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Research article

Olfactory marker protein contributes to the evaluation of odour values by olfactory glomerular processing

Akiko Nakashima [a], Taku Nakagawa [a,b], Makoto Takano [a], Noriyuki Nakashima [a,*]

[a] Department of Physiology, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka, 830-0011, Japan
[b] Department of Anaesthesiology, Graduate School of Medicine, Kyushu University, Fukuoka, 812-8582, Japan

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ABSTRACT

Olfaction starts from olfactory receptor neurons (ORNs) that express olfactory marker protein (OMP). OMP deficit results in various behavioural phenotypes indicating olfactory dysfunction due to the impaired responses of ORNs. Recently, OMP was demonstrated to maintain strong olfaction by buffering olfactory cAMP signalling. However, the impact of OMP on olfaction behaviours, the assessment of which requires time to evaluate odour values, remains largely unexplained.

Here, we examined the behaviour of heterozygous *OMP*$^{+/GFP}$ (HET) mice vs. homologous GFP-knock-in OMP-deficient *OMP* $^{GFP/\ GFP}$ (KI) mice during the olfactory investigation of odours with different values. When a swab containing an organic odour was presented, both HET and KI mice swiftly approached and investigated the swab with gradual habituation over test sessions. However, when another similar odour was presented, KI mice investigated the new swab much less intensively than HET mice. Next, mice were placed in a chamber with an aversive odour source in one corner of a test chamber. KI mice more frequently approached the compartment containing the aversive odour source than HET mice. Finally, we trained mice to associate two odours with solutions by utilizing reward-penalty values. HET mice stayed close to the reward-associated odour, while KI mice initially approached the reward-associated odour, occasionally turned towards the penalty-associated odour source and eventually stayed in the reward-odour compartment. Histologically, c-Fos-expressing juxtaglomerular cells were fewer and more broadly distributed around glomeruli in KI mice than HET mice.

In conclusion, OMP contributes to the evaluation of odour values by glomerular processing during an olfactory investigation task.

1. Introduction

Olfaction starts with olfactory receptor neurons (ORNs), utilizing cAMP as a second messenger [1]. The investigation of olfaction has been facilitated by the discovery of olfactory marker protein (OMP), which labels peripheral mature ORNs [2–5]. Despite its utility as a genetic marker for ORNs, the roles of OMP in olfaction remain largely unexplained.

Each ORN expresses one odorant receptor (OR) out of hundreds of members of the OR gene superfamily [6], while OMP is expressed ubiquitously in ciliated ORNs [7–9]. Thus, OMP has been inferred to operate in common physiological processes for olfaction within ORNs regardless of OR type.

Close examinations have revealed that OMP-deficient mice show various olfactory dysfunction-related phenotypes. Behaviourally, OMP-deficient mice show impaired odour discrimination ability [10–12]. ORNs present prolonged responses mediated by Ca^{2+}-permeable cyclic nucleotide-gated (CNG) channels and delay Ca^{2+} extrusion via the Na^{+}-Ca^{2+} exchanger [13–17]. Based on these findings, OMP has been proposed to participate in the regulation of olfaction sensitivity, possibly due to its interaction with olfactory adenylate cyclase or phosphodiesterase [17].

On the other hand, OMP deficiency is also known to affect refined neuronal map formation in the olfactory bulb [18,19]. During development, olfactory neuronal map formation is governed by neuronal activity, cAMP-dependent kinase activity and the extracellular gradient

Abbreviation: CNG channel, cyclic nucleotide-gated channel; HET, heterozygous *OMP*$^{+/GFP}$; KI, GFP-knock-in *OMP*$^{GFP/GFP}$; L(+), *R*-(+)-limonene; L(-), *S*-(-)-limonene; OMP, olfactory marker protein; OR, odorant receptor; ORN, olfactory receptor neuron.

* Correspondence author.
E-mail address: nakashima_noriyuki@med.kurume-u.ac.jp (N. Nakashima).

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of repulsion/attraction molecules [19–23]. The roles of OMP are not restricted to the determination of olfaction signalling processes but are likely also relevant to the cellular machinery for olfactory information coding in various temporal scales. In the neural system, olfactory information is coded spatially in the loci of olfactory glomeruli in the bulb and temporally through the firing patterns of ORNs [24–26]. However, ORNs lacking OMP still retain the capacity to respond to odours along with potential mates.

Recently, the cAMP-binding motif was determined in OMP [27]. OMP buffers the cAMP surge in ORNs upon sensory stimulation and sharpens CNG channel activities; otherwise, the prolonged opening of CNG results in the silencing of ORNs and deteriorates odour source localization [27]. In this study, we investigated the contribution of OMP in olfactory investigation tasks by using odours with various values, including similar odours, urine-derived social odours and innately aversive odours.

2. Materials and methods

2.1. Experimental animals

Animals were treated in accordance with the guidelines of ARRIVE, the Animal Experiments Committee and the Ethics Committee of Kurume University, under the committee's approval. Male knock-in (KI) mice in which the OMP gene was replaced by GFP ($OMP^{GFP/GFP}$-*KI* mice; RBRC02092) [5] were obtained from RIKEN with permission from Dr. Mombaerts (Max Planck Institute). KI mice were crossbred with $OMP^{+/+}$ wild-type mice (C57BL/6NCrSlc; Japan SLC, Inc., Shizuoka, Japan) to generate heterozygous $OMP^{+/GFP}$ mice (HET mice). HET mice were crossbred for more than 10 generations to generate HET and KI mice. HET mice were used as controls. The animals for breeding were housed in groups of six per cage under a 12/12-h light/dark photoperiod at 24°C. The animals used for behavioural experiments were housed individually after weaning and were fed ad libitum. Only males were used in these experiments.

2.2. Genotyping

Animals were blindly genotyped by PCR after the behavioural tests. The primers used were as follows: Forward-1, 5′-CTGCAGTTCGATCACTGGAAC-3′; Forward-2, 5′-GAG AAGCGCGATCACATGGTCCT-3′; Common reverse, 5′-AAAGGCCTCTACAGTCTATAG-3′. Amplification was carried out with Advantage 2 Polymerase mix (Clontech, CA, USA) using 35 cycles of 95°C for 20 s, 63°C for 20 s and 72°C for 30 s.

2.3. Odour discrimination test

Odour discrimination tests were based on previous methods [27–30]. The odorants used for the fine discrimination test were 10 mM *R*-(+)-limonene vs. *S*-(-)-limonene as enantiomers (Tokyo Chemical Industry, Tokyo, Japan) and 100 mM butanol vs. pentanol (Wako Pure Chemical, Osaka, Japan); all the chemicals were dissolved in mineral oil. Social odours were prepared by wiping a cotton swab across the bottom of a dirty cage that had not been cleaned of excrement for three days. For the test, mice were handled every day for at least one minute. Prior to the behavioural assay, the mice were acclimated to a new cage for one hour and then habituated to presentation of the swab containing 100 μL of mineral oil for several sessions. In the test phase, the mice underwent sequential sessions as follows: 4 sessions with *R*-(+)-limonene, 1 session with *S*-(-)-limonene, 4 sessions with butanol, 1 session with pentanol and finally, 4 sessions with the social odour. The motivation for mice to investigate particular odorants is an intrinsic interest in a novel stimulus, followed by a habituation to one stimulus with repeated presentations. Each session consisted of one minute of odour presentation with a two-minute inter-session interval. The cumulative time spent sniffing was measured as the time the mouse spent with its nose oriented within 2 cm of the swab.

2.4. Odour aversion test

2,4,5-Trimethylthiazole (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in 100 μL of water at 2 v/v%. Two 1.5-mL test tubes containing either the solution or water were placed on one side of the experimental chamber (18 cm × 27 cm). The mice were left free to move for 120 s. The 120-s video data were separated into pictures at the rate of one frame per 2 s in JPEG format, converted into binary data and stacked into one image to construct a heatmap for time spent in the assay chambers. The maximal signals were levelled down to correspond to 15 s or more.

2.5. Odour-source navigation test

An eight-week-old male mouse deprived of water for 8 h was allowed to drink the 1 mM sucrose solution for 1 min and the 300 μM quinine solution for the next 1 min at 1-minute intervals. At each presentation, a swab containing 50-μL mineral oil (Sigma-Aldrich, MO, USA) with limonene or butanol prepared at 10 v/v% was presented close to the drinking spout [27] to train the mouse to associate the sucrose reward with the odour of *R*-(+)-limonene and the quinine (Nacalai Tesque, Kyoto, Japan) penalty with the odour of butanol. Training comprising this session was repeated 5 times a day. After training, the mouse was given free access to a water bottle until the next day, and this training was repeated for 8 days. The odour-source navigating ability was tested in a cage (width × depth × height = 25 cm × 40 cm × 20 cm), wherein a metal container with limonene odour-emitting filter paper was suspended in one corner, and a container with butanol-emitting filter paper was suspended in the opposite corners of the cage. A trained mouse deprived of water for 8 h prior to the test was placed in the middle of the cage and allowed to move freely to search for visible but empty bottles with odours as discriminating clues. Because the bottles were empty, the mouse kept searching by olfactory investigation. The time spent in the reward-associated half of the cage was analysed for a 2-min test session.

2.6. Immunohistochemistry

One hour after the 2-min odour-source navigation test, mice were anaesthetized with a 200-μL mixture of medetomidine, midazolam and butorphanol (0.075 mg/mL, 2 mg/mL and 0.1 mg/mL, respectively) and perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). The olfactory bulb was extracted, cryoprotected overnight in PBS containing 30 w/v% sucrose, mounted in OCT Compound (Sakura Finetek, Tokyo, Japan) and frontally sectioned at 35-μm thickness using a cryostat (CM3050S, Leica Microsystems, Wetzlar, Germany). Sections were incubated at room temperature overnight in an appropriate blocking solution containing antibodies against c-Fos (PC38, Merck Millipore, MA, USA), washed with PBS containing 0.3 v/v% Triton X-100 (PBS-X), incubated with the appropriate secondary antibody (ab150064, Alexa Fluor 594 conjugated, 1:200; Abcam, CA, USA) for 1.5 h, washed in PBS-X, mounted onto MAS-coated glass slides (Matsunami Glass, Tokyo, Japan), coverslipped using Vectashield antifade reagent (Vector Labs, CA, USA) and tightly sealed. The fluorescence signals were detected and analysed using a BX50 fluorescence microscope with cellSens image analysis software (Olympus, Tokyo, Japan). The images were captured by exposing the samples for 100 ms and were analysed offline.

2.7. Statistical analysis

Statistical analyses were performed using KaleidaGraph 4 (Synergy Software, PA, USA). See Supplementary materials for details.

3. Results

3.1. OMP$^{GFP/GFP}$–KI mice showed an olfactory deficit in locating the aversive odour source

We first re-evaluated the ability of KI mice to discriminate non-innate odours by repeatedly presenting cotton swabs containing various odours to freely moving mice. Mice first were habituated with a swab containing only solvent (Fig. 1A), which was presented from the cage ceiling for 1 min, and the sniffing time was cumulatively measured. Both HET and KI mice investigated the swab by sniffing for approximately 500 ms at the end of the training (4th trial, Fig. 1A). Successively, we presented an odour of excrement to show innate social interest. KI mice initially extensively investigated the swab with an odour, but this behaviour showed a gradual decline in duration with acclimation, similar to HET mice (1st-4th trials, Fig. 1B), indicating that both HET and KI mice were capable of smelling and showed fundamentally similar preferences towards social odours as acclimation.

Next, we applied two pairs of odours, limonene enantiomers and one-carbon-different alcohols, to investigate the fine odour discrimination ability. When *R*-(+)-limonene was present, both HET and KI mice initially sniffed the novel odour swabs for approximately 2 s in total and then subsequently for a shorter period of time (1st-4th trials of L(+), Fig. 1C). Then, HET mice attentively sniffed the next isomeric *S*-(-)-limonene as a novel odour, whereas KI mice did not (1 st trial of L(-), Fig. 1C).

Mice showed similar discriminative behaviour towards the butanol vs. pentanol odour pair (comparison of the 4th trial of butanol with the 1 st trial of pentanol, Fig. 1D), indicating that KI mice had impaired odour discrimination ability, consistent with previous reports [10,11].

HET mice displayed virtually no investigation behaviour during the 4th session, while KI mice sniffed the odours for a relatively longer duration of time (arrow, Fig. 1D) and lingered near the odour. Hence, we suspect that the KI mice tried to find the source by sniffing but could not locate the exact source of the odour by sniffing. As the cumulative sniff time was as short as a few seconds after habituation, it was not a reliable parameter to monitor the process of olfactory behaviour. We next performed an odour-clued test to evaluate the differences in olfactory behaviour between KI and HET mice.

3.2. OMP$^{GFP/GFP}$–KI mice were incapable of locating the aversive odour source

Next, we examined the behavioural phenotypes of KI mice using an aversive odorant. Innately, mice show avoidance behaviour when exposed to a synthetic analogue of the fox anal gland odour (2,4,5-trimethylthiazole; predator odour), even in the absence of the predator itself [27,29,31]. To eliminate visual and auditory aids by presenting a swab, we placed a mouse into a cage pre-filled with an aversive odour that was passively emitted from either side. Mice were intrinsically motivated to sniff continually to sense the odour gradient within the cage and avoid the source. HET mice displayed avoidance behaviour, with occasional immobilization at the furthest distance away from the odour source (red spot on the heatmap in Fig. 2A,B), whereas KI mice did not display complete immobilization but continued to wander around the cage (Fig. 2A,B). In the control condition without the predator odour, mice from both groups spent equal amounts of time in compartments 1 and 3, showing no interest in the odour-free test tubes on the floor (Fig. 2C,D). Thus, the presence of the test tubes did not bias the average time spent in each compartment. Meanwhile, KI mice exposed to the predator odour spent significantly less time (26.2 ± 6.6%, Fig. 2A,B) in compartment 1 than those not exposed to the predator odour (34.8 ± 4.5%, Fig. 2C,D). These results indicate that KI mice cannot maintain an adequate distance from the aversive odour source.

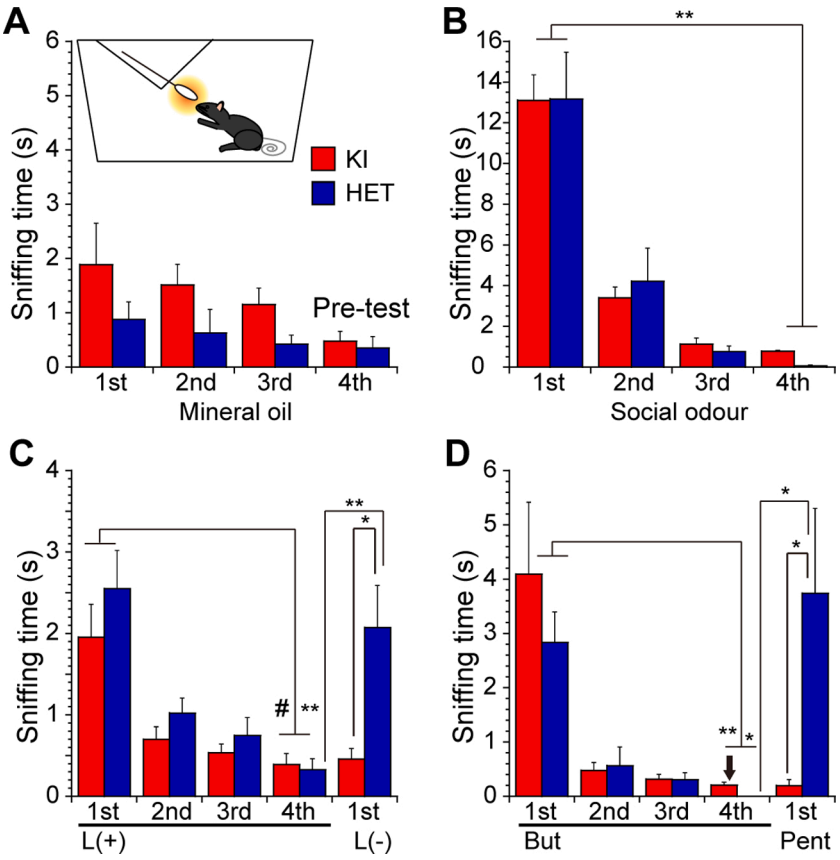

Fig. 1. Odour discriminationwasimpaired in KI mice.
(**A**) Acclimation session using an odour solvent (mineral oil). After the 4th trial with the presentation of mineral oil (a pre-test condition), mice were used for subsequent behavioural tests. (**B**) Sensitivity to social odours. (**C**) Discrimination between enantiomers. (**D**) Discrimination between one-carbon-different alcohol species. Abbreviations: L(+), *R*-(+)-limonene; L(-), *S*-(-)-limonene; But, butanol; and Pent, pentanol. Mean ± s.e.m; n = 5 and 7 animals for KI and HET mice, respectively. #, P < 0.03, *P < 0.01, **P < 0.001.

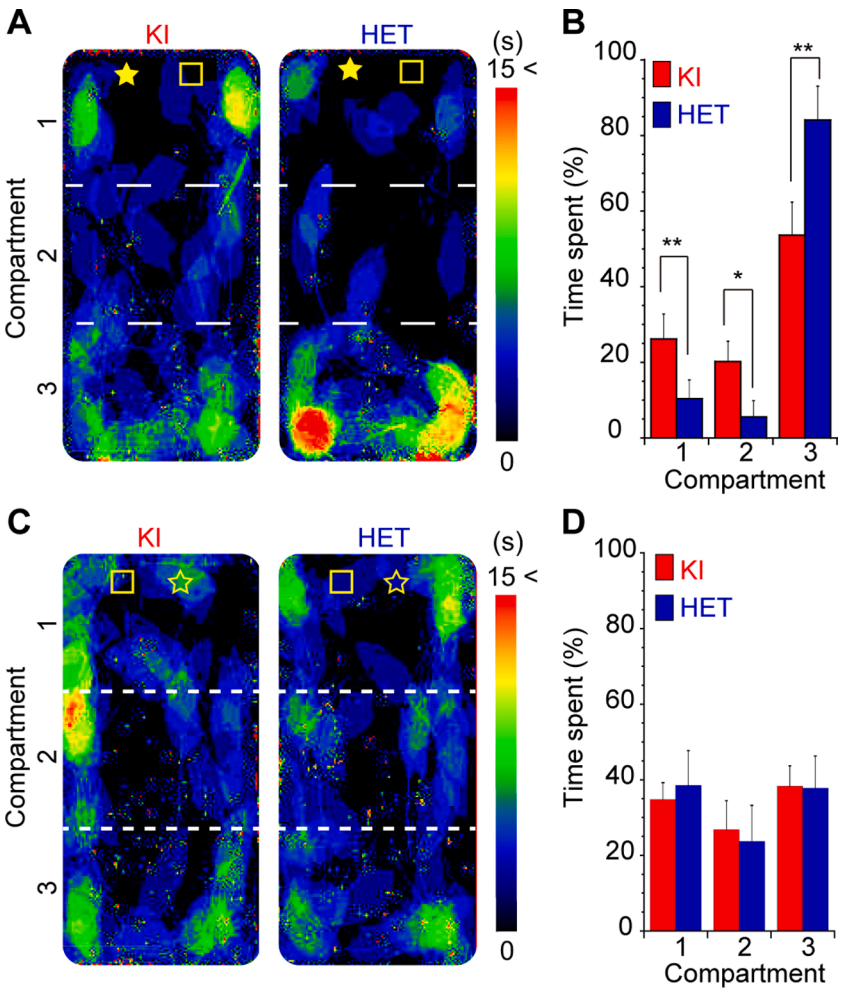

Fig. 2. Odour aversion was impaired in KI mice.
(**A**) Heat maps of the time spent by subject mice during the predator odour aversion test. The filled yellow star indicates the tube containing the fox odour. The open square indicates an empty tube used as a control novel object. (**B**) Time spent in the compartments shown in (**A**). n = 12 and 6 animals for KI and HET mice, respectively. (**C**) Heat maps for the time spent by the subject mice in each compartment during the control session for the predator odour aversion test. The open squares indicate the empty test tubes. The open stars indicate the test tubes containing distilled water. (**D**) The time spent in the compartments shown in (**C**). n = 10 and 7 animals for KI and HET mice, respectively. Time is shown as a percent, with 60 s as 100 %. Mean ± s.e.m. *, P < 0.01. **, P < 0.001.

3.3. Odour-value determination was impaired in OMP$^{GFP/GFP}$–KI mice

To highlight the odour-source navigation ability of mice, we first trained mice to associate odours with reward-penalty values to compare locations scented with preferred or aversive odours. The preference for sucrose and quinine was measured by lickometry (Fig. 3A-E). Both KI and HET mice preferred the 1 mM sucrose solution and avoided the 300 μM quinine solution, indicating that these solutions can operate as a reward and a penalty, respectively. Then, mice were trained to associate 1 mM sucrose solution and 300 μM quinine solution with limonene and butanol, 10 v/v% in mineral oil, respectively, for 8 days (Fig. 3E). Next, the trained mice were placed in a cage with a fenestrated steel container with paper dipped in mineral oil with limonene in one corner and a container with butanol in the other corner (Fig. 3F). Mice were allowed to freely move in the cage for 120 s to sense the odours and approach the bottles. As the bottles were empty, mice continued olfactory investigations during the test session. In the initial 30 s, both HET and KI mice showed a preference towards the area with the limonene odour (Fig. 3G). KI mice started to wander into the other half of the cage with the butanol odour longer in the next 30 s and finally stayed in the limonene area, whereas HET mice stayed longer in the half of the test cage with the limonene odour until the end of the test (Fig. 3G).

Because OMP deficiency also disturbs olfactory glomerular refinement [12,18,19], we examined the activity-dependent expression of the immediate early gene c-FOS in the olfactory bulb. After odour-source navigation, the olfactory bulbs of KI mice showed fewer responsive juxtaglomerular cells around more glomeruli than those of HET mice (Fig. 4A-C). These results indicate that weaker and less selective olfactory information is delivered to olfactory glomeruli in KI mice than HET mice.

4. Discussion

OMP appears to be dispensable for noticing ambient odour because KI mice tried to avoid the aversive odour even without visual cues. However, the olfactory dysfunction of KI mice seemed most apparent in discriminating odour values in olfactory stimulation during sniffing; it took longer for KI mice to display odour-associated behaviours in the odour aversion test and reward-penalty test. Previous behavioural studies have also shown that OMP is required for odour valence and source identification by using mother mice, food or structurally similar odours [10,11,27]. Although the limitations of relying on the intrinsic motivation of subjects to investigate olfactory stimuli after habituation should be noted, OMP seems to be fundamentally essential for animals in determining the values of aversive, food or environmental odours. This phenotype is especially disadvantageous in situations where animals are confronted with predators and must escape for their survival.

Sniffing behaviour is a process of not only inhaling odorants but also coding information based on mechanical stimulation from ORNs to glomeruli. In fact, active olfaction by sniffing causes odorant inhalation on pace with air intake into the nasal cavity, which provides mechanical information regarding the nature and location of odours and enables the scenting of foods and predators [25,27,32–34]. Previous reports on cell physiology indicate that OMP is involved in determining olfactory signalling kinetics via cAMP and is necessary to sharpen the activity of cAMP-gated channels to maintain neuronal sensitivity [15,17,27].

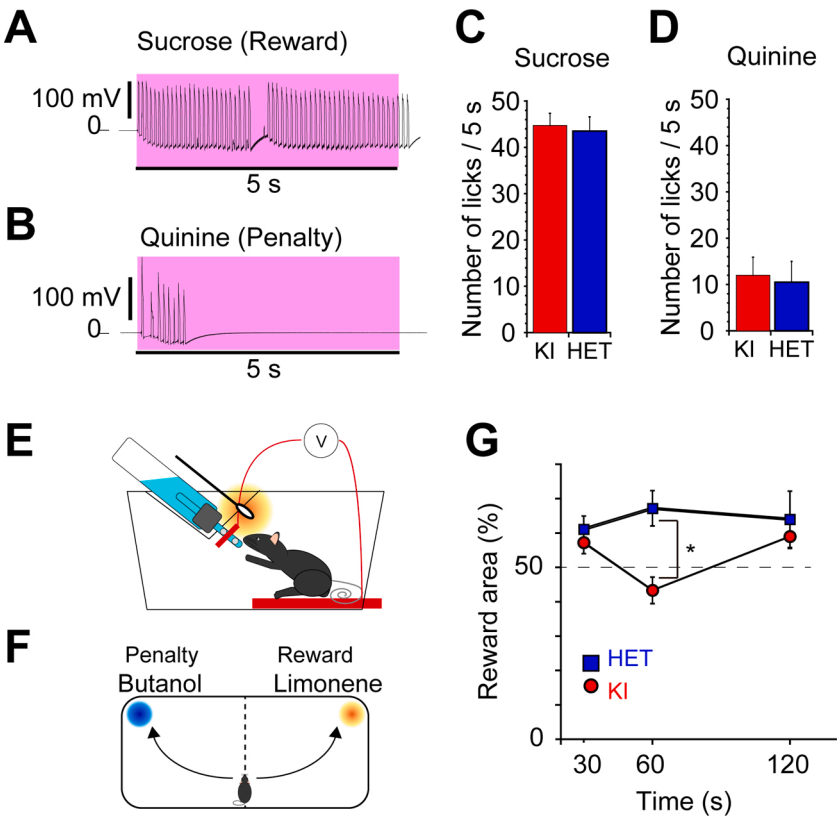

Fig. 3. Odour-source navigationwas impairedin KI mice. (**A**, **B**) Preference of mice for (**A**) sucrose and (**B**) quinine. Licking was counted for 5 s from the first lick. (**C**, **D**) Summary of licks for (**C**) sucrose and (**D**) quinine during the initial 5 s from the first lick. n = 6 animals each for KI and HET mice. (**E**) Schematic representation of the odour-association training. (**F**) Schematic representation of the odour-association test. (**G**) Summary of the time spent in half of the compartment scented with reward-associated *R*-(+)-limonene over the total time including the compartment with penalty-associated butanol elapsed during the test session n = 6 animals each for KI and HET mice. Mean ± s.e.m. *, P < 0.01.

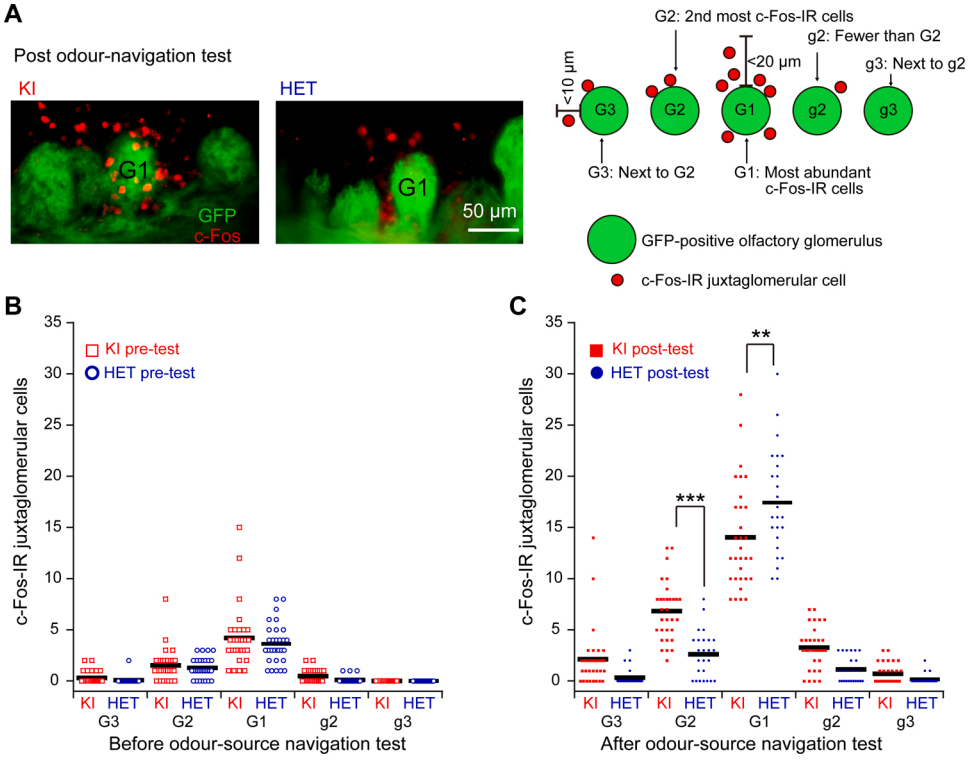

Fig. 4. Odour-source navigation activated broader juxtaglomerular cells in KI mice. (**A**) c-FOS immunoreactivity (IR) in juxtaglomerular cells per responding olfactory glomerulus (G1). (**B, C**) The number of c-FOS-IR cells per responding glomerulus (**B**) before and (**C**) after the odour-source navigation test; cells within 10–20 μm of the glomerular perimeter were counted. Bars, means. n = 25–30 glomeruli from 3 mice each for HET and KI mice. **, P < 0.001; ***, P < 0.0001.

During sniffing, ORNs in the main olfactory epithelium receive mechanical input, which is thought to provide information related to odour quality [24,25,27,33,34]. Recently, odorant receptors have been proposed to be polymodal sensors for chemical and mechanical stimuli that share the cAMP-associated signalling pathway [27,34–36]. Thus, it is more likely that the roles of OMP are related to the temporal processing of odour information in ORNs.

Histologically, odour-source navigation evoked broader glomeruli in

KI mice. OMP is ubiquitous in mature ORNs, but OMP expression levels are diverse across ORNs and possibly change depending on the amount of sensory stimulation [37], suggesting that OMP might balance neuronal sensitivity to odorants in the long run. OMP is proposed to directly buffer cAMP to sharpen odour-evoked signalling and elevate basal cAMP actions [17,27,38–40], which might also account for the long-term functions of OMP in forming the histological neural map from ORNs to the olfactory bulb [17–19,21]. Thus, OMP is also important for establishing the neural network to discriminatively code odour information [12,17–19,21,27,39].

OMP reportedly enters the nucleus to interact with transcription factors [41] and affect cell proliferation by unknown mechanisms [38]. However, how OMP expression and the associated signalling modules are regulated physiologically and pathologically at certain maturation stages of selective cell populations remains unknown [2,42,43]. Determining how ORNs functionally mature along with the expression mechanisms of OMP requires further investigation to understand the temporal dynamics of olfactory information processing.

Author contributions

A.N. performed the odour-association test and immunohistochemistry. A.N. and T.N. maintained the strain and genotyped mice blindly. A.N. analysed and interpreted the data with N.N., T.N., and M.T. A.N. and N.N. discussed the results and wrote the manuscript with M.T.

Declaration of Competing Interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2020.135445.

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