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Dense representation of natural odorants in the mouse olfactory bulb

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In mammals, odorant molecules are thought to activate only a few glomeruli, leading to the hypothesis that odor representation in the olfactory bulb is sparse. However, the studies supporting this model used anesthetized animals or monomolecular odorants at limited concentration ranges. Using optical imaging and two-photon microscopy, we found that natural odorants at their native concentrations could elicit dense representations in the olfactory bulb. Both anesthesia and odorant concentration were found to modulate the representation density of natural odorants.

In the olfactory bulb, odorant molecules evoke complex spatiotemporal patterns of activated glomeruli[1–10] that substantially vary in density with stimulus concentration[1,6,7,10,11]. To better understand how odorants are normally coded in the glomeruli array, it is important to use concentrations that are behaviorally relevant. Mixed odorants from natural products come, by definition, in behaviorally relevant concentrations and ratios. Such complex odorants activate only a few glomeruli[12], providing evidence for a general theory of sparse glomerular coding[12,13]. However, the studies supporting the model of sparse coding were performed on anesthetized animals or used monomolecular odorants in a limited range of concentrations.

Using intrinsic optical signal imaging, we investigated how natural odorants are represented in awake mice (**Fig. 1a**,**b**). We presented the stimuli either directly to the mouse snout (**Fig. 1b**) or using an olfactometer (**Fig. 1a**). In the latter case, the concentrations were reduced at least twofold compared with the concentrations that a mouse would experience when being close to the odor source (**Fig. 1c**,**d** and Online Methods), leading to a reduced number of activated glomeruli ($n = 6$ odorant-mouse pairs, paired t test, $P < 0.005$; **Fig. 1e**).

We then applied 40 different natural odorants using the olfactometer to minimize the animal's stress and to have a better temporal control of the odorant application. In all of the mice that we tested ($n = 8$), every odorant evoked a specific dense pattern of glomerular activity (**Fig. 2a** and **Supplementary Fig. 1**). For each odorant, we analyzed the image sequence (Online Methods and **Supplementary Fig. 2**) and quantified the number of activated glomeruli (**Fig. 2b** and **Supplementary Fig. 3**). We found that the number of activated glomeruli for each stimulus ranged between 10 and 40 glomeruli (**Fig. 2b**). For the same set of 30 natural stimuli, the total number of activated glomeruli per olfactory bulb was 443 ± 15 glomeruli ($n = 5$ mice; **Fig. 2c**,**d**). By merging the glomeruli activated by several odorants (Online Methods), we obtained a map composed of glomeruli with a unique identity showing that the natural odorants that we used activated a large portion of the dorsal olfactory bulb (127 ± 3 glomeruli; **Fig. 2c**,**d**).

We then addressed the issue of the fraction of the total number of glomeruli activated by the odorants. Using two-photon imaging

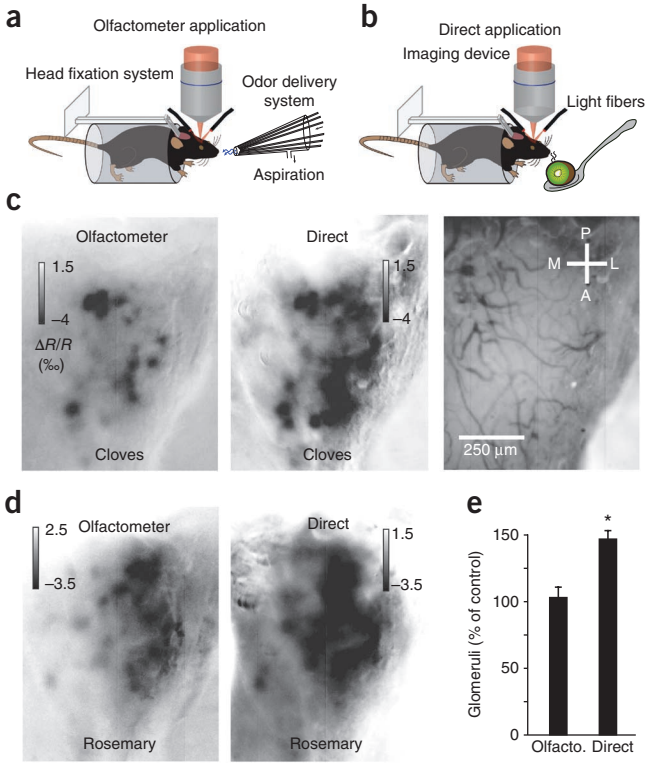

Figure 1 Intrinsic optical signal imaging of glomerular-evoked activity in awake mice. (**a**,**b**) Schema showing natural odorants delivered by an olfactometer in **a** and natural products drawn up to the mouse snout in a spoon in **b**. (**c**,**d**) Glomeruli maps evoked by two natural odorants presented either by an olfactometer or directly. The blood vessel pattern is shown in **c** (right image). P, posterior; A, anterior; L, lateral; M, medial. (**e**) Comparison of the number of glomeruli activated in the two mode of applications. The number of glomeruli activated by the olfactometer application served as a control (control, 100%) for each natural odorant-mouse pair ($n = 6$; $^*P < 0.005$, paired t test). Data are presented as mean ± s.e.m.

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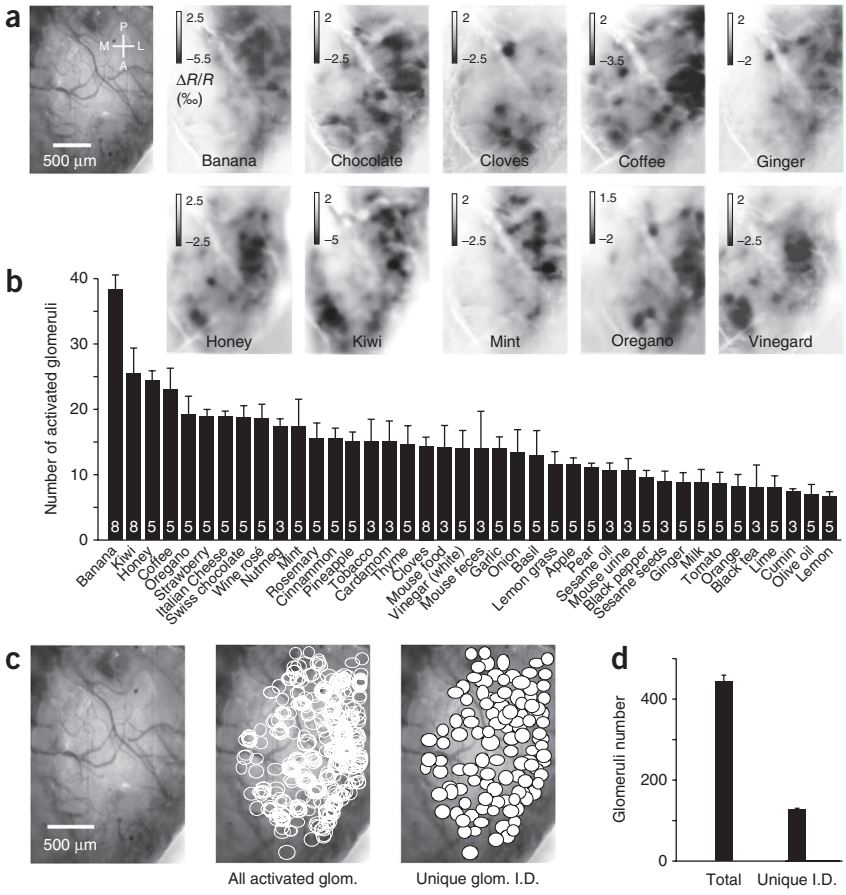

Figure 2 Dense representation of natural stimuli in the olfactory bulb of awake mice. (**a**) Glomerular activity patterns evoked by a variety of natural odorants in a single mouse. The images represent the average of all frames during odor application. (**b**) Number of glomeruli activated by each natural odorant tested (number of mice tested in white). (**c**) Total number (middle image; glomeruli activated by multiple odorants and plotted several times) and individual number (right image; Unique I.D.: that is, each activated glomerulus is only plotted once) of glomeruli activated by 30 natural odorants presented to one mouse and overlaid on the blood vessel pattern (left image) after realignment with the blood vessels. (**d**) Quantification of the total and unique I.D. numbers of activated glomeruli (n = 5 mice that shared the same 30 natural odorants). Data are presented as mean ± s.e.m.

of *Omp*-GFP[14] mice, we estimated the dorsal olfactory bulb accessible to imaging contains ~200 glomeruli (**Supplementary Fig. 4**). Thus, natural odorants activated 10–30% of the total glomeruli number (including the correction factor of 1.5 calculated in **Fig. 1e**), although this value may have been underestimated (Online Methods).

Our observations differ considerably from a previous report in isoflurane anesthetized animals[12]. We tested the hypothesis that isoflurane may alter odorant-evoked patterns. Used as a regular smell, isoflurane specifically and stereotypically activated glomeruli in the posterior-lateral portion of the olfactory bulb in awake mice (**Fig. 3a**,**b** and **Supplementary Fig. 5a**,**b**). We then assessed the potential effect of constant isoflurane application on odorant maps. All the odorants tested consistently activated more glomeruli in the awake condition than during isoflurane anesthesia (**Fig. 3c**–**h** and **Supplementary Fig. 5c**–**f**). The effect was independent of odorant concentration (**Fig. 3i**,**j**), reversible after ending gas anesthesia (**Fig. 3i**,**j**) and not homogenous across the olfactory bulb surface (**Supplementary Fig. 5d**,**f**). Thus, gas anesthesia led to previous underestimations[12] of the number of glomeruli normally that are activated by natural odorants (**Supplementary Fig. 6**).

We identified two mechanisms that could explain the isoflurane-mediated glomeruli suppression. First, long-term exposure to the anesthetic may alter normal neuronal function (pharmacological effect). Second, constant application of isoflurane may induce adaptation and/or desensitization of a large number of olfactory receptor neurons, as could result from constant application of a non-anesthetic odorant (odorant effect; **Supplementary Fig. 7**). Further work will be necessary to identify the exact mechanisms underlying the isoflurane-mediated glomeruli suppression.

Natural odorants evoked denser patterns than were previously reported for monomolecular odorants[13]. In this regard, one important parameter is the odorant concentration that is used[6,7,9–11]. Natural odorants are complex mixtures of monomolecular components, each of which can bind different olfactory receptors. A monomolecular odorant could either activate as many glomeruli as a multimolecular odorant or only a few glomeruli. In the latter case, natural odorant–evoked patterns could represent the summation of multiple monomolecular odorant–evoked patterns[12]. We compared natural odorants with monomolecular odorants at concentrations that are perceived by humans to smell the same and as strong as the natural odorants (**Supplementary Fig. 8a**–**c**). The number of activated glomeruli was comparable between natural and monomolecular odorants (**Supplementary Fig. 8d**). Thus, even a monomolecular odorant can activate a large number of glomeruli in the olfactory bulb at some concentrations. This result differs from the sparse patterns that have been reported in studies that used weaker concentrations of monomolecular odorants[13]. However, decreasing the concentrations of odorants (up to similar concentrations as those used previously) led to a sparsening of activated glomerular patterns and a decrease in the glomerular response amplitude (**Supplementary Fig. 9**).

We conclude that the odorant representation can vary in density with concentration and anesthesia. Natural odorants at their native concentrations evoke dense patterns of activated glomeruli in awake mice. Given that coactivation of multiple glomeruli is required to fire a pyramidal neuron in the piriform cortex *in vivo*[15], a dense representation should improve processing of odorants from the olfactory bulb to the olfactory cortex.

METHODS

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

Note: Supplementary information is available on the Nature Neuroscience website.

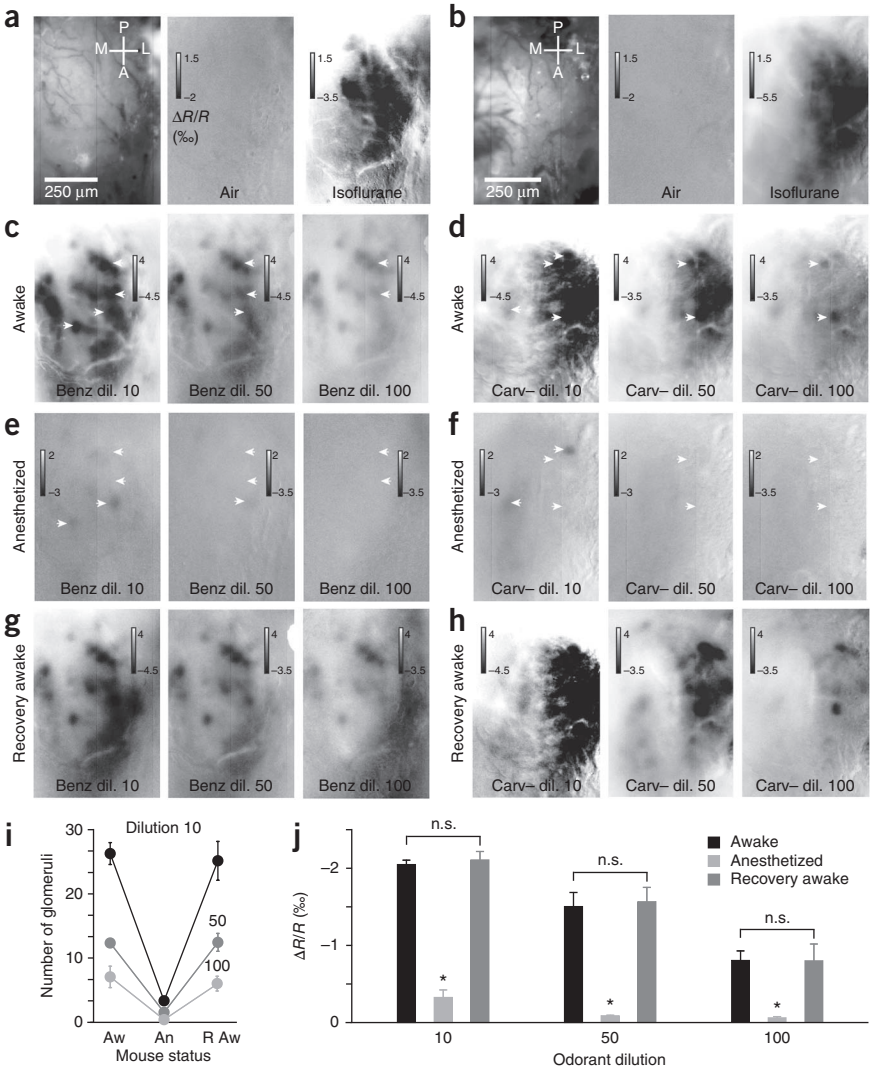

Figure 3 Gas anesthesia reversibly suppresses odorant-evoked response. (**a**,**b**) Blood vessel patterns of the olfactory bulb in two mice (left images). Glomerular maps induced by air and isoflurane when applied as an odorant. (**c**,**d**) Glomerular patterns induced by benzaldehyde (Benz) and carvone-(–) (Carv–) during wakefulness at three odorant dilutions. (**e**,**f**) Constant isoflurane anesthesia suppressed glomerular responses. White arrows indicate some glomeruli that disappeared or displayed a reduced response following anesthesia. (**g**,**h**) Recovery of the glomerular patterns when animals were awake again (10 min after ending the anesthesia). (**i**) Quantification in different conditions (Aw, awake; An, anesthetized; R Aw, awake after anesthesia) of the average number of glomeruli activated by different odorants ($n = 24$ odorant-mouse pairs) at three dilutions (10, 50 and 100 dilutions: black, dark grey and light grey curves, respectively). Awake versus anesthetized, for all paired t test, at least $P < 0.05$; awake versus awake after anesthesia, for all paired t test, $P > 0.05$. (**j**) Quantification of the glomeruli responses amplitude. Gas anesthesia mediated a significant suppression of activity at all of the dilutions that we used (* represents paired t test between awake and anesthetized states, at least $P < 0.05$). The effect of the gas anesthesia was reversible (paired t test between awake and recovery awake states, $P > 0.05$; n.s. = not significant). Data are presented as mean ± s.e.m.

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AUTHOR CONTRIBUTIONS

A.C., R.V., K.B., O.G. and J.B. carried out the study conceptualization and performed experiments or analysis. A.C., R.V. and O.G. wrote and edited the manuscript with comments from J.B. and K.B.

COMPETING FINANCIAL INTERESTS

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ONLINE METHODS

Animals and initial preparation. All experiments were performed on 12–20-week-old male C57BL/6J mice (Charles River France; for intrinsic imaging and tetrodes recording) and hemizygous *Omp*^{tm3Mom} (for *in vivo* two-photon laser-scanning microscopy, referred to as *Omp*-GFP)[14]. All experiments were in accordance with the Swiss Federal Act on Animal Protection and Swiss Animal Protection Ordinance. Our experiments were approved by the university and the state of Geneva ethics committee.

A few days before performing any experiment, a custom-made head-fixation system was mounted on the mouse's head. Mice were anesthetized with isoflurane (4% induction and 1–2% maintenance; Baxter AG). A local anesthetic, carbostesin (AstraZeneca), was subcutaneously injected before any incision, and the eyes were protected with artificial tears to prevent dryness. Body temperature was monitored and maintained at ~37 °C using a heating pad (FHC). The skin overlaying the skull was removed and a custom-made metallic head post was then fixed on the bone by embedding its base in dental cement (Omni-Etch Dentin). The rest of the skull was covered with dental cement excepting the olfactory bulb. The bone overlying one of the two bulbs was carefully thinned to allow good light transmission and a dental cement well was constructed around it. To avoid direct contact of the thinned bone with air, we applied a small amount of silicon elastomer (Kwik-Cast and Kwik-Sil).

A few days after recovery, we placed the animal in a tube and head-fixed it by screwing the head post into a home-made metal device fixed on the air table (**Fig. 1a**). The mice were trained in this restrained condition for 2–4 sessions (30 min each) over 2 d.

Odorants preparation and stimulation. All natural stimuli (banana, honey, ripe kiwi, coffee, oregano, strawberry, Italian cheese (parmesan), Swiss chocolate, wine rosé, nutmeg, rosemary, mint, thyme, cinnamon, tobacco, cardamom, cloves, vinegar, garlic, onion, pineapple, basil, lemongrass, apple, pear, sesame oil, black pepper, sesame seeds, ginger, milk, tomato, orange, black tea, lime, cumin, olive oil and lemon) except mouse food, urine and feces were purchased from local grocery stores. C57Bl/6 male mouse urine was collected and stored at −80 °C until use. The mouse feces were collected every day from male mice cages. Natural odorants were prepared fresh each experimental day (for example, fruits were freshly pressed and used for a few hours maximum). Either 4 g of dry solid (for mouse feces, 1 g) or 4 ml (for mouse urine, 1 ml) of natural stimuli or 4 ml of pure artificial odorant (including isoflurane) were placed in glass vials. They were at natural partial pressure.

All monomolecular odorants (0.22 mM amyl acetate, 0.63 mM ethyl butyrate, 0.28 mM isoamyl acetate, 33 µM eugenol, 11 µM citral, 22 µM carvone-(−), 0.76 mM 3-hexanone, 24 µM acetophenone, 11 µM geraniol, 0.11 mM benzaldehyde, 0.11 mM cineol) were from Sigma-Aldrich. The molar concentrations of the monomolecular odorants were calculated using the ideal gas law $n = PV/RT$. Odorants were delivered through a custom-made olfactometer as described previously[2,3]. Odorants were delivered for 5 s (2 s after recording onset). An air flow passed through the vials containing the odorants. Depending on the experimental conditions, the odorants were differently diluted with clean dry air before being sent to the nose: 50% (**Figs. 1** and **2** and **Supplementary Figs. 1–3** and **6**) dilution for natural odorants, and 10% (**Fig. 3**), 5% (**Supplementary Figs. 5** and **7–9**), 2% or 1% (**Fig. 3** and **Supplementary Figs. 6** and **9**) dilution for monomolecular odorants and isoflurane stimuli. The total flow was constant (0.4 l min^{-1}). To maintain a stable odor concentration during the entire stimulus application, we ensured that flows were stationary with a 5-s preloading before the odorant was delivered.

For the experiments using natural products presented directly to the mouse (**Fig. 1**), the odorants were drawn up close to the snout for ~5 s with the help of a spoon containing 2 g of solid natural products. For comparison, the concentrations of natural odorants sent by the olfactometer were reduced at least twofold (minimum of two times dilution in air due to the olfactometer design) to the concentrations reached when presenting directly the product in the spoon, equivalent to the concentrations that a mouse would experience when being close to the odor source.

Intrinsic optical imaging. For intrinsic signal imaging, the olfactory bulb was illuminated with red light at 700 nm (BP 20 nm) using a stable 100 W halogen lamp and a light guide system. Images were acquired at 5 Hz for 10 s (2 s before, 5 s during and 3 s after the stimulus) using the Imager 3001F system (Optical Imaging) mounted both on a custom-built macroscope (Navitar 17 mm, bottom lenses; Nikon 135 mm, upper lens; total magnification 7.9×). Images were acquired at 512 × 512 pixels and further 2 × 2 binned. The images were cropped to show the dorsal olfactory bulb. The blood vessel pattern was taken using green light (546-nm interference filter) at the beginning and the end of each experimental session, to assess the focus and to minimize drift. For the analysis, all the image were bandpass filtered ($\sigma1 = 2$, $\sigma2 = 100$) and realigned by comparing the blood vessels pattern between images using a custom script running in Matlab (MathWorks). Each odor was presented four times. For illustration, the images shown are the average of all the frames acquired during the stimulation (5 s) plus the frame acquired during the post-stimulus phase (3 s).

We analyzed the image time sequence, offering a better identification of individual glomeruli in single frame than in an average image (**Supplementary Figs. 2** and **3**). The glomerulus detection procedure (that is, ovoid region of interest exhibiting decrease in reflectance) was done on individual time frames by drawing regions of interest (the process was done independently by three experienced persons). We excluded every region that appeared in only a single frame or that looked like blood vessels. For the unique identification glomeruli number (**Fig. 2d**), the overlapping glomeruli were removed using a script running in Matlab (The MathWorks). After calculating the average diameter of the glomeruli (103 ± 6.04 µm), if two partially overlapping glomeruli had a distance between centers < 51.5 µm (half of the average diameter) they were counted as one; if instead the two glomeruli had a distance between centers > 51.5 µm, they were counted as different. All statistical testing was done in Statistica or Excel.

The number of activated glomeruli that we observed may be an underestimate of the actual activation pattern, as intrinsic optical signal imaging may not be sensitive enough to detect some weak glomerular responses or may not report all strongly activated glomeruli[16]. Furthermore, as glomeruli strongly vary in size, small glomeruli often overlap with larger ones (**Supplementary Fig. 4**), limiting the possibility of discerning all activated glomeruli.

Imaging in awake mice constrained us to acquiring only dorsal olfactory bulb activity. However, this limitation does not change our conclusions for several reasons. First, olfactory receptor neurons expressing the same olfactory receptor converge on ~two glomeruli located in different parts of the olfactory bulb[17,18]. Thus, if one glomerulus is activated in the dorsal part, an equivalent glomerulus should be activated elsewhere on the olfactory bulb surface as well. Second, certain stimuli are known to activate more glomeruli in regions inaccessible to imaging[19–22]. Taken together, similar patterns should be triggered by odorants across the entire olfactory bulb surface.

Two-photon laser-scanning microscopy. *Omp*-GFP mice were anesthetized with an intraperitoneal injection of 3.1 µl per g of body weight of a mixture consisting of 60 µl Medetomidin (Dormitor, Pfizer AG; 1 mg ml^{-1}), 160 µl Midazolam (Dormicum, Roche Pharma AG; 5 mg ml^{-1}) and 40 µl Fentanyl (Sintenyl, Sintetica S.A.; 50 µg ml^{-1}). The bone overlying one hemibulb was thinned and gently removed, leaving the dura mater intact. A small drop of 1% agarose was added on top of the dura; a circular cover glass was then placed on top of the agarose and sealed with a thin layer of dental cement. After the surgery the animals were immediately transferred to the two-photon microscope for image acquisition.

Two-photon imaging was performed using a trimscope scanning head (LaVision BioTec GmbH) mounted on a BX51WI microscope (Olympus). The specimen was illuminated with 915-nm light from a tunable pulsed Ti:sapphire femtosecond Chameleon laser with an approximate laser intensity of 50 mW (Coherent). A macroscopic image was initially taken under a 4× objective to obtain a global image of the entire bulb and eventually locate the extremities of the structure for the first *z* stack. The bulb was then divided into successive square windows (field of view) of 500 × 500 µm with a spatial resolution of 970 × 970 pixels. All *z* stacks were acquired with a 20× 0.95 NA water-immersion objective (Olympus). Emitted light was collected using a photomultiplier tube (Hamamatsu). Starting from the posterior and medial corner consecutive 200-µm-thick *z* stacks were acquired with a step size of 2 µm. Scanning and image acquisition were controlled using Imspector Pro software (LaVision BioTec GmbH). Subsequent image processing was performed in ImageJ (US National Institutes of Health). The glomerulus counting procedure was done by delineating regions of interest (the process was done independently by three experienced persons).

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