The role of adult-born neurons in olfactory bulb plasticity

Thepekhem shel te'amim hnelidim b'moh b'olafkiyot shel
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Abstract

The adult mammalian olfactory bulb (OB) is continuously supplied with new neurons throughout life. This unique population has been hypothesized to play an important role in the network's plasticity by the mere addition of neurons to the network. However, experimental studies seeking evidence for the causal role of adult-born neurons in olfaction have yielded contradictory results. Furthermore, there have been only few studies directly exploring the adult-born neurons themselves in the context of OB plasticity, thus limiting the understanding of their potential involvement in it. The general goal of this thesis is to examine the role of adult-born neurons, if any, in the OB. We will take a two-step approach. The first step will be to characterize the time window in which adult-born neurons change in response to manipulations of sensory experience. Based on these results, the second step will be to test if adult-born neurons are essential for a specific OB function - figure-background separation. To this end, combining genetic labeling methods with in vivo time-lapse two-photon imaging and intrinsic signal imaging, we provide evidence for experience-dependent plasticity of both developing and mature adult-born neurons. Interestingly, we found that young and mature adult-born neurons exhibit distinct signatures of plasticity. We also provide preliminary in vivo electrophysiology data. These experiments will expand our knowledge of the functional role of adult-born neurons in the mammalian brain.
Introduction

Olfaction is a central sense in the daily life of most animals, serving diverse functions, such as foraging, mating, kin recognition, maternal care, and more. The first station of olfactory processing is the OB. Axons of epithelial sensory neurons expressing the same odorant receptor converge into distinct anatomical and functional units in the OB called glomeruli (Fig. 1; Shepherd, 2004). The circuit of the OB combines signals from these input channels into the responses of mitral cells, whose axons project to downstream brain areas. Each mitral cell sends a single apical dendrite into a primary glomerulus, where it receives excitatory input from receptor neurons and receives inhibition from local interneurons. Mitral cells also extend a broad field of lateral dendrites that form reciprocal synapses with granule cells (GCs; Fig. 1; Shepherd, 2004). While the basic information flow in the OB is fairly understood, little is known about the functions it serves, and the mechanisms by which it performs them. Although the OB probably serves different functions, the goal of this thesis is to focus on one that may be shaped by ongoing adult neurogenesis.

Throughout life, new neurons are constantly born and supplied to the OB (Altman, 1969; Alvarez-Buylla and Garcia-Verdugo, 2002; Lledo et al., 2006). In the OB of rodents, adult-born neurons give rise to different subtypes of interneurons, mainly GCs and periglomerular neurons (PGNs; Adam and Mizrahi, 2010). The mere addition of adult-born neurons has been hypothesized to play an important role in the OB network’s plasticity (Lazarini and Lledo, 2011; Lledo et al., 2006). However, there have been only few studies directly addressing the plasticity of the adult-born neurons themselves in vivo. Furthermore, thus far there has been mostly circumstantial evidence supporting the role of adult-born neurons in olfactory plasticity (Alonso et al., 2006; Magavi et al., 2005; Moreno et al., 2009). Recent studies have tried to find evidence for the causal role of adult-born neurons in olfactory behaviors, but the results have been contradictory (reviewed in Lazarini and Lledo, 2011). Most of these studies focused on basic olfactory behaviors (e.g., detection, discrimination), and did not systematically examine OB physiology. Our goal is to link changes in adult-born neurons and manipulations of adult neurogenesis to changes in sensory processing.
One of the most basic aspects of olfactory processing is the ability to detect an "important" odor, while ignoring background odors, a process known as figure-background separation (FBS; Gottfried, 2010; Hopfield, 1999; Kadohisa and Wilson, 2006). The current notion is that FBS is a form of "higher order" processing which occurs in the olfactory cortex, but not in the OB (Gottfried; Kadohisa and Wilson, 2006). However, by mere anatomical considerations (e.g., widespread inhibitory networks and massive centrifugal inputs; Shepherd, 2004) the OB has the potential to be involved in complex computations such as FBS. In addition, computational modeling has suggested that FBS can arise from the properties of the input to the OB (Hopfield, 1999). Furthermore, recent experiments have started to reveal that the OB plays a role in higher processing, reminiscent of an associative cortex (Doucette et al.; Restrepo et al., 2009; Shea et al., 2008). The neural mechanisms of OB computations, both "lower" and "higher", are only now beginning to be unveiled and are largely not understood. For these reasons we think it is well worth the effort to reassess some of the older notions about the function of the OB.
Research Plan

The general goal of this thesis is to examine the role of adult-born neurons in OB processing, focusing on dynamic processes that may be shaped by experience. To this end, we will take a two-step approach. The first step will be to characterize the structural dynamics of adult-born neurons' synapses, \textit{in vivo} – are they plastic? If so, “how” and “when” are they plastic? (See below). The second step will be to test if adult-born neurons are essential for FBS and FBS plasticity.

Part 1

Our first goal is to characterize structural plasticity of adult-born neurons at the single cell level. We will focus on the following questions: what types of structural dynamics do these neurons undergo during their development, and after they mature?

\textit{Plasticity of young adult-born neurons}

We will first establish a model for experience-dependent plasticity, \textit{in vivo}. To do so, we will combine genetic tools with \textit{in vivo} time-lapse two-photon imaging and intrinsic signal imaging. We will genetically label putative synapses and dendrites of adult-born neurons, allowing us to examine their structure and synaptic dynamics (see Fig. 3 below). As a simple model for manipulating sensory experience we will use odor enrichment. Odor enrichment has been shown to increase adult-born neurons' survival, and to cause changes in the odor responses of mitral cells and OB interneurons (reviewed in Mandairon and Linster, 2009). Furthermore, odor enrichment has been shown to enhance olfactory perception (Mandairon and Linster, 2009; Moreno et al., 2009). Due to these considerations, we hypothesize that adult-born neurons will undergo some form of \textit{in vivo} experience-dependent plasticity during their development in response to odor enrichment.

\textit{Plasticity of mature adult-born neurons}

The experience-dependent plasticity of developing adult-born neurons can be an important source of OB plasticity. However, since developing adult-born neurons have only a
~50% chance to survive, developmental plasticity may have short-lived effects on determining their survival, rather than shaping the OB network in the long run. Accordingly, we hypothesize that adult-born neurons remain similarly plastic after they mature and integrate into the network, and thus serve as an important source of long-term plasticity to the OB. To test this hypothesis, we will study mature adult born neurons using the same experimental system mentioned above for young adult-born neurons.

Part 2

In part 1, we will establish that adult-born neurons can undergo experience-dependent plasticity during development and after maturation. Accordingly, our second goal is to explore the role of young and mature adult-born neurons in OB processing and plasticity. To this end, we will first establish an experimental system for studying FBS in the OB. Then, we will use behavioral manipulations to investigate FBS plasticity in the OB. Finally, we will selectively silence adult-born neurons and characterize the effects of this manipulation on FBS and FBS plasticity.

Do OB neurons perform FBS?

One of the least studied but yet most important aspect of olfactory processing is the ability to distinguish between meaningless background odors and a meaningful odor (e.g., predator odor, food odor, etc.). We will use a previously established experimental protocol for studying FBS (Gottfried, 2010; Kadohisa and Wilson, 2006). This protocol

\[ \text{Figure 2: FBS - concept and experimental design. A. Conceptual demonstration of the phenomenon and experimental design. Prolonged exposure to pear tree leaves will induce a specific adaptation to them, while enhancing detection of a novel stimulus (pears). B. Experimental protocol for FBS (see text). Taken from Gottfried (2010), based on Kadohisa and Wilson (2006).} \]
is based on the assumption that a certain odor can become "background" due to prolonged exposure to it (Goyert et al., 2007). On top of this background, a new, "figure", odor is added (Fig. 2A-B). Using this protocol one can then test if neurons respond to simultaneously presented odors simply as the inhaled stimulus (i.e., the mixture) under any condition, or if they can separate a novel odor from an odor background under certain conditions. Using this protocol (Fig. 2B), neurons that perform FBS, will respond to A+B differentially between the two conditions (Fig. 2B, left panel vs. right panel). This difference can arise from changes (increase or decrease) in the response to B, to A, or to both. There are many possible combinations for FBS to take form in neuronal response profiles and we plan to test these in our dataset. Notably, this is a reexamination of (or even a challenge to) the current notion that OB neurons do not perform FBS (Gottfried, 2010). Unlike a previous report, which used biased extracellular recordings (Kadohisa and Wilson, 2006), here we will use more sensitive and unbiased measurements – juxtacellular and whole-cell recordings. Juxtacellular recording is an unbiased method to sample spiking activity, and whole-cell recording reports sub-threshold information. The same previous report also used overly simple analyses (Kadohisa and Wilson, 2006). Here, we will use more sensitive analyses, which also take into account other parameters in neuronal output like the timing of spiking/sub-threshold activity with respect to the respiration cycle (Bathellier et al., 2008; Dhawale et al.; Fantana et al., 2008).

**FBS plasticity in the OB**

After showing that OB neurons do perform FBS, we will investigate FBS plasticity. To do so we will first train mice to associate a specific odor with a food reward. We hypothesize that this behavioral contingency will lead to enhanced FBS, with this odor as the "figure". This enhancement could, for example, be in the form of a stronger response to the figure odor, or a larger proportion of cells exhibiting FBS to this odor. Next, the same mice will undergo odor enrichment with the same rewarded odor (same enrichment protocol as in part I). This type of enrichment has been shown to decrease odor responsiveness of OB mitral cells (Buonviso and Chaput, 2000). Accordingly, we hypothesize that enrichment will transform this odor from "figure" to "background". We will thus record neuronal responses of OB neurons to this odor in three experimental conditions: "naive" (no manipulation), "figure", and "background". We expect that even if OB neurons will not respond to this odor as to a "figure" in the "naive"
condition, the abovementioned behavioral manipulations will transform the response profiles to be similar those to a "figure" odor (Fig. 2C). We also expect that subsequent enrichment with the same odor will transform the response profile to a “figure” odor to be similar to those to a "background" odor (Fig. 2C). All these changes will controlled for possible non-FBS changes in the general response profiles of OB neurons due to the behavioral manipulation alone.

The role of adult-born neurons in FBS and FBS plasticity

After establishing that OB neurons perform FBS, and that this capacity can be modified by behavioral contingencies, we will next test if adult-born neurons are essential for FBS and FBS plasticity. To this end, we will utilize genetically engineered mice, in which adult-born neurons' presynaptic output is genetically silenced by inducible expression of tetanus toxin light chain (Imayoshi et al., 2009; Kim et al., 2009; collaboration with Prof. Kageyama, Kyoto University). From the time of the activation of this genetic switch all differentiating adult neural stem cells will be silenced. Accordingly, all neurons born from the time of the genetic switch onwards will be silenced. We will perform the FBS protocol on mice with silenced adult-born neurons either 14 days after induction (only immature adult-born neurons will be silenced), or 75 days after induction (mature adult-born neurons are also silenced), and compare them to control mice. This will test if adult-born neurons are essential for basic FBS. Next, we will use the same mouse model to test if adult-born neurons are essential for FBS plasticity using the abovementioned behavioral manipulations. We hypothesize that this approach will reveal that adult-born neurons are essential for FBS plasticity, either during development and maturity, or only during maturity.
Preliminary Results

Part 1

This part has been mostly completed (Livneh et al., 2009; Livneh and Mizrahi, under revision) and will be described here only in brief.

In order to explore the plasticity of adult-born neurons at the single cell level, we developed an experimental system for imaging in vivo their dendritic structure and synapses. We transduced adult-born neurons at the stem cell niche (subventricular zone and/or rostral migratory stream) with a lentivirus encoding a fluorescently-tagged postsynaptic marker (PSD95-GFP; Fig. 3A). This enabled us to image, in vivo, the dendrites and putative synapses of adult-born neurons (Fig. 3B-C). To verify that PSD95-GFP puncta are a good proxy for synapses we also performed targeted immunoelectron microscopy (Fig. 3D) and immunohistochemistry experiments (data not shown, see Livneh et al., 2009). As a simple model for manipulating sensory experience we used a 10 day odor enrichment protocol.

Figure 3: An experimental system for imaging the synapses and dendritic structure of adult-born neurons, in vivo. A. Schematic experimental protocol. Lentil. Inj.: lentivirus injection; 2PI: in vivo two-photon imaging; SVZ: subventricular zone; RMS: rostral migratory stream. B-C. Two-photon micrographs of an adult-born GC dendrite (B) and PGN (C), expressing PSD95-GFP. Notice the fluorescence in the spine head in the GC, but on the dendritic shaft in the PGN. Scale bar are 5 μm (B) and 20 μm (C). D. Immunoelectron micrograph of a PSD95-GFP labeled synapse between an adult-born PGN and an olfactory receptor neuron. Arrow: anti-GFP immuno-labeling. Scale bar: 200 nm.
Plasticity of young adult-born neurons

In order to explore the plasticity of young adult-born neurons we combined time-lapse two-photon imaging with intrinsic signal imaging (ISI) and odor enrichment. This approach allowed us to achieve experimental control of sensory activity, as only certain regions of the OB are enriched. Thus, we could test experience-dependent plasticity of developing adult-born neurons. We first injected a PSD95-GFP expressing lentivirus to label and birth-date adult-born neurons. Immediately thereafter, we performed odor-evoked ISI, to locate odor-active regions (Fig. 4A-B). We then enriched the mice for 10 days with the same odor. After 10 days of odor enrichment we performed in vivo time-lapse two-photon imaging and compared neurons in enriched and non-enriched regions of the OB. Using this approach, we compared enriched and non-enriched neurons in the same tissue (Fig. 4C).

Odor enrichment caused a two-fold increase in the dendritic structure and number of putative synapses of young adult-born PGNs (Fig. 4D-G). This caused enriched immature PGNs to be indistinguishable in overall size and number of synapses from mature PGNs (45 days old; Fig. 4D-G). Non-enriched PGNs were indistinguishable from PGNs from naïve mice, validating that they can serve as an inner control. Similar data were obtained for GC spine density (data not shown). Thus, developing adult-born neurons exhibit robust plasticity in face of manipulations of sensory experience. Because this enrichment increases their number of synapses, it will most likely affect their synaptic connectivity, potentially affecting OB function.
Figure 4: Developmental plasticity of adult-born PGNs. A. Schematic representation of the experimental protocol (upper panel), and time-course of the ISI-targeted 2P imaging experiment (lower panel). (B-C) A representative ISI-2P experiment. (B) Odor map (upper left panel), and blood vessel map (upper right panel), of the intrinsic signal response to the odor mixture (IS-active domains are circled in black in the blood vessel map). Two regions of interest (ROIs), containing the neurons shown in 'C', are circled in both maps. ROI 1 is in an IS-non-active domain, while ROI 2 is within an IS-active domain. Bottom panel: the intrinsic signal time-course of a single trial in ROIs 1 and 2. Notice the intrinsic signal response in ROI 2, but no response in ROI 1. Black trace: pure oxygen, grey trace: odor. Horizontal black line shows the stimulus duration (4 sec). A: anterior, P: posterior, L: lateral, M: medial; scale bar: 1 mm. (C) Two adult-born PGNs from the same experiment shown in 'B', one from a non-enriched domain (IS-non-active, ROI 1) and another from an enriched domain (IS-active, ROI 2). Top, maximum projection images of the original Z stacks. Bottom, two-dimensional view of the reconstructed neurons at the top. Scale bar: 20 μm. (D-G) Quantitative morphological comparisons of neurons from enriched domains and non-enriched domains (black and white bars, respectively). Neurons from enriched domains had significantly greater total dendritic branch length (TDBL; D), number of branch points (E), and number of puncta per neuron (F). (G) Sholl analysis, showing that the significant differences between neurons in enriched domains vs. those in non-enriched domains, were centered between 20–50 μm from the cell body. All higher values of enriched neurons were similar to those of mature neurons from naïve mice (dashed grey bars and lines). The lower values of neurons from non-enriched domains were similar to those of immature neurons from naïve mice (dashed grey bars and lines). n=9 PGNs in enriched domains and n=8 PGNs in non-enriched domains. Morphological data of immature and mature neurons from naïve mice are the same as in Figure 4, and are presented here as dashed grey bars and lines. All values are mean ± s.e.m. *p<0.001 (Kruskal-Wallis test, followed by Mann-Whitney test).Imm.- naïve: immature PGNs from naïve mice, mat.- naïve: mature PGNs from naïve mice, imm.- enrich.: immature PGNs from enriched domains in enriched mice, imm.- non-enrich.: immature PGNs from non-enriched domains in enriched mice.

Plasticity of mature adult-born neurons

To test the plasticity of mature adult-born neurons, we used a similar experimental approach with a few variations. We used a modified version of our cranial window procedure, which allowed long-term time-lapse imaging with multiple imaging sessions (Adam and Mizrahi, 2011, In press). We injected lentivirus, implanted a chronic cranial window, and approximately 60 days later (i.e., long after the neurons had matured) we performed ISI (Fig. 5A-C). We then performed time-lapse two-photon imaging before, during and after enrichment (Fig. 5). We did not detect any substantial changes in the general structure of adult-born neurons due to enrichment (Fig. 5D-E), indicating that there is a developmental critical period for this form of experience-dependent plasticity. As a more sensitive measure for plasticity we then tested synaptic dynamics, which are a well-established measure for experience-dependent
plasticity in mature neocortical neurons (Holtmaat and Svoboda, 2009). Specifically, each neuron was imaged 8 times in total, twice before enrichment, twice during a short enrichment period (2 days), twice during a longer enrichment period (10 days), and twice 1 week after enrichment ended (Fig. 5A-B). Each of these 4 imaging epochs was used to assess the short-term synaptic dynamics during the course of the experiment. This approach allowed us to detect significant changes, which could not be detected otherwise due to large inter-neuronal variability.
Figure 5: The experimental design for studying experience-dependent plasticity of mature adult-born neurons. A-B. Schematic experimental setup and time-line of the experimental protocol. C. Activation map of coffee odor from a representative experiment. The locations of one enriched (D) and one non-enriched (E) abPGNs are indicated by asterisks. Scale bar: 0.5 mm. D-E. Two-photon micrographs (top) and reconstructions (bottom) of abPGNs from enriched (D) and non-enriched (E) loci (same location shown in 'C'). Two of eight imaging sessions are shown (left: before enrichment; right: after a 10 day enrichment period). Red dots correspond to PSD95-GFP puncta. Scale bar: 20 μm. Odor enrichment did not cause any substantial change in the general dendritic structure of either neuron. RMS: rostral migratory stream.

A two day enrichment period was enough to induce a marked stabilization of puncta dynamics, in both types of adult-born neurons (Fig. 6). Surprisingly, puncta dynamics did not revert back to baseline even 1 week after ending enrichment (Fig. 6). These data suggest that mature adult-born neurons switch to a mode of stable synaptic dynamics in response to enrichment. Notably, this effect was specific to adult-born neurons in enriched OB loci, as the puncta dynamics of non-enriched adult-born neurons remained continuously dynamic throughout the course of the experiment (Fig. 6). This demonstrates that mature adult-born neurons can undergo experience-dependent plasticity well after they have matured and integrated into the network. As a result, mature adult-born neurons may endow the network with a reliable, stable and long-lasting substrate for plasticity. Our results demonstrate that both developing and mature adult-born neurons undergo experience-dependent plasticity. Accordingly, we hypothesize that adult-born neurons (developing or mature, or both) may be essential for dynamic sensory processing in the OB, particularly processing that is shaped by sensory experience. In the next part, we will test this hypothesis using FBS as an example of OB processing.
Figure 6: Odor enrichment stabilizes PSD95-GFP puncta dynamics of mature adult-born neurons. A,C. Two-photon micrographs from 8 consecutive imaging sessions of an enriched and non-enriched PGNs (A) and GCs (C). Green, red and blue arrowheads show representative stable, lost and new puncta, respectively. Scale bar: 5 μm. B, D. Quantitative analysis of the short-term PSD95-GFP puncta dynamics in enriched (blue) and non-enriched (red) PGNs (B) and GCs (D), normalized to the initial dynamics before enrichment. Thin lines show individual neurons, thick lines show means. Odor enrichment significantly stabilized PSD95-GFP puncta dynamics in odor-enriched neurons but not in non-enriched ones. N=10 enriched and 7 non-enriched PGNs, 13 enriched and 11 non-enriched GCs from 5 mice. All values are mean±s.e.m. *p<0.01, **p<0.004, ns: not significant. Wilcoxon Signed Rank test, corrected for multiple comparisons.
Part 2

*In vivo recordings of odor responses in OB neurons*

In order to test the role of adult-born neurons in FBS, we first established a system for recording odor evoked activity in OB neurons. To date, we have only designed the setup and performed *in vivo* juxtacellular and whole-cell current clamp recordings from a few putative mitral cells and GCs in anesthetized mice (Fig. 7). An example of a single unit recording is shown in figure 7A. This putative mitral cell had a delayed excitatory response to the presented odor, and its firing pattern was only partially locked to respiration. Figure 7B shows an example of a single unit recording from a GC. This GC's firing rate was substantially lower than that of the mitral cell in figure 7A, and its firing pattern was locked to respiration. In addition, this GC responded to the odor with an inhibitory delayed response. These changes in firing rates were stimulus specific, reliable and

*Figure 7:* *In vivo* recordings from OB neurons. A. Juxtacellular recording from a putative mitral cell. Top: voltage trace, bottom: respiration. B. Juxtacellular recording from a granule cell. Top: voltage trace, bottom: respiration C. Whole-cell current clamp recording from a granule cell.

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were notable even after a single trial across many recordings (data not shown). To demonstrate our ability to gain access to sub-threshold events, we also performed whole-cell current clamp recordings. Figure 7C shows an example of a recording from a GC. This GC responded to both odors with a rapid depolarization, followed by a hyperpolarization. This odor response pattern occurred in response to all four examined odors (only 2/4 odors shown). These results provide a "proof of concept" that we are technically capable of performing the FBS experiments.
Methods

Animals

We used Balb/C and C57B6 mice (8-13 weeks old at the beginning of the experiment). Animal care and experiments were approved by the Hebrew University Animal Care and Use Committee.

Adult-born neuron labeling

To label adult-born neurons and synapses for in vivo imaging, we injected a lentivirus expressing PSD95-GFP into the subventricular zone (SVZ) and/or rostral migratory stream (RMS). PSD95-GFP is a postsynaptic density scaffold protein, which is largely restricted to synapses and is a postsynaptic marker commonly used in vitro and in vivo in numerous preparations (e.g., Minerbi et al., 2009; Niell et al., 2004). PSD95-GFP has been previously used by us and others to explore synapses of adult-born OB neurons (Kelsch et al., 2008; Kelsch et al., 2009; Livneh et al., 2009). PSD95-GFP puncta in adult-born OB neurons label synapses and does not affect the morphological, biophysical and synaptic properties of adult-born neurons (Kelsch et al., 2008; Kelsch et al., 2009; Livneh et al., 2009). PSD95-GFP labeling patterns in PGNs and GCs reflect the fact that most PGN synapses are on the dendritic shaft, while most GC synapses are on dendritic spines. We used a lentivirus encoding PSD95-GFP, controlled by the Elongation–Factor–1–α promoter. Virus titers were >10⁸ transducing units/ml.

Surgical procedure, two-photon imaging, and analysis

Lentivirus injections into the RMS were performed as described earlier (Livneh et al., 2009). Mice were anesthetized using ketamine (100 mg per kg of body weight) and medetomidine (0.83 mg per kg of body weight). Depth of anesthesia was assessed by monitoring the pinch withdrawal reflex. Injections were done stereotaxically using pressure (coordinates relative to Bregma: anterior-3.3 mm, lateral-0.8 mm, ventral-2.9 mm). Part of the skull overlying both OBs was carefully removed, leaving the dura intact. A 3-mm diameter circular glass cover slip (no. 1) was positioned over the opening and sealed in place using histoacryl (TissueSeal, USA) and dental cement. A 0.1 g metal bar was glued to the skull for repositioning the animal’s head under the microscope in consecutive imaging sessions. After surgery, mice fully recovered and returned to the animal facility under normal housing conditions until imaging. Imaging was performed in anesthetized freely breathing mice through the cranial window.

Imaging was performed on an Ultima microscope (Prairie Technologies) equipped with a 40X (0.8 NA) IR–Achroplan water-immersion objective (Olympus). A femtosecond laser (Mai–Tai, Spectra Physics) was used to excite GFP at 920 nm. Images (512 x 512 pixels) were acquired at 0.25 μm/pixel resolution in the XY dimension and 0.9 μm steps in the Z dimension.
Reconstructions were performed manually from the complete three dimensional image stacks using Neurolucida (MicrobrightField). Puncta dynamics were analyzed from the complete 3D stacks using ImageJ (http://rsb.info.nih.gov/ij/) with the ‘Cell-Counter’ and ‘Sync-Windows’ plugins. For display purpose only, image stacks were filtered, projected as standard deviation projections, and adjusted for contrast and brightness using ImageJ. Additionally, we have previously validated that shorter imaging intervals (~20 min) do not hold substantial dynamics, and that lost puncta are not simply puncta that underwent photo-bleaching (Livneh et al., 2009).

**Intrinsic signal imaging and analysis**

For each mouse, we used one of two odors - either an odor composed of a low-concentration mixture of butanal or methyl-benzoeate (1:1; 20 ppm each; Sigma-Aldrich), or grained instant coffee. A neuron, imaged in vivo, was considered “within” an ISI-active locus (and thus classified as "enriched") if its locus obeyed both threshold and temporal dynamics criteria of the ISI signal (see below). ISI maps remain stable before and after enrichment, precluding the possibility of major physiological changes in olfactory receptor neurons input (Livneh et al., 2009).

Intrinsic signal imaging (ISI) of the dorsal surface of the bulb was performed using an Imager 3001 (Optical Imaging) via the cranial window. Before each ISI experiment, the surface blood vessel pattern was acquired under green light illumination (546 nm). Light reflectance from the surface of the OB (630 nm wavelength light illumination) was captured using a CCD camera (Dalsa 1M60P). Images were acquired with a spatial resolution of ~20 μm/pixel (full frames were 1024x1024 pixels and binned (3x3) for analysis). IS maps were analyzed off-line, using a Matlab-based software which was developed and kindly provided to us by H. Spors (Max Planck Institute of Biophysics, Frankfurt, Germany). Normalized ISI signal was obtained by dividing the ISI maps by the first 5 frames. ISI maps were the averaged response to the odorant in 4-8 trials. ISI images were first analyzed in a course manner (by signal threshold) to designate active or non-active areas for reference during two-photon imaging. The surface blood vessel pattern was later used to align intrinsic signal images with two-photon images as described previously (Livneh et al., 2009). A more detailed analysis was later used at the exact location of all the imaged PGNs and GC dendrites. Specifically, each location was examined based on its time course and signal strength. Active areas exhibited a time course that was coupled to the stimulus onset and showed a significant decrease in intrinsic signal (dR/R>4X10⁻⁴). Non-active areas did not meet both conditions.
Odor enrichment

Odor-enriched mice were housed in their home cages with an “odor pot” (i.e., tea ball) hanging from the top of the cage. This odor pot contained either 150μl of 20 ppm odor mixture (methyl-benzoate and butanal, 1:1 diluted in mineral oil) on a swab, or instant coffee grains. The odorant was replaced approximately every 24 h. The odor pot was present in the cage throughout the experiment, from the first imaging session, to avoid novelty effects.

Immuno-Electron Microscopy

Mice were perfused transcardially using 300 ml of 0.25% glutaraldehyde and 2% formaldehyde in 0.1M PB, pH 7.4 at room temperature (RT), at a rate of 10 ml/min. OBs were dissected out and first cut into 60 μm coronal slices using a vibratome (Leica VT1000). Vibratome sections were screened for labeled PGNs using standard fluorescent microscopy. Once the labeled PGNs were located, the slices were aligned and photographed (using a still camera), and a region corresponding to ~5 glomeruli was then isolated for further targeting of the cells. Isolated regions, normally containing one labeled PGN, were then processed for TEM separately. To abolish endogenous peroxidases, the sections were incubated for 10 min in 0.015% H2O2 (in PBS). Sections were cryoprotected in a solution of 15% sucrose/5% glycerol in PBS for 20 minutes in RT and then in a solution of 30% sucrose 10% glycerol in PBS in 4°C, overnight. Then, sections were frozen in liquid nitrogen-cooled isopentane, followed by liquid nitrogen and thawed in PBS. For immunolabeling, the sections were incubated in a blocking solution containing 5% normal rabbit serum (NRS), 2% bovine serum albumin (BSA), 0.5% glycine, 0.5% lysine, 0.01% triton X-100 and 0.13% sodium azide in PBS for 1 hour (at RT). Slices were incubated overnight in an anti-GFP primary antibody (chicken anti-GFP 1:600, Millipore, USA) in 2% NRS and 0.01% triton X-100 in PBS, and then with a biotinylated secondary antibody (1:500 rabbit anti-chicken F(ab')2 fragment, Jackson Labs). Labeling was revealed using the avidin-biotin peroxidase complex (ABC Elite, Vector Labs) and 3, 3'-diaminobenzidine tetrachloride. Briefly, slices were incubated for 10 min at 60°C in a solution containing 2.6% hexamethylene tetramine, 0.2% AgNO3, and 0.2% Borax, washed in 2% sodium acetate and incubated for 5 min in 0.05% HAuCl4 at RT, washed in 2% sodium acetate and then incubated for 5 min in 3% Na2SO3 at RT. The sections were then post-fixed with 1% osmic acid and 1.5% potassium ferricyanide in 0.1M cacodylate buffer (pH 7.4), for 1 hour in RT, dehydrated in a graded series of ethanol (30-100%) and embedded in increasing concentrations (50%-100%) of Agar 100 resin (Agar Scientific. Essex, England). Once cured in a 60°C oven for 48 hours, ultrathin sections of the region, in which a labeled PGN was located, were sectioned using an LKB-3 ultramicrotome, collected onto thin bar 200# cuprum grids, and then stained with saturated aqueous uranyl acetate and lead citrate solutions. Control sections were processed as described above except that the primary antibody was
omitted. Control sections had no specific staining. Ultrathin sections were observed with a Tecnai 12 (Phillips, Eindhoven, the Netherlands) TEM equipped with MegaView II CCD camera and AnalySIS® version 3.0 software (SoftImaging System GmbH, Münster, Germany). Slices from one mouse were processed as described, but without the intensification and substitution with silver/gold particles. These slices were used as a control to verify that the intensification and substitution with silver/gold particles did not cause any alterations in the tissue.

Electrophysiology

Juxtacellular recordings were obtained using blind patch-clamp recording. Electrodes (4 - 7 MΩ) were pulled from filamented, thin-walled, borosilicate glass (outer diameter, 1.5 mm; inner diameter, 1.0 mm; Hilgenberg GmbH, Malsfeld, Germany) on a vertical two-stage puller (Narishige, EastMeadow, NY). Internal solution contained (in mM):140 K-gluconate, 10 KCl, 10 HEPES, 10 Na2-Phosphocreatine, 4 MgATP, 0.4 Na2GTP, 0.5 EGTA adjusted to PH 7.25 with KOH) and 2-3% low melting agar (type IIIa, Sigma-Aldrich, St. Louis, MO) was placed over the craniotomy to minimize pulsations. An increase of the pipette resistance to 10 - 200 MΩ resulted in most cases in the appearance of spikes. For whole-cell recordings negative pressure was applied to reach a GΩ seal and the membrane was breached by an abrupt application of negative pressure. All recordings were acquired using an intracellular amplifier in current clamp mode (Multiclamp 700A, Molecular Devices), acquired at 10 kHz (Digidata 1440A, Molecular Devices, Sunnyvale, CA) and filtered with a 50 Hz high pass filter for spike detection. Odors were presented using a custom-made olfactometer.
References


