The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger NCKX4 governs termination and adaptation of the mammalian olfactory response

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Sensory perception requires accurate encoding of stimulus information by sensory receptor cells. We identified NCKX4, a potassium-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, as being necessary for rapid response termination and proper adaptation of vertebrate olfactory sensory neurons (OSNs). Nckx4\textsuperscript{−/−} (also known as Slc24a4) mouse OSNs displayed substantially prolonged responses and stronger adaptation. Single-cell electrophysiological analyses revealed that the majority of Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} exchange in OSNs relevant to sensory transduction is a result of NCKX4 and that Nckx4\textsuperscript{−/−} mouse OSNs are deficient in encoding action potentials on repeated stimulation. Olfactory-specific Nckx4\textsuperscript{−/−} mice had lower body weights and a reduced ability to locate an odorous source. These results establish the role of NCKX4 in shaping olfactory responses and suggest that rapid response termination and proper adaptation of peripheral sensory receptor cells tune the sensory system for optimal perception.

Accurate encoding of spatial and temporal properties of sensory stimuli by peripheral sensory receptor cells is a prerequisite for accurate sensory perception. Tight and fast regulation of sensory transduction is necessary for the proper activation, termination and adaptation of sensory responses, with Ca\textsuperscript{2+} often having a crucial role in all three processes. In rod and cone photoreceptors, changes in cytoplasmic Ca\textsuperscript{2+} levels are responsible for regulating the sensitivity and dynamics of phototransduction to background light\textsuperscript{1}, whereas in vertebrate olfactory sensory neurons (OSNs), Ca\textsuperscript{2+} has dual but seemingly opposing roles in the signaling cascade\textsuperscript{2,3}. On odorant stimulation, Ca\textsuperscript{2+} enters OSN cilia through the olfactory cyclic nucleotide-gated (CNG) cation channel, which is opened via the olfactory G protein-mediated signal transduction cascade\textsuperscript{2,3}. Ca\textsuperscript{2+} in OSN cilia triggers a depolarizing Cl\textsuperscript{−} current, which serves as an amplification step for membrane depolarization\textsuperscript{4−6}. Ca\textsuperscript{2+} also adapts the transduction pathway, presumably by negatively regulating the activities of several transduction components, which leads to reduced sensitivity to repeated odor exposure\textsuperscript{7}. The time course over which ciliary Ca\textsuperscript{2+} accumulates and is removed influences not only the sensitivity but also the rates of activation and termination of the olfactory signaling pathway. Thus, proper regulation of ciliary Ca\textsuperscript{2+} dynamics should be crucial for encoding olfactory stimuli.

As OSN cilia do not contain intracilial vesicular organelles\textsuperscript{8}, Ca\textsuperscript{2+} homeostasis is believed to be achieved by plasma membrane Ca\textsuperscript{2+} transporters, including ATP-dependent Ca\textsuperscript{2+} pumps and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers\textsuperscript{2,9}. Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers are transmembrane proteins that harness the energy stored in the Na\textsuperscript{+} electrochemical gradient across the plasma membrane to actively transfer Ca\textsuperscript{2+} against its electrochemical gradient. There are three families of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers in mammals\textsuperscript{10}. The solute carrier 8 (SLC8) family contains three NCX proteins, which exchange three Na\textsuperscript{+} for one Ca\textsuperscript{2+}. The SLC24 family contains five NCKX proteins, which exchange four Na\textsuperscript{+} for one Ca\textsuperscript{2+} and one K\textsuperscript{+}. The CCX family contains one member, NCLX, which has not been fully characterized. Both NCXs and NCKXs are known to have crucial roles in regulating compartmental cytoplasmic Ca\textsuperscript{2+} in sensory receptor cells, particularly in vertebrate\textsuperscript{11} and Drosophila melanogaster\textsuperscript{12} photoreceptors. Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} exchange has been observed in OSNs electrophysiologically and by Ca\textsuperscript{2+} imaging\textsuperscript{13−17}. Inhibition of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in OSNs by replacement of extracellular Na\textsuperscript{+} results in prolonged receptor currents owing to a prolonged increase in levels of intracellular Ca\textsuperscript{2+} and continuous activation of the Cl\textsuperscript{−} channel. Low levels of extracellular Na\textsuperscript{+} also cause stronger adaptation, suggesting the importance of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers in regulating olfactory transduction\textsuperscript{13,14,17}. Expression of several Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers, including members of both the NCX and NCKX families, has been reported in OSNs\textsuperscript{9,15}. However, the molecular identity of the particular Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (or exchangers) involved in olfactory transduction is still undetermined. We identified NCKX4 as the principal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger that governs response termination kinetics and adaptation of the OSN, and that subsequently influences how odor information is encoded and perceived.

RESULTS

We previously conducted a proteomic screen of OSN ciliary membranes to identify previously unknown olfactory signaling components\textsuperscript{18}. In this screen, a single Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, NCKX4, was identified. To determine the expression pattern of Nckx4 in the olfactory epithelium, we performed in situ hybridization and found that Nckx4 mRNA is expressed specifically in the layer of mature OSNs (Fig. 1a). Consistent with these findings, previous microarray evidence...
indicated that Nckx4 was the only Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger to be enriched substantially in the olfactory epithelium19, and specifically in OSNs20. Together, these data implicated NCKX4 to be the leading candidate Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger for regulating the OSN response.

**Generation of Nckx4\textsuperscript{-/-} mice**

To determine the function of NCKX4 in the olfactory system, we generated mutant mice for the Nckx4 gene. We flanked exon 5, which encodes the first of two ion-exchanger domains, with loxP sequences. Cre recombinase-mediated deletion of this highly conserved exon also causes a frame shift in the remaining transcript sequence, and should therefore cause a fundamentally null mutation of NCKX4 (Supplementary Fig. 1a–c). Straight knockout (Nckx4\textsuperscript{-/-}) mice that were generated by early embryonic Cre expression were viable. The deletion of exon 5 from Nckx4 transcripts was confirmed by reverse transcription PCR (RT-PCR) using cDNA from olfactory epithelium of Nckx4\textsuperscript{-/-} mice (Fig. 1b).

We found that loss of NCKX4 did not affect the overall histology of the olfactory epithelium; the relative proportion of mature (olfactory marker protein (OMP) positive) and immature (growth-associated protein 43 (GAP43) positive) OSNs was unchanged, and no observable difference in OSN proliferation or death was detected, as assessed by 5-ethynyl-2′ deoxyuridine (EdU) nucleotide incorporation and activated caspase 3, respectively (Fig. 1c and Supplementary Fig. 1d). Furthermore, the cilia localization of the olfactory transduction components adenylyl cyclase III (ACIII) and the B1 subunit of the CNG channel (CNGB1b) were unaltered (Fig. 1d). We also found that the loss of NCKX4 did not affect the overall histology of the olfactory bulb; the total number of glomeruli remained unchanged (Supplementary Fig. 1e–f).

**Nckx4\textsuperscript{-/-} OSNs exhibit slowed response termination**

To analyze the effect of NCKX4 loss on OSN responses, we recorded the electroolfactogram (EOG), an extracellular field potential at the surface of the olfactory epithelium resulting from a summation of individual OSN responses.\textsuperscript{21,22} On stimulation with a brief (100 ms) odorant pulse, wild-type EOG signals show a rapid activation phase followed by a termination phase. The signals peak within several hundred milliseconds and recover to baseline within seconds.\textsuperscript{23,24} The peak amplitudes and activation kinetics of EOG signals reflect the overall sensitivity of OSNs. We found that Nckx4\textsuperscript{-/-} mice had peak EOG amplitudes equal to wild-type mice across all odorant concentrations to two commonly used odorants, heptanal (Fig. 2a,b) and amyl acetate (Supplementary Fig. 2a,b). We found that the response latency and the time to peak—two metrics for EOG activation kinetics—were unchanged in Nckx4\textsuperscript{-/-} mice (Fig. 2c–e). Similar results were seen for responses to amyl acetate (Supplementary Fig. 2c–e). Because both the response amplitude and the activation kinetics were unaffected in the mutant mice, NCKX4 is unlikely to control the sensitivity of OSNs in response to a single odorant exposure.

Although Nckx4\textsuperscript{-/-} mice showed normal sensitivity, they exhibited remarkably slowed termination kinetics of EOG signals (Fig. 2c,f and Supplementary Fig. 2c,f). The time constants (\(\tau\)) of the EOG termination phase, which were obtained by fitting the traces to a single exponential equation, were twofold to threefold longer in Nckx4\textsuperscript{-/-} mice than in their wild-type littermates. Furthermore, even Nckx4\textsuperscript{+/+} (heterozygous) mice showed significantly prolonged termination to stimulation at odorant concentrations near the EC\textsubscript{50} (HP 10\textsuperscript{-4} M, \(P = 0.025\); AA 10\textsuperscript{-4} M, \(P = 0.017\); Fig. 2c,f and Supplementary Fig. 2c,f), suggesting a gene dose-dependence for controlling the response termination rate.

We further analyzed the effect of NCKX4 loss on OSN response termination using single-cell suction recordings. The cell bodies of single dissociated OSNs were sucked into a recording pipette electrode with only the cilia and the dendritic knob exposed to a controlled extracellular solution.\textsuperscript{17} A phosphodiesterase inhibitor that increases cilia cyclic AMP levels, 3-isobutyl-1-methylxanthine (IBMX, 1 mM), was applied to mimic odorant stimulation and induced rapidly increasing receptor currents in both wild-type and Nckx4\textsuperscript{-/-} OSNs (Fig. 3a). IBMX has been widely used as a surrogate for odorants in OSN single-cell studies owing...
Figure 2  EOG analysis of Nckx4−/− mice. (a) Representative EOG traces to a 100-ms pulse of 10−3 M heptanal. The trace color codes also apply to b–f. (b) Dose–response relationships of EOG peaks evoked by single, 100-ms pulses of heptanal. For b–e: wild type, n = 9 animals; Nckx4−/–, n = 10; Nckx4−/−, n = 9. (c) Averaged EOG traces to a 100-ms pulse of 10−7 to 0.05 M heptanal. The traces were normalized relative to their peaks to allow comparison of activation and termination kinetics. (d) EOG activation latency, defined as the time from odor stimulation to 1% peak amplitude. (e) EOG rise time, defined as the time from 1% to 99% peak amplitude. (f) EOG termination rates. The termination time constant (τ) is determined by fitting a single exponential function to the termination phase of the EOG trace (y is the amplitude at time t, A is the initial amplitude, and c is an arbitrary vertical offset). For odor concentrations of 10−4 M and 10−3 M, n = 16 for wild type, n = 19 for Nckx4+/+, and n = 17 for Nckx4−/−. For other concentrations, n = 9 for wild type, n = 10 for Nckx4+/+, and n = 9 for Nckx4−/−. Error bars indicate 95% confidence intervals. Asterisks indicate P values for wild type versus Nckx4−/− (*P < 0.05, ***P < 0.001), and diamonds indicate P values for wild type versus Nckx4−/− (•, P < 0.05).

to the low probability of a given OSN being responsive to a given odorant. It has been shown that the termination kinetics of OSN response under IMBX stimulation closely resemble the kinetics under odorant stimulation25. In wild-type OSNs, the current in regular Ringer’s solution recovered to baseline in a short time period after IBMX removal (τRinger = 146 ± 13 ms, mean ± s.e.m.; Fig. 3a,b). By contrast, in Nckx4+/+ OSNs, the current recovered over a significantly longer time period (τRinger = 612 ± 53 ms, P < 0.001; Fig. 3a,b), consistent with the EOG data. Together, our results demonstrate that NCKX4 is necessary for efficient termination of the olfactory signaling cascade.

NCKX4 is the principal olfactory Na+/Ca2+ exchanger

We next examined whether, in addition to NCKX4, other Na+/Ca2+ exchangers also regulate OSN response termination. Single-cell suction recording allows precise inhibition of Na+/Ca2+ exchange17 by switching the extracellular bath solution to a Na+−free solution (Na+ was replaced equimolarly with choline+, which does not support Na+/Ca2+ exchange) to eliminate all Na+-dependent Ca2+ extrusion. When wild-type OSNs were switched to a Na+−free solution immediately after IBMX stimulation, the termination phase was greatly prolonged (τNa−free = 600 ± 71 ms; Fig. 3a,b, see also ref. 17). This prolonged current component is due to slowed Ca2+ extrusion, which results in a prolonged increase in cilial Ca2+ levels and continued opening of the Ca2+-activated Cl− channel14,26. By contrast, the already prolonged response termination phase of Nckx4−/− OSNs was prolonged further only slightly (albeit significantly, P < 0.001, see below) by removal of external Na+ (τNa−free = 646 ± 55 ms; Fig. 3a,b), suggesting that NCKX4 contributes the vast majority of Na+/Ca2+ exchange to regulate OSN response termination. Because response termination can vary widely between individual OSNs, we plotted τRinger/τNa−free for each OSN. The τNa−Free/τRinger ratio remained mostly constant among wild-type OSNs, as well as among Nckx4−/− OSNs (Fig. 3c).

We further calculated the τNa−Free/τRinger for each OSN and calculated

Figure 3  Single-cell analysis of Na+/Ca2+ exchanger-dependent response termination. (a) Single-cell suction electrode recordings from a dissociated wild-type (top panel) and Nckx4−/− (bottom panel) OSN stimulated with a 0.3-s pulse of IBMX. For each neuron, two recordings were performed: one with extracellular Ringer’s solution applied throughout the recording time period (black trace for wild type, red trace for Nckx4−/−), the other with a Na+−free Ringer’s solution applied during the termination phase of the OSN response (blue traces). (b) Termination time constants (see Online Methods). Error bars represent s.e.m.; ***P < 0.001 for wild type in Ringer’s solution versus all other categories. Cell numbers (n) are indicated in the bars. (c) Plot of the termination time constants in a Na+−free Ringer’s solution versus a standard Ringer’s solution for wild-type (black squares) and Nckx4−/− (red squares) OSNs. Each data point represents a single neuron. (d, e) Ca2+ dependency of termination rates in Nckx4+/+ OSNs. (d) Recordings from a wild-type (top) and Nckx4−/− (bottom) OSN stimulated with a 0.3-s pulse of IBMX. For each neuron, two recordings were performed: one in standard Ringer’s solution (containing 2 mM Ca2+), the other in low-Ca2+ Ringer’s solution (containing 20 μM Ca2+). The traces were normalized relative to their peaks for comparison of response kinetics. (e) The termination time constants are plotted for each genotype and condition. Error bars represent s.e.m.; N.S., not significant, P > 0.05; **P < 0.01, ***P < 0.001. Cell numbers (n) are indicated in the bars.
the average for all wild-type and Nckx4−/− OSNs. Exposure to Na+-free solution prolonged the response 4.5 ± 0.6 (mean ± s.e.m.)-fold in wild-type OSNs but only 1.08 ± 0.03-fold in Nckx4−/− OSNs (P < 0.001). The slight response prolongation on Na+ removal in Nckx4−/− OSNs was statistically significant (P < 0.001), indicating that a small contribution of another Na+-dependent Ca2+ extrusion mechanism (or mechanisms) remains in Nckx4−/− OSNs.

Previously, it has been shown that prolonged Ca2+ transients in OSNs extend activation of the Ca2+-activated Cl− channel. To determine whether the effects of prolonged termination in the Nckx4−/− OSNs were indeed dependent on Ca2+, we performed single-cell suction electrode recordings in the presence of regular levels of Ca2+ (2 mM) or low levels of Ca2+ (20 µM) in the extracellular solution. This low concentration of Ca2+ was chosen so that Ca2+ influx would be minimized but would not be low enough to entirely unblock the CNG channel, which would yield large Na+ currents and fast cell deterioration. In wild-type OSNs, the termination τ in the regular Ringer’s solution (τ = 150 ± 20 ms) and in 20 µM extracellular Ca2+ solution (τ = 156 ± 30 ms) were comparable (Fig. 3d,e). This result is consistent with the notion that NCKX4 allows an efficient removal of ciliary Ca2+ and allows a normal rate of termination under a broad range of Ca2+ concentrations. In Nckx4−/− OSNs, the response displayed prolonged termination in comparison to wild-type OSNs both in the regular Ringer’s solution (τ = 600 ± 90 ms) and in the 20 µM extracellular Ca2+ solutions (τ = 288 ± 40 ms), but the termination τ in the 20 µM Ca2+ solution was significantly shorter than that in the regular Ringer’s solution (P < 0.001; Fig. 3d,e). The less-severe defect in 20 µM Ca2+ solution in Nckx4−/− OSNs is consistent with the idea that there is a smaller amount of Ca2+ entering the cilia and activating the Cl− channel. Although the amount of Ca2+ entry is reduced in low Ca2+ conditions, NCKX4 is still required to ensure a fast response termination. Together, these results demonstrate that NCKX4 is necessary for nearly all Na+-dependent Ca2+ exchange that governs OSN response termination, probably by removing ciliary Ca2+ and thus closing the Ca2+-activated Cl− channel.

Nckx4−/− OSNs over-adapt to repeated odorant exposure

As Ca2+ mediates olfactory adaptation, we suggested that NCKX4 is required for OSNs to adapt properly to odor. To test this hypothesis, we performed EOG recordings using a paired-pulse protocol, where the olfactory epithelium was stimulated with two equal 100 ms odorant pulses. In wild-type mice, the response to the second pulse was reduced relative to the first, signifying that the OSNs were adapted (Fig. 4a–c and Supplementary Fig. 3a–c). In Nckx4−/− mice, the response to the second pulse was even further reduced, indicating that Nckx4−/− mice exhibited enhanced OSN adaptation (Fig. 4a–c and Supplementary Fig. 3a–c). In wild-type mice, the reduction of the second response largely recovered if the interpulse interval was lengthened to 4 s or beyond, whereas in Nckx4−/− mice, significant reduction of the second response was observed even when the interpulse interval was extended to 15 s (P < 0.001; Supplementary Fig. 3e,f). In addition to an amplitude reduction, another manifestation of adaptation is slower onset kinetics of the EOG signal. Using the metric of onset kinetics, the same phenomenon was seen: OSNs adapt to a greater extent in Nckx4−/− mice relative to wild-type mice (Fig. 4d and Supplementary Fig. 3d). As was seen for the termination kinetics, there was a gene dose-dependent effect of NCKX4 on adaptation. Nckx4+/− mice (heterozygotes) showed an adaptation enhancement compared to wild-type mice, but this enhancement was less pronounced in Nckx4−/+ mice than in Nckx4−/− mice (Fig. 4a–d and Supplementary Fig. 3a–d).

We further characterized the effects of NCKX4 loss on OSN adaptation using single-cell suction pipette recordings. In these experiments, pairs of 1 s IBMX (1 mM) stimulations were given with varied interpulse intervals (Fig. 4e). Adaptation was assessed as a reduction in peak current to the second pulse relative to the first pulse. In wild-type and Nckx4−/− OSNs, the effects of adaptation were strongest when the interpulse interval was shortest and became weaker as the interval was lengthened (Fig. 4f). In Nckx4−/− OSNs, the adapted responses were smaller than those observed in wild-type OSNs for each tested interval, indicating over-adaptation. Wild-type OSNs recovered from adaptation within 4 s after the first pulse (Fig. 4f). In Nckx4−/− OSNs,
however, the effects of adaptation persisted even when the interpulse interval extended beyond 10 s (Fig. 4f). Together, these results demonstrate that NCKX4 activity moderates the extent of, and supports recovery from, adaptation to repeated stimulation.

**Nckx4−/− OSNs fire fewer action potentials when adapted**

In OSNs, the odor-evoked receptor potential is further transduced into action potentials, which usually occur during the activation phase of the receptor current\(^7\). Given that Nckx4−/− OSNs displayed slower response termination and stronger adaptation, we asked how this altered receptor potential might affect the encoding of action potentials.

We analyzed the single-cell paired-pulse recordings under a filter setting that allows action currents to be observed (Fig. 5a). Wild-type OSNs quickly regained full ability to generate action potentials when the interpulse period was lengthened; by 0.25 s after the first stimulation, ~45% of OSNs were able to fire action potentials to a second stimulation, and by 2 s, 100% of OSNs fired action potentials (Fig. 5b). By contrast, Nckx4−/− OSNs did not generate any action potentials to the second stimulation until the interpulse interval reached 1 s and never gained 100% firing probability even after a 10 s interval (Fig. 5b). These results demonstrate that NCKX4 poises OSNs to relay odor information under adapting stimulation.

**Nckx4−/− mice exhibit a reduced ability to locate odors**

Given that Nckx4−/− mice displayed a defect in relaying odor information from repeated stimulation, we investigated whether the loss of NCKX4 affects the ability of mice to perceive odors. We initially observed that Nckx4−/− mice weighed less than their wild-type littermates (Fig. 6a,b). In mice, olfaction is required for pups to suckle. Reduced body weight is a hallmark that is characteristic of olfactory defects in mice owing to impaired ability to locate the teat: anosmic mice die in the first few postnatal days unless special care is provided\(^27–29\). Nckx4−/− mice were viable through weaning without special care, thus suggesting that they retain at least some functional olfactory perception. But consistent with an olfactory impairment, they weighed the same as their wild-type littermates at birth and developed defects in body weight during the nursing period. Their body-weight defects were most pronounced at the time of weaning (21–28 days after birth) and became progressively less pronounced through 4 months of observation (Fig. 6b). To determine whether the body-weight reduction was more likely to be due to an olfactory defect rather than to pleiotropic effects, we generated olfactory-specific knockout mice in which Nckx4 was conditionally knocked out by the expression of Cre recombinase under the control of the OMP promoter\(^30\). OMP shows restricted expression in sensory neurons of the main olfactory epithelium and of other olfactory subsystems. EOG recordings of these conditional Nckx4-knockout mice (Nckx4\(^{loxP/loxP}^{OmpCre}\)) showed response defects that were

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**Figure 5** Single-cell analysis of action potential generation in Nckx4−/− OSNs. (a) Single-cell suction electrode recordings from a Nckx4\(^{+/+}\) (top) and a Nckx4−/− (bottom) OSN stimulated with paired 1-s pulses of IBMX (1 mM) separated by a 1-s interval. Traces were filtered with the wide bandwidth of DC to 5,000 Hz to monitor action potential firing. Action-potential currents are indicated by arrows. (b) Percentage of OSNs firing action potentials, plotted against the interpulse time. Error bars indicate 95% confidence intervals; N.S., not significant, \(P > 0.05\); **\(P < 0.01\); ***\(P < 0.001\).

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**Figure 6** Effects of NCKX4 loss on olfactory-mediated behaviors. (a) Body size of a Nckx4−/− mouse relative to a Nckx4\(^{+/+}\) mouse at 24 days postnatal age. (b) Time course of average body weights for Nckx4\(^{+/+}\) (n = 10–54), Nckx4\(^{−/−}\) (n = 17–85) and Nckx4\(^{−/−}\) (n = 5–31) mice before (left) and after (right) weaning. Error bars indicate 95% confidence intervals. Statistical significance for Nckx4\(^{+/+}\) versus Nckx4−/− mice is indicated over each time point: N.S., not significant, \(P > 0.05\); *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\). (c) Average body weights for mice of all combinations of Nckx4 (+ or \(^{loxP}\), indicated by +) and Omp (+ or Cre) alleles at 24 days postnatal age. The red color highlights the olfactory conditional Nckx4 knockout (Nckx4\(^{loxP/loxP}^{OmpCre}\)). Error bars indicate 95% confidence intervals. All genotypes weighed significantly more than the double knockout (\(P < 0.001\)). All other pairwise \(P\) values that are less than 0.05 are also indicated. Mouse numbers (n) are indicated in the bars. (d) Buried food pellet test for Nckx4\(^{+/+}\); Omp\(^{Cre}\) and Nckx4\(^{loxP/loxP}^{OmpCre}\) mice (n = 20 mice per genotype). The average time to locate the food pellet is plotted against the trial day. On day 6, the pellet was left on the surface of the bedding to be visible to the mouse. Error bars indicate 95% confidence intervals. See Supplementary Movie 1 for a video of both genotypes’ behavior in this task.
similar to those found in the straight-knockout (Nckx4−/−) mice (data not shown). We found that the reduced-body-weight phenotype persisted in these conditional knockout mice (Fig. 6c).

To further test whether slower response termination and enhanced adaptation of OSNs affect the ability of mice to perceive odors, we conducted the ‘buried food pellet test’. In this test, food-restricted wild-type mice learned to rapidly locate a buried food pellet, showing daily performance improvements through 5 trial days (Fig. 6d and Supplementary Movie 1). By contrast, most olfactory conditional Nckx4-knockout mice failed to locate the buried pellet in the allotted 200 s (Fig. 6d and Supplementary Movie 1). Over the course of 5 trial days, these conditional Nckx4-knockout mice showed no substantial improvements in their ability to locate the pellet. On trial day 6, when the food pellet was made visible by placing it on the surface, both wild-type and olfactory conditional Nckx4-knockout mice rapidly located the pellet in a similar time frame, controlling for possible secondary locomotor, motivational or cognitive defects. These results demonstrate that NCKX4, which confers rapid olfactory response termination and moderates OSN adaptation, is necessary for mice to optimally locate odorous sources.

DISCUSSION

In this study, we identified NCKX4 as the principal Na+/Ca2+ exchanger that allows rapid response termination and proper adaptation of the OSNs, presumably by mediating Ca2+ extrusion from OSN cilia. Furthermore, by modulating the peripheral olfactory response, we showed that NCKX4 is crucial for an animal to accurately encode and react to olfactory stimuli in the environment.

NCKX4 is a component of the olfactory transduction pathway

NCKX4 was initially chosen as the leading candidate for the olfactory Na+/Ca2+ exchanger on the basis of our previous proteomic analysis, which aimed to identify previously unknown olfactory transduction components.18 Historically, molecular identification of many olfactory transduction components was first deduced by the abundance and localization of mRNAs through the use of either cDNA library screening31–35 or PCR-based approaches36–38. Many of these components were later functionally confirmed by gene-knockout experiments in mice32,28,29,39–41. By directly detecting the proteins at the site of their predicted function, cilial membrane proteomics have proven fruitful in identifying the remaining long-sought olfactory transduction components, including the olfactory Ca2+-activated Cl− channel Anoctamin 2 (ANO2)18 and, here, the olfactory Na+/Ca2+ exchanger NCKX4 (Supplementary Fig. 4).

The Nckx4 gene was first cloned from human and mouse brains42, and the NCKX4 protein was shown to display K+-dependent Na+/Ca2+ exchanger activity43,44. However, no specific physiological role has been definitively attributed to NCKX4. Through loss-of-function analysis, this current study defines a physiological role for NCKX4 in olfactory transduction. We showed that NCKX4 is needed to allow rapid olfactory termination and to prevent over-adaptation to repeated stimulation. These effects of NCKX4 are most likely due to the Ca2+ transporter activity of NCKX4. However, one cannot exclude the possibility that NCKX4 may have additional functions in the olfactory system. Future technological improvements that allow imaging of Ca2+ dynamics in mammalian olfactory cilia will further clarify the precise function of NCKX4 in olfactory transduction.

It has been suggested that active extrusion of Ca2+ from olfactory cilia is mediated by both Na+/Ca2+ exchangers and plasma membrane Ca2+ ATPases (PMCA). Na+/Ca2+ exchangers have been favored as a mechanism for extruding Ca2+ from OSNs for olfactory transduction13,14,17. Na+/Ca2+ exchangers have a much greater capacity than PMCA to extrude Ca2+ (ref. 44), and are thus better suited for rapidly reducing intraciliary Ca2+ to fulfill the need for rapid OSN response kinetics. In comparison to NCX proteins, NCKX proteins harness additional driving force for Ca2+ by coupling the transport of an additional Na+ and a K+, which can maintain the driving force for Ca2+ removal even at depolarized membrane potentials and can allow the reduction of Ca2+ to nanomolar levels55,46. NCKX4 is therefore well suited for its role in regulating olfactory responses.

NCKX4 regulates the olfactory response

The loss of NCKX4 could influence the sensitivity of the OSN response positively by increasing Ca2+-activated Cl− current, negatively by increasing phosphodiesterase 1C (PDE1C) activity23, negatively by increasing (inhibition of the CNG channel and negatively by loss of the electrogenic effect47. However, we found that both the amplitude and activation kinetics of the EOG response were not changed in Nckx4−/− mice (Fig. 2). This unchanged sensitivity in Nckx4−/− OSNs could result from a cancellation of these positive and negative effects. Alternatively, NCKX4-mediated Ca2+ extrusion might not affect these mechanisms until after the response has peaked, so that the loss of NCKX4 does not alter response sensitivity to a brief odorant exposure.

A pronounced effect of NCKX4 loss is stronger OSN adaptation (Figs. 4 and 5; see also Supplementary Discussion). This result provides in vivo demonstration that Ca2+ mediates olfactory adaptation. Although it is thought that Ca2+-mediated adaptation is achieved by negative feedback of Ca2+ on olfactory signaling components, the exact molecular target (or targets) of this feedback remain to be determined24.

The most pronounced effect of NCKX4 loss on the olfactory response is a prolonged termination phase (Fig. 2). Loss of one Nckx4 allele (Nckx4+−) caused a mild prolongation, whereas loss of both Nckx4 alleles (Nckx4−/−) caused a severe prolongation. Termination of the OSN response requires the closure of both the CNG channel and the ANO2 channel. Degradation of cAMP by PDE1C, which leads to closure of the CNG channel, was once thought to determine the rate of response termination. However, Pde1c−/− mice do not show a prolonged olfactory response, indicating that regulation of cAMP dynamics does not dominate the rate of response termination23. The slower termination seen in Nckx4−/− mice could be best attributed to a slower clearance of Ca2+ from OSN cilia, and thus prolonged activation of the Cl− current (however, see ref. 48). Together, these studies establish that regulation of Ca2+ dynamics, rather than degradation of cAMP, has the leading role in determining the rate of OSN response termination.

Olfactory behavior requires controlled response kinetics

It has always been assumed that rapid termination of peripheral sensory responses is necessary to prepare animals to sense recurring stimulation. However, in the olfactory system this assumption has never been fully testable owing to the fact that most mutations in core and modulatory signal transduction components cause either complete loss26,29,41 or severe reductions in the sensitivity of the OSN response23,39,49. This loss or reduction of OSN sensitivity precluded investigations and confounded interpretations of whether alterations in peripheral response properties affect the ability of animals to encode and react to sensory stimuli.

Nckx4-knockout mice, which display defects in response termination and adaptation but have unchanged sensitivity, provided a unique opportunity to address this question. We found that defects in termination and adaptation of the OSN receptor potential led to a defect in encoding action potentials (Fig. 5). Further, olfactory-specific Nckx4-knockout mice displayed lower body weights, which is consistent with a nursing defect as a result of impaired olfactory function (Fig. 6c and Supplementary Discussion). Finally, these mice were less efficient at
performing a food-finding task (Fig. 6d). Thus, by identifying NCKX4 as a regulator of OSN responses, these studies suggest that rapid response termination and proper regulation of adaptation in OSNs are essential for the proper encoding of and reactions to olfactory stimuli.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

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AUTHOR CONTRIBUTIONS

A.B.S. and H.Z. designed and performed the initial experiments to identify NCKX4. A.B.S. generated the targeted deletion of Nckx4 in mice and designed and conducted the in situ hybridizations, immunostaining, EOG analyses and behavioral analyses. S.T. set up crosses and weighed the conditional knockout mice, performed the olfactory bulb neural cell adhesion molecule staining and performed the adaptation time course EOG measurements. J.R. designed and performed the single-cell recordings. M.D. performed the Ca2+-free single-cell recordings. C.M.W. analyzed glomerular formation using antibodies to odorant receptor (data not shown). A.B.S., H.Z. and J.R. wrote the initial manuscript draft. All authors discussed the results and the contents of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Online Methods

Animals. For all experiments involving mice, the animals were handled and killed in accordance with methods approved by the Animal Care and Use Committees of Johns Hopkins University and the Monell Chemical Senses Center. Unless otherwise noted, all analyses involving animals were performed on adult (>40-d-old) mice.

Generation of Nckx4−/− mice. The 85-bp exon 5 of the Nckx4 gene on chromosome 12 was selected to be flanked by loxp sites, with a 4.0 kb fragment upstream and a 5.2 kb fragment downstream of this exon used as homologous arms for embryonic stem (ES) cell gene targeting. pBluescript KS (Clontech) was PCR amplified with primers ACTGGGCCCTGGTACAGGGCTTACTAAGGGAGAGGAGCAGAAGGTGATCACAGCTGTAGGGA and CTCAAAAATAAAGGGGAAACAATGTAGGAGAAACATCTGATAGAGAATCTGTAACAGGCTG, which contain 50 bp homologous arms (underlined) for the Nckx4 genomic DNA, and the resulting PCR product was used to retrieve the genomic DNA from bacterial artificial chromosome (BAC) RP135-11 (Children’s Hospital Oakland Research Institute BACPAC Resource Center) in SW102 cells using a cell-based recombineering procedure. A loxp–neomycin resistance cassette–loxp sequence amplified from plasmid pL452 (ref. 50) with primers CCGTGGCAGGCTCTAGCCATCTGCTCTGCTAGGAGCTTGTCCGATCCGCAACATTGCAATATTCG and AGGAGACAGCGCAGCTCCGGCAGGGTGCTCTACTAGAAGAGGTGG, which contain 50 bp homologous targeting arms, was recombined into the 5′ to exon 5 in SW102 cells. The neomycin cassette was removed by arabinose-inducible Cre recombinase expression in SW106 cells, leaving a single loxp sequence upstream of exon 5. A flippase recognition target (Frt)–neomycin resistance cassette–Frt–loxp sequence amplified from plasmid pL451 (ref. 50) with primers ATGGCAGAGAGCCAGGGGCTTACGGGCGACAGTTACGGGAGGTTAGAACCATCTGATATCCGAGAAGGCTGTACAGGTGTAGGGA and CTCAAAAATAAAGGGGAAACAATGTAGGAGAAACATCTGATAGAGAATCTGTAACAGGCTG, which contain 50 bp homologous targeting arms, was recombined into 3′ to exon 5 in SW102 cells. The targeting vector was assessed by extensive restriction digestion and sequencing analysis.

ES cell gene targeting was performed in a 129/SvEv ES cell line (MC1 line, Johns Hopkins University transgenic core). Three hundred and eighty-four independent colonies were screened for homologous recombination using a phosporylase kinase (pgk) promoter-specific primer CTACAATCTTTCGCTACTAGT and upstream primer CTACAATCTTTCGCTACTAGT, which were confirmed with homologous combination: 1A12 and 2B2. Each ES cell clone was injected into blastocysts (Johns Hopkins University transgenic core facility). The chimera with the highest fraction of agouti fur color and positive PCR for the mutant allele. Only ES cell clone 2B2 resulted in germline transmission. For germline (straight) knockouts, the F1 heterozygote mice were mated to animals from the mouse line B6.FVB-Tg(FIa-e)creCS579Lingd/J (The Jackson Laboratory), which express Cre recombinase at an early stage in the embryo. The F2 mice were back-crossed with C57BL/6 mice to eliminate the Cre allele, and then the F3 mice were inbred to obtain homozygous animals. The F2 mice were back-crossed with C57BL/6 mice to eliminate the 5′ exon. The targeting vector was assessed by extensive restriction digestion and sequencing analysis.

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RT-PCR analysis of olfactory epithelium RNA. Total olfactory epithelial RNA was isolated using TRIzol Reagent (Invitrogen). cDNA was made by reverse transcriptase reaction using total RNA using random decamer primers (Ambion). To amplify each specific gene, lower PCR cycles were used to preserve semi-quantitative information regarding relative transcript abundance. The primers used for Nckx4 were TCCAAACAAAGAAGCAGCA and GTTCCTCACCCGCACACTTGC (245-bp product with exon 5, 160 bp without exon 5). The primers used for Cog2 were TGCCCTTAAAGGGGCCATGTG and AGACAGCTCCTAATCTTGCGGTGTC (596-bp product). The primers used for Ano2 were AGTCTACCTGTGGTACATCCGAG and CAGGTGTTGTTGAAGTCACA (233-bp product).

In situ hybridization. The Nckx4 probe template was PCR amplified from mouse olfactory epithelial cDNA with primers TACACTCTAGCGTCTGATTTGGC TCGATTCG and AGATGGCCCGCCCGTGAACACCATCGTCCGGCT, and cloned into pBluescript KS(+) (Clontech). This 655-bp fragment of Nckx4 was chosen to minimize cross-reactivity of the probe with other Nα/Gα2 genes. Antisense and sense digoxigenin-labeled RNA probes were then generated by transcription from the T3 and T7 promoters. The in situ hybridization was performed on 14-µm-thick olfactory epithelium cryosections.

Immunohistochemistry. The olfactory epithelium and olfactory bulb tissues were fixed in 4% (wt/vol) paraformaldehyde at 4 °C followed by cryoprotection in 30% (wt/vol) sucrose. The tissues were cut into 18-µm-thick coronal cryosections (olfactory epithelium) and 20-µm-thick coronal cryosections (olfactory bulb). Sections were incubated at 4 °C overnight with primary antibodies in phosphate-buffered saline (pH 7.4) containing 0.1% (vol/vol) Triton X-100 and 1% (vol/vol) goat serum. Primary antibodies were used at the following dilutions: anti-OMP (chicken antibody, provided by Q. Gong, University of California, Davis), 1:1000; anti-GAP43 (Chemicon MAB347), 1:500; anti-AICIII (Chemicon AB3403), 1:200; anti-CNGB1β, 1:300; anti-NCAM (Developmental Studies Hybridoma Bank (DSHB), 588 supernatant), 1:1. After washing, the sections were incubated with fluorescent secondary antibodies, mounted in Vectashield (Vector Labs) containing DAPI stain and imaged.

To assess cell death, 30-µm-thick sections were stained with anti-cleaved caspase 3 antibody (Cell Signaling Technology #9661) at a dilution of 1:200 as the primary antibody. The activated caspase 3 staining was performed on 28-day-old mice.

Cell proliferation assay. Mice were injected with 125 µg EdU (Invitrogen) every 2 h for 12 h (7 injections total). Two hours after the final injection, the tissue was fixed and cryoprotected as described above. Olfactory epithelium tissue was cut into 30-µm sections. EdU-labeled cells were detected using Click-it EdU Alexa Fluor 488 Imaging Kit (Invitrogen). Sections were mounted in Vectashield containing DAPI stain and imaged. The EdU labeling was performed on 28-day-old mice.

EoG recording. Mouse EoG recordings were performed as described. Adult mice, aged 3–5 months, were used. Heptanal and amyl acetate were diluted in DMSO (Sigma) to result in a 50× stock solution series. The stock solutions were then diluted to 1 in water to 5 ml final volume in a sealed 60 ml glass bottle to generate vapor-phase odorant. EoGs were recorded from a consistent position on turbinate IIB. One set of mice was used in single-pulse dose–response recordings. Another set of mice was used for single, paired-pulse and extended-pulse recordings at 10−4 and 10−3 M odorant concentrations. The data were collected and analyzed using AxoGraph Software (Axon Instruments) at a sampling rate of 1 kHz. All recordings were digitally filtered at 25 Hz before analysis. For measuring termination time constants, the time windows used for the fit were 2.5–4 s for 10−2 M odorant, 2.4–4 s for 10−3 M, 2.4–4.5 s for 10−4 M, 2.4–10 s for 10−5 M, 2.4–15 s for 10−6 M and 2.4–20 s for 10−7 M.

Single-cell suction recording and analysis. The suction pipette technique was used to perform single OSN recordings. The cell body of an isolated OSN was sucked into the tip of a recording pipette, leaving the cilia and the dendritic knob (but not the cell body) accessible for solution changes. Solution exchanges were achieved by transferring the tip of the recording pipette across the interface of neighboring streams of solution using the Perfusion Fast-Step SF–77B solution changer (Warner Instruments). As the intracellular voltage is free to vary
in this recording configuration, the recorded current has two components: fast biphasic current transients resembling action potentials and the slower receptor current. Currents were recorded with a PC-501A patch clamp amplifier (Warner Instruments) and digitized using a Micro1401 data acquisition unit and Signal acquisition software (Cambridge Electronic Design). Suction currents were sampled at 10 kHz. The recorded signals were filtered from DC to 50 Hz to display the suction current alone, and filtered from DC to 5,000 Hz to display action potential currents. Mammalian Ringer’s solution contained (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 0.01 ethylenediaminetetraacetic acid (EDTA), 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 glucose. The pH was adjusted to 7.5 with NaOH. All chemicals were purchased from Sigma. Experiments were performed at 37 °C.

The decay time constant was calculated by integrating the current generated after cessation of IBMX exposure. This overall charge was normalized to the current at the cessation of IBMX exposure. The obtained time constant is mathematically equivalent to the time constant obtained from a single exponential decay (in cases in which the current decay follows a single exponential decay).

Body mass measurement. Mouse pups were weighed daily to an accuracy of 10 mg. After weaning, mice were weighed weekly to an accuracy of 100 mg. All weight measurements were carried out at the same time of day.

Buried food pellet test. The buried food pellet test was performed using adult Nckx4+/+;OmpCre/+ (n = 20) and Nckx4fl/fl;OmpCre/+ (n = 20) mice at Zietgeber time 7–12. All animals were housed individually with wood-shaving bedding for the duration of the experiment with free access to water. Mice were deprived of food for 24 h before the experiment and subsequently restricted to 2.5 g of Rodent Chow (Harlan Teklad) per day. The testing chambers were clean cages of dimensions 30 × 19 × 13 (L × W × H, in cm) filled with ~800 cm³ of wood-shaving bedding. Two 40–60 mg pieces of Oreo Cookies (Nabisco) were buried just below the surface of the bedding in the following manner. The cage area was designated into halves length-wise. One cookie piece was buried in a randomized location in the left half, the other in the right half. Including two pellets in the experiment reduced the effective search area by half and better controlled for variance in the depth at which the pellet was buried. In a single trial, a mouse was placed in the center of the cage and was given 200 s to locate either of the two pellets. Latency in finding the first pellet was recorded when the mouse touched the pellet. After the mouse located the first pellet, it was allowed to consume the cookie, and was given another 200 s to locate the second pellet. If a mouse failed to find a pellet in the allotted 200 s, the cookie pellet was exposed and presented to the mouse for subsequent consumption. After the trial, each mouse was returned to its respective cage. Mice were tested in a single trial per day for 5 consecutive days. The testing order of the animals was randomized for each day. Fresh bedding was used for each mouse, each day.

Statistical analyses. For the comparison of percentage of OSNs firing action potentials (Fig. 5b), the P values were calculated using a chi-squared test. For all other comparisons that only involved wild-type mice versus Nckx4−/− mice, P values were calculated with Student’s t test at each odorant concentration or time point. When three or more genotypes were compared, pairwise P values were calculated with the Tukey-Kramer ANOVA post-test at each odorant concentration or time point. Statistical analyses were performed using R (http://www.r-project.org/).

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