit any odorant-evoked excitatory response.

Adaptation in olfactory neurons is mediated largely by calcium influx (Kurahashi and Shibuya, 1990). Since most of the inward current through the olfactory CNG channel is carried by calcium ions (Kurahashi and Shibuya, 1990; Frings et al., 1995), the properties of this channel are sufficient to account for both excitatory signaling as well as calcium-mediated modulation of olfactory signal transduction.

Experimental Procedures

Derivation of Mice Harboring a Targeted Disruption in the Gene Encoding the α Subunit of the Olfactory CNG Channel

A sequence encoding a portion of the mouse olfactory CNG channel α subunit gene was isolated by reverse transcriptase–PCR, using mouse olfactory epithelium RNA as a template. The PCR product, which corresponds to amino acids 289–560 of the rat olfactory CNG channel protein (Dhallan et al., 1990), was subcloned into pBluescript and subjected to DNA sequencing to confirm its identity. Genomic clones containing the mouse olfactory CNG channel α subunit gene were isolated from a mouse 129/Sv genomic library using the mouse CNG channel PCR clone as a probe. DNA fragments from the olfactory CNG channel α subunit gene were then used to construct a targeting vector in which the neomycin resistance gene (driven by the PGK promoter) is flanked by ~2 kb of genomic DNA as well as the herpes virus thymidine kinase gene on one side and ~1 kb of genomic DNA on the other side (see Figure 1B). Homologous recombination of the targeting vector with the olfactory CNG channel gene results in the replacement of channel coding sequence corresponding to amino acids 304–470 of the rat olfactory CNG channel (Dhallan et al., 1990) with the PGK–neomycin resistance gene.

ES cells derived from the 129/Sv strain of inbred mice (a gift from Dr. C. Stewart) were transfected with the targeting construct by electroporation and placed under double selection with G418 and FIAU (Ramirez-Solis et al., 1993). Clones were isolated and screened for homologous recombination by Southern blot analysis. Embryonic stem cell lines containing the mutant olfactory CNG channel gene were expanded and used to generate chimeric mice by aggregation with morulae derived from outbred CD1 mice (Stewart, 1993). A total of seven chimeric mice were obtained from aggregations using two independent cell lines.

Chimeras were mated with either 129/Sv or FVB female mice to produce F1 offspring. Genotyping of offspring was performed by Southern blotting as well as by PCR for the neomycin resistance gene on genomic DNA from F1 offspring. The following PCR primer pairs were used to detect the neomycin resistance gene: 5′-AGAGG CTATTCGGCTATGACTG-3′/5′-CCTGATCGACAAGACCGGCTTC-3′ or 5′-GTGTTCCGGCTGTCAGCGCA-3′/5′-GTCCTGATAGCGGTCC GCCA-3′. Pups were sexed by PCR using primers for the Y chromosome–specific SRY gene (5′-GAGAGCATGGAGGGCCAT-3′/5′-CCA CTCCTCTGTGACACT-3′; Gubbay et al., 1992).

One male chimera transmitted the mutation through the germline when crossed with wild-type females. To obtain F2 generation mice on the inbred 129/Sv background, heterozygous female F1 mice derived from crosses between the original chimera and 129/Sv females were mated with wild-type 129/Sv males. To obtain F2 generation mice on the FVB background, heterozygous F1 females derived from crosses between the chimera and FVB females were mated with wild-type FVB males. Females resulting from these latter crosses were then mated with wild-type FVB males to produce F3 generation mice on the FVB background.

In Situ Hybridization and Immunohistochemistry

Coronal sections were prepared from the snouts of 1-day-old F2 male pups that were derived on the FVB background. Localization of OMP and olfactory CNG channel α subunit RNAs was performed on 20 μm thick fresh frozen sections using digoxigenin-labeled RNA probes (Schaeren-Wiemers and Gerfin-Moser, 1993). For OMP, a rat cDNA was used as a template for probe synthesis (Dancinger et al., 1989); for the olfactory CNG channel, a probe was synthesized from a mouse sequence corresponding to amino acids 289–560 of the rat olfactory CNG channel coding region. Odorant receptor RNAs were localized within 20 μm thick fresh frozen sections using ^{33}P-labeled antisense RNA probes for mouse odorant receptors M50 and K4 (Ressler et al., 1993), essentially as described (Wilkinson et al., 1987).

Immunohistochemistry was performed on 10 μm thick frozen sections prepared from snouts that were fixed in 4% paraformaldehyde and cryopreserved in 30% sucrose prior to embedding. Tissue sections were reacted with rabbit polyclonal anti-peptide antibodies specific for type III adenylyl cyclase or G_{so}/G_{olf} (Santa Cruz Biotechnology, Incorporated) according to conditions recommended by the manufacturer. Specifically bound primary antibody was localized with a horseradish peroxidase–conjugated anti-rabbit IgG, followed by a chromogenic reaction using diaminobenzidine/H_2O_2 as a substrate.

Electrophysiology

One-day-old mouse pups were sacrificed by decapitation; each head was bisected through the septum with a razor blade to expose the medial surfaces of the olfactory turbinates; the right or left sides were chosen at random for electrophysiological recording. The remainder of each animal was frozen and later genotyped and sexed by PCR analysis, using primers for the neomycin resistance gene and the male-specific SRY gene; these analyses were performed blind with regard to phenotype. Odorant stimulation and EOG recording were carried out as described (Wang et al., 1993). Switching between humidified clean and odorized air streams was accomplished by a pneumatically actuated four-way slider valve. Odorized air was produced by blowing clean air through a horizontal glass cylinder that was half filled with the odorant solution. Peanut butter (Skippy Creamy) was spread on a piece of filter paper that covered most of the inside surface of the glass tube. Odorant concentrations are expressed as the concentration of odorant in the liquid phase contained within the evaporation tubes (see figure legend to Figure 6 for concentrations) and were chosen to produce as large a response as possible in wild-type mice without causing an excessively long recovery time. Each odorant was presented only once to a preparation with a 1 min interval between different stimuli. No difference in response amplitudes was observed if the interval was increased to 2 min. The mineral oil used as a diluent for water-insoluble odorants was deodorized by passing through two 50 cm long silica gel columns. This reduced the amplitude of the response to mineral oil by about 8-fold.

Recording stability was improved by placing a drop of saline on the epithelial surface prior to recording, and any excess was removed by aspiration. This procedure was repeated during the recording session if the baseline became unstable. Applying saline to the mucus layer probably introduced some variability in the thickness of the mucus layer, which may have also increased the variability of the EOG amplitude. The recorded signals were low pass filtered at 30 Hz and digitized at 125 Hz.

Cell-attached patch-clamp recordings (Hamill et al., 1981) were performed on individual olfactory neurons in tissue slices, using solutions as described (Lowe and Gold, 1993b). Recordings were low pass filtered at 2 kHz and digitized at 8 kHz.

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