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Cortical representations of olfactory input by trans-synaptic tracing

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In the mouse, each class of olfactory receptor neurons expressing a given odorant receptor has convergent axonal projections to two specific glomeruli in the olfactory bulb, thereby creating an odour map. However, it is unclear how this map is represented in the olfactory cortex. Here we combine rabies-virus-dependent retrograde mono-trans-synaptic labelling with genetics to control the location, number and type of 'starter' cortical neurons, from which we trace their presynaptic neurons. We find that individual cortical neurons receive input from multiple mitral cells representing broadly distributed glomeruli. Different cortical areas represent the olfactory bulb input differently. For example, the cortical amygdala preferentially receives dorsal olfactory bulb input, whereas the piriform cortex samples the whole olfactory bulb without obvious bias. These differences probably reflect different functions of these cortical areas in mediating innate odour preference or associative memory. The trans-synaptic labelling method described here should be widely applicable to mapping connections throughout the mouse nervous system.

The functions of mammalian brains are based on the activity patterns of large numbers of interconnected neurons that form information processing circuits. Neural circuits consist of local connections—where pre- and postsynaptic partners reside within the same brain area—and long-distance connections, which link different areas. Local connections can be predicted by axon and dendrite reconstructions[1], and confirmed by physiological recording and stimulation methods[2]. Long-distance connections are more difficult to map, as commonly used methods can only trace bulk projections with a coarse resolution. Most methods cannot distinguish axons in passing from those that form synapses, or pinpoint the neuronal types to which connections are made[1,2]. Trans-synaptic tracers can potentially overcome these limitations[2]. Here we combine a retrograde rabies-virus-dependent mono-trans-synaptic labelling technique[3] with genetic control of the location, number and cell type of 'starter' neurons to trace their presynaptic partners. We systematically mapped long-distance connections between the first olfactory processing centre, the olfactory bulb, and its postsynaptic targets in the olfactory cortex including the anterior olfactory nucleus (AON), piriform cortex and amygdala (Supplementary Fig. 1).

Genetic control of trans-synaptic tracing

Rabies virus can cross synapses from postsynaptic to presynaptic neurons with high specificity[4], without notable defects in the morphology or physiology of infected neurons for extended periods of time[3,5]. Recent genetic modifications of rabies virus have permitted mono-trans-synaptic labelling[3]. Specifically, the rabies envelope glycoprotein (G) required for viral spread was replaced with a fluorescent marker[6]. Further, the virus was pseudotyped with EnvA, an avian virus envelope protein that lacks an endogenous receptor in mammals, and thus cannot infect wild-type mammalian cells. However, it can infect cells expressing the EnvA receptor TVA, and can subsequently produce infectious particles if TVA-expressing cells also express G to complement the *ΔG* rabies virus (Fig. 1a, bottom). The new viral particles can cross synapses to label presynaptic partners of starter neurons. As trans-synaptically infected neurons do not express G, the modified virus cannot spread from them to other neurons. Paired recordings in cultured brain slices support the efficacy and specificity of this strategy[3].

To extend this method to a limited number of starter cells of a defined type and at a precise location *in vivo*, we combined mouse genetics and viral infections (Fig. 1a, b). We created a transgenic mouse (*CAG-stop-tTA2*) that conditionally expresses the tetracycline transactivator tTA2 under the control of a ubiquitous *CAG* promoter only upon Cre-mediated excision of a transcriptional stop cassette. After crossing these mice with transgenic mice expressing the tamoxifen-inducible Cre (CreER), a small fraction of CreER$^+$ cells also express tTA2 following tamoxifen induction. We then used stereotactic injections to deliver into specific regions of the brain an adeno-associated virus (AAV) serotype 2 expressing three proteins: histone–GFP, TVA and G, under the control of a tetracycline-response element (*TRE*). Expression of TVA and G allows infected, tTA2$^+$ cells to be receptive to infection by the modified rabies virus, which we injected into the same location two weeks later. We define starter cells as those infected by both AAV and rabies virus, and therefore labelled by both histone–GFP and mCherry; their presynaptic partners are infected only by rabies virus and therefore express only mCherry.

We tested our strategy by using a ubiquitously expressing *actin-CreER*[7] in combination with *CAG-stop-tTA2* in the neocortex. Starter cells could be unambiguously identified by histone–GFP expression (Supplementary Fig. 2). In all but one case, we observed more than one starter cell (Supplementary Fig. 3 shows the example of a single starter cell). In a typical example, 35 starter cells in the motor cortex expressed histone–GFP and mCherry (Fig. 1c (3)), demonstrating that AAV and rabies virus can infect the same cells *in vivo*. In addition to many locally labelled cells (Fig. 1c (1)), mCherry$^+$ cells were

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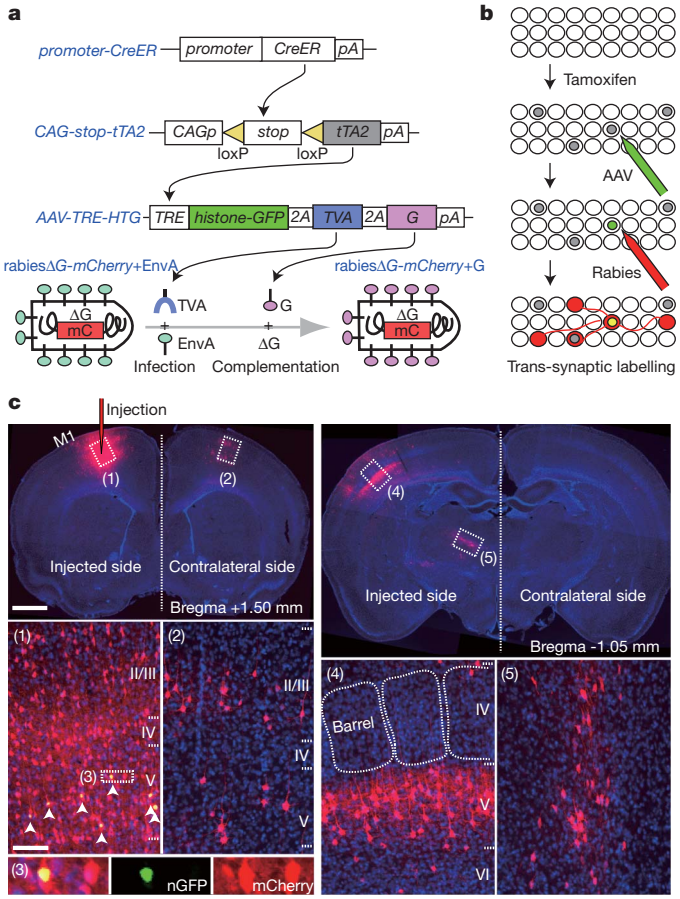

Figure 1 | Genetic control of rabies-mediated neural circuit tracing. **a**, **b**, Schematic representation of the methodology used to control the location, number and type of starter cells for rabies-virus-mediated trans-synaptic labelling. tTA2 is expressed in a small subset of CreER$^+$ cells (grey nuclei in **b**). tTA2 activates an AAV-delivered transgene to express: (1) a histone–GFP marker to label the nuclei of starter cells in green; (2) EnvA receptor (TVA) to enable subsequent infection by EnvA-pseudotyped rabies virus (rabies*ΔG-mCherry*+EnvA); and (3) rabies glycoprotein (G) to initiate trans-synaptic labelling. **c**, Top left, a 60-μm coronal section that includes the injection site in the motor cortex (M1). Cells labelled with both histone–GFP (nGFP) and mCherry (arrowheads in **c** (1), magnified in **c** (3)) can be distinguished from cells labelled with mCherry alone, which are found near the injection site (**c** (1)), in the contralateral motor cortex (**c** (2)), in the somatosensory barrel cortex (top right; magnified in **c** (4)), and in the motor-specific ventrolateral nucleus of the thalamus (**c** (5)). Scale bars, 1 mm for low-magnification images at the top, 100 μm for high-magnification images at the bottom.

enriched in layers II, III and V in the contralateral motor cortex (Fig. 1c (2)), consistent with layer specificity of callosal projections[8]. mCherry$^+$ cells were also found in layers III and V of the ipsilateral somatosensory cortex (Fig. 1c (4)) and in motor-specific thalamic nuclei (Fig. 1c (5)), which are known sources of monosynaptic inputs to the motor cortex[9].

In all experiments, histone–GFP$^+$ cells were found within 450 μm of the injection sites, consistent with a previous report that AAV serotype 2 predominantly infects neurons locally[10]. Omitting AAV or tamoxifen yielded no trans-synaptically labelled neurons (Supplementary Fig. 4). Moreover, our strategy labelled neurons only through synaptic connections but not through axons in passage (Supplementary Fig. 5). Finally, rabies virus spread was restricted to neurons directly connected to starter cells, and only in the retrograde direction (Supplementary Fig. 6). Together, these experiments validated our genetic strategy for retrograde mono-trans-synaptic labelling *in vivo*.

AON maintains the dorsal–ventral topography

In the mouse, olfactory receptor neurons that express a single type of odorant receptor send convergent axonal projections to a specific pair of glomeruli in the lateral and medial olfactory bulb[11–13]. Odorants are detected by combinations of olfactory receptor neuron classes[14], and are represented as spatiotemporal activity patterns of glomeruli[15]. Each mitral cell sends its apical dendrite to a single glomerulus and thus receives direct input from a single olfactory receptor neuron class. Mitral cell axons relay information to the olfactory cortex (Supplementary Fig. 1a). Previous axon tracing studies showed that individual mitral cells send axons to distinct cortical areas, and that small cortical regions receive broad input from the olfactory bulb[16–19]. However, understanding the principles underlying odour perception and odour-mediated behaviours requires systematic and quantitative analysis of connection patterns of mitral cells with their cortical target neurons.

We first established that mitral cells throughout the olfactory bulb can be infected by rabies virus via their axons (Supplementary Fig. 7). We then applied our strategy (Fig. 1a, b) to specific areas of the AON, piriform cortex and cortical amygdala (Supplementary Fig. 1b), and examined the distribution of trans-synaptically labelled mitral cells. In a typical example, 11 clustered starter cells in the AON (Fig. 2a) resulted in 69 labelled mitral cells distributed widely across the olfactory bulb (Supplementary Fig. 8 and Supplementary Movie 1). Bright mCherry fluorescence from rabies virus allowed us to unequivocally follow the primary dendrites of the labelled mitral cells to single target glomeruli (Fig. 2b). Each mitral cell sent its apical dendrite into a single glomerulus. Four glomeruli were each innervated by two labelled mitral cells (Fig. 2b, right, and Supplementary Table 1).

To quantitatively compare the patterns of labelled glomeruli from different animals, we established a three-dimensional (3D) reconstruction protocol for the olfactory bulb, and aligned each olfactory bulb to a standard olfactory bulb model (Fig. 2c). To test the accuracy of this procedure, we reconstructed and aligned olfactory bulbs from three *P2-IRES-tauGFP* transgenic mice[20]. These GFP-labelled glomeruli were located within a distance of a few glomeruli from each other (Supplementary Fig. 9), consistent with the natural variability of olfactory receptor neuron axon targeting[21]. This precision of our 3D reconstruction enables the comparison of olfactory bulbs from different animals.

The AON has been proposed to provide feedforward modification of information from the olfactory bulb to the piriform cortex[22]. Little is known about its organization except for a small and distinct AON pars externa, which maintains dorsal–ventral olfactory bulb topography[23–25]. We injected AAV and rabies virus to different areas of the AON (Supplementary Table 1), and established an AON 3D-reconstruction protocol analogous to that for the olfactory bulb (Fig. 2c, left). Labelled glomeruli from AON injections were distributed widely in the olfactory bulb (Fig. 2c, middle). However, starter cells from the ventral and dorsal AON preferentially labelled ventral and dorsal glomeruli, respectively (Fig. 2c). To quantify the spatial distributions of starter cells in the AON and trans-synaptically labelled glomeruli in the olfactory bulb, we introduced a cylindrical coordinate system into the olfactory bulb and AON models, where Z represents the position along the anterior–posterior axis and θ represents the angle from the polar axis (Fig. 2c). No correlations were found between Z_{AON} and θ_{OB} (where OB is olfactory bulb), Z_{AON} and Z_{OB}, or θ_{AON} and Z_{OB} (Supplementary Fig. 10a). However, we found a strong positive correlation ($R^2 = 0.79$) between θ_{AON} and θ_{OB} (Fig. 2d), which correspond to the dorsal–ventral axes of the AON and olfactory bulb, respectively. Thus, the AON maintains the dorsal–ventral topography of the olfactory bulb.

A coarse topography exists between olfactory receptor neuron cell-body positions in the olfactory epithelium and target glomeruli in the olfactory bulb along the dorsal–ventral axis[26]. Specifically, the olfactory cell adhesion molecule (OCAM) is expressed in a subset of olfactory receptor neurons[27] that project to the ventral ~55% of glomeruli in the olfactory bulb. In the olfactory bulb, ~25° clockwise rotation of the

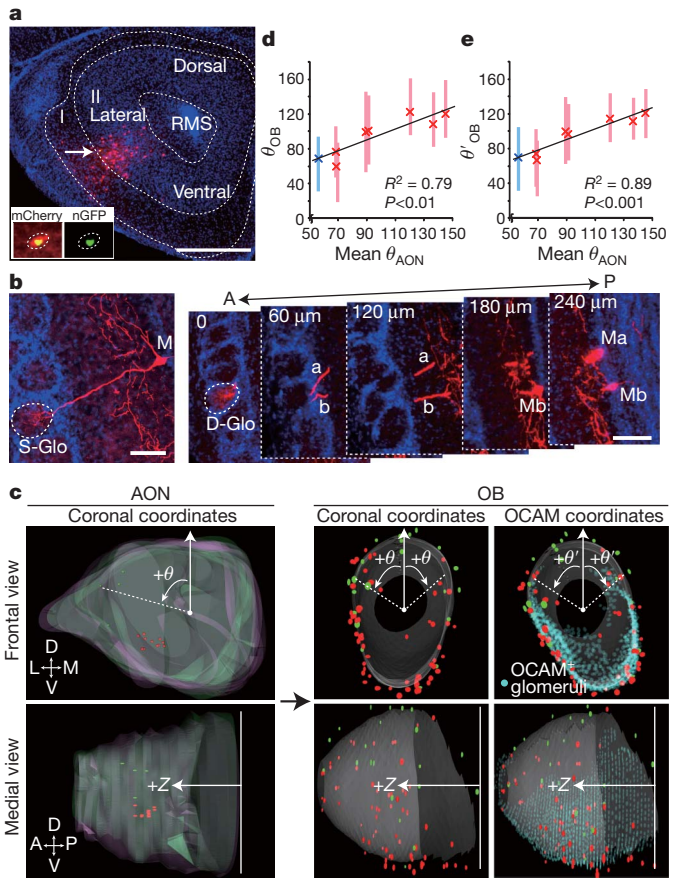

Figure 2 | The olfactory bulb to AON connections show a dorsal–ventral topography. a, A 60-µm coronal section with two starter cells located in layer II of the ventrolateral AON, one of which (arrow) is magnified in the inset. RMS, rostral migratory stream. Scale bar, 500 µm. **b**, Typical examples of trans-synaptically labelled mitral cells from cortical starter cells. Left, a 60-µm coronal section that captures both the cell body and the apical dendrite of a mitral cell. Right, more frequently, a mitral cell apical dendrite spans several consecutive 60-µm coronal sections. S-Glo, glomerulus innervated by a single labelled mitral cell (M). D-Glo, glomerulus innervated by two labelled mitral cells (Ma, Mb). A, anterior; P, posterior. Scale bar, 100 µm. **c**, Superimposed 3D reconstructions of the AONs and olfactory bulbs (OBs) from two injected brains. Eleven red and four green starter cells from two AONs labelled red and green glomeruli, respectively. Light red and green, contours of two superimposed AONs. D, dorsal; L, lateral; M, medial, V, ventral. **d**, **e**, Correlations between θ_{AON} and θ_{OB} (**d**) and θ_{AON} and θ'_{OB} (**e**). Crosses represent mean θ_{AON} (x-axis) and mean θ_{OB} or θ'_{OB} (y-axis). Error bars represent 50% of the distribution surrounding the mean θ_{OB} or θ'_{OB}. R^2, square of Pearson's correlation coefficient; P, statistical significance tested against the null hypothesis assuming no correlation between θ_{AON} and θ_{OB} or θ'_{OB}. Red and blue, experiments using *actin-CreER* and *CaMKII-CreER*[47], respectively.

polar axis around the z-axis maximized the separation of OCAM$^+$ and OCAM$^-$ glomeruli (Supplementary Fig. 11). In this new OCAM coordinate system represented by θ'_{OB} (Fig. 2c, right), the correlation coefficient between θ'_{OB} and θ_{AON} increased to $R^2 = 0.89$ (Fig. 2e), showing that adjusting the dorsal–ventral axis of the olfactory bulb according to a biological marker improved the AON and olfactory bulb topographic correspondence. Thus, the topography between the olfactory epithelium and the olfactory bulb further extends to the AON.

Dorsally biased olfactory bulb input to amygdala

Mitral cell axons project to the anterior and posterolateral cortical amygdala[28,29]. The organization of this axonal input is unknown. We injected AAV and rabies virus to small areas within these regions, and mapped starter cells onto a common schematic drawing based on anatomical landmarks (Fig. 3a). Trans-synaptically labelled mitral cells and glomeruli from amygdala starter cells were broadly distributed in the olfactory bulb. However, the labelled glomeruli were enriched in the dorsal olfactory bulb (Fig. 3c). For quantification, we compared the mean experimental θ'_{OB} for each injection with mean θ'_{OB} values produced by computer simulation from the same number of glomeruli distributed randomly throughout the olfactory bulb ($^{sim}\theta'_{OB}$). For the AON experiments, the mean experimental θ'_{OB} values for the majority of the samples were significantly larger or smaller than the corresponding mean $^{sim}\theta'_{OB}$ values (Fig. 3e, left), reflecting the dorsal–ventral topography between the olfactory bulb and the AON. By contrast, none of the mean θ'_{OB} values from the amygdala was significantly larger than the corresponding mean $^{sim}\theta'_{OB}$ (Fig. 3e, middle). Six out of ten mean θ'_{OB} values from the cortical amygdala fell significantly below the corresponding mean $^{sim}\theta'_{OB}$ values. For these dorsally biased samples, the density of labelled glomeruli gradually decreased along the dorsal–ventral axis without a sharp boundary (Supplementary Fig. 12). Simple spatial correspondence between starter-cell locations and the degree of dorsal bias was not evident (Supplementary Fig. 10b). In summary, the cortical amygdala overall receives biased input from the dorsal olfactory bulb.

Less organized olfactory bulb input to piriform cortex

The piriform cortex is the largest cortical area in the olfactory cortex. Recent physiological analysis[30,31] found that neurons activated by specific odours are apparently not spatially organized; the underlying anatomical basis is unclear. We injected AAV and rabies virus into several areas in the anterior and posterior piriform cortex, and mapped starter cells from different brains onto a common schematic drawing of the entire piriform cortex based on anatomical landmarks (Fig. 3b). Labelled glomeruli were broadly distributed throughout the olfactory bulb, regardless of starter-cell locations in the piriform cortex (Fig. 3d). In sharp contrast to trans-synaptic labelling from the AON or amygdala, where different samples showed highly variable mean θ'_{OB}, mean θ'_{OB} values from the piriform cortex tracings were much less variable and closely resembled a random distribution (Fig. 3e). Only one out of ten samples had a mean θ'_{OB} slightly above the 95th percentile of the mean $^{sim}\theta'_{OB}$. Further, no strong spatial correspondence was evident in correlation analyses of θ'_{OB}, Z_{OB} and the location of starter cells in the piriform cortex (Supplementary Fig. 10c). These data indicate that highly restricted areas of the piriform cortex receive direct mitral cell input representing glomeruli that are distributed throughout the olfactory bulb with no apparent spatial organization.

Convergence of mitral cell input

Convergent inputs from different glomeruli to individual cortical neurons could allow the olfactory cortex to integrate combinatorial odour representations in the olfactory bulb. In support of this, previous studies have shown that odour receptive ranges of AON cells are broader than those of mitral cells[32], and that some piriform cortex neurons are activated by a binary odour mix but not individual components[31]. However, a large fraction of inputs in these studies could come from other cortical neurons through extensive recurrent connections (Figs 2a and 3a, b). Direct convergence of mitral cell axons onto individual cortical neurons is implied in physiological studies of piriform cortical neurons in slices[33–35]. Our trans-synaptic labelling enabled a direct examination of mitral cell convergence to individual cortical neurons *in vivo*.

The convergence index, defined by the number of labelled mitral cells divided by the number of the starter cells in the cortex, exceeded 1 in all experiments using *actin-CreER* (Fig. 4a and Supplementary Table 1). This finding demonstrates that individual cortical neurons receive direct inputs from multiple mitral cells *in vivo*. As the vast majority of labelled mitral cells corresponded to different glomeruli (Supplementary Table 1), individual cortical neurons must receive direct inputs representing multiple glomeruli. This convergence index

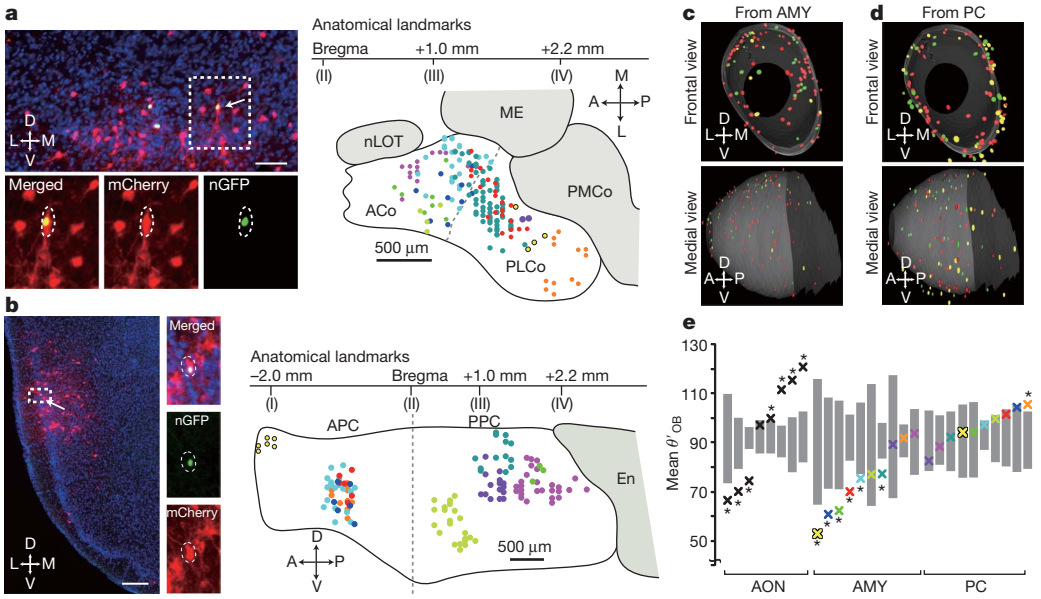

Figure 3 | Representations of olfactory bulb input in the amygdala and piriform cortex. **a**, **b**, Starter cells from the cortical amygdala and piriform cortex. Left, single coronal sections at the injection sites in the posterolateral cortical amygdala (**a**) and the posterior piriform cortex (**b**). Arrows point to starter cells magnified in insets. Scale bars, 100 µm in **a**, 200 µm in **b**. Right, schematic representations of ten independent injections each into amygdala (**a**) or piriform cortex (**b**). Starter cells from each injection are labelled with a specific colour. The dotted line denotes the rough border between the anterior cortical amygdala (ACo) and the posterolateral cortical amygdala (PLCo) based on anatomical landmarks according to a mouse brain atlas[48]. APC, anterior piriform cortex; En, lateral entorhinal cortex; ME, medial amygdala; nLOT, nucleus of lateral olfactory tract; PMCo, posteromedial cortical amygdala; PPC, posterior piriform cortex. **c**, **d**, Superposition of three independent 3D reconstructions of glomerular maps with starter cells from the cortical amygdala (AMY; **c**) or the piriform cortex (PC; **d**). **e**, Mean θ'_{OB} values (crosses) from each experiment are plotted in the same column with the 95% confidence intervals for corresponding ${}^{sim}\theta'_{OB}$ values (grey bars). Samples with experimental mean θ'_{OB} outside the 95% confidence intervals are labelled with asterisks ($^*P < 0.05$). Colours in **a** (scheme), **c** and **e** (amygdala) are matched to represent the same samples, and so are the colours in **b** (scheme), **d** and **e** (piriform cortex).

is probably an underestimate, as not all starter cells necessarily received direct mitral cell input (overestimation of the denominator), and not all cells presynaptic to starter cells were trans-synaptically infected by the rabies virus (underestimation of the numerator; see Supplementary Fig. 3).

The convergence indices varied widely in different experiments, and did not differ substantially in the three cortical areas we examined. However, in experiments that contained starter cells located in layer I, which is mostly composed of GABAergic local interneurons[36], the convergence indices were greater (Fig. 4a, red). Assuming all starter cells in a given layer contribute equally to mitral cell labelling, multiple regression analyses indicate that layer I neurons receive direct input from more mitral cells than layer II/III neurons (Fig. 4b).

To confirm the higher convergence index for layer I GABAergic neurons, we replaced the ubiquitous *actin-CreER* with *GAD2-CreER*, which is expressed only in GABAergic interneurons (Supplementary Fig. 13). We found that GABAergic neurons located in layer II or III of the piriform cortex received little direct mitral cell input, whereas those located in layer I showed a much greater convergence index (Fig. 4b, right, and Supplementary Table 1). Thus, cortical GABAergic neurons are highly diverse with respect to mitral cell innervation. These observations are in accordance with recent physiological studies[30,34], and suggest different physiological roles for these GABAergic neurons; layer I and deeper layer GABAergic neurons provide global feedforward and feedback inhibition to cortical pyramidal neurons, respectively.

Sister mitral cells connect independently

Each glomerulus is innervated by the apical dendrites of ~25 electrically coupled mitral cells[37]. We refer to these cells as 'sister' mitral cells. Sister mitral cells may preferentially connect to the same cortical postsynaptic target neurons compared to 'non-sister' mitral cells that receive direct input from different glomeruli. Such an organization could increase the signal-to-noise ratio in information transmission from mitral cells to cortical neurons. Alternatively, sister mitral cells may connect to cortical neurons independently to deliver olfactory information widely across different cortical neurons.

We used the frequency of dually labelled glomeruli from our data set and statistical simulation to distinguish between these possibilities. Dually labelled glomeruli (D) could result from a single starter cell (Ds) or two starter cells (Dt). Assuming that an individual starter cell can receive input from any of the 2,000 glomeruli, we compared the distribution of Ds derived from our data and from a simulation according to the null hypothesis that sister mitral cells connect independently with postsynaptic targets. If sister mitral cells share significantly more postsynaptic targets than at random, then the 'data Ds' distribution should be significantly higher than the simulated 'random Ds' distribution. In all but two cases, these two distributions were not statistically different (Fig. 4c). Both exceptions came from trans-synaptic labelling from the AON, which showed dorsal–ventral topography, so the original assumptions were not accurate. When we reduced the number of accessible glomeruli to 1,500, no sample showed significant differences. Thus, our analysis indicates that individual mitral cells innervating the same glomerulus act independently in making connections with their cortical targets.

Discussion

Our study revealed several general principles that define cortical representations of the olfactory bulb input. First, individual cortical neurons receive direct input from mitral cells originating from multiple glomeruli. On average, each excitatory neuron receives direct input from four mitral cells, but this number is likely to be an underestimate. Convergence of mitral cell inputs enables cortical neurons to integrate information from discrete olfactory channels. The lower bound of four already affords $\sim 10^{12}$ glomerular combinations for 1,000 olfactory channels, far exceeding the number of neurons in the mouse olfactory cortex. Thus, the olfactory cortical neuron repertoire samples only a

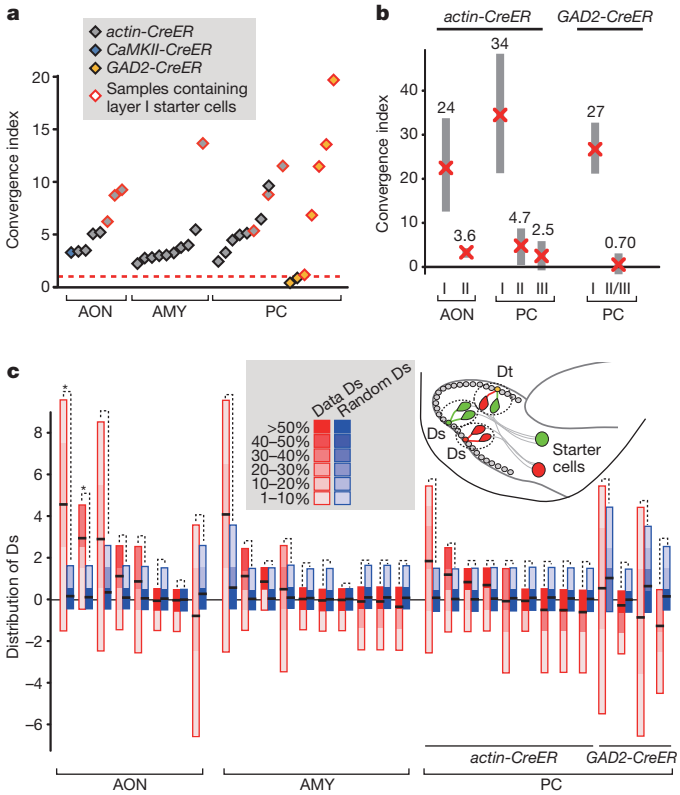

Figure 4 | Convergence and independence of mitral cell inputs. **a**, Convergence index for each cortical injection experiment is represented by a diamond, with the type and layer of starter cells specified by the colour code above. AMY, amygdala; PC, piriform cortex. **b**, Multiple regression analysis to estimate the convergence indices of starter cells located in different layers of the AON and piriform cortex. Estimated mean convergence indices (red crosses) and the corresponding 95% confidence intervals (grey bars) are shown. Data from *actin-CreER* and *GAD2-CreER* were analysed separately. Injections into amygdala produced only one sample that contained layer I cells and were therefore excluded. **c**, Schematic of dually labelled glomeruli (D) resulting from two starter cells (Dt) or a single starter cell (Ds). Comparison of the distributions of Ds derived from experimentally observed frequency of D (Data Ds; red) and from simulated D based on the null hypothesis detailed in Methods (Random Ds; blue). For each sample, the distributions of 'data Ds' and 'random Ds' are shown by coloured heat maps. $*P < 0.05$.

small fraction of all possible combinations of direct olfactory bulb inputs.

Second, neurons restricted to small olfactory cortical regions receive input from glomeruli that are broadly distributed in the olfactory bulb. Although similar findings were reported previously[16–19], our study provides a higher resolution analysis of direct connectivity between mitral cells and cortical neurons, rather than inferring connection from the presence of axons, which could be a major caveat of previous tracing studies (see Supplementary Fig. 5). At the same time, mitral cells representing the same glomerulus connect independently to post-synaptic cortical neurons, thus maximizing the spread of olfactory information originating from individual olfactory channels. Our finding is consistent with analyses of axon arborization patterns of singly labelled mitral cells (S. Ghosh and colleagues; manuscript submitted).

Third, different cortical areas receive differentially organized olfactory bulb input (Supplementary Fig. 1c). The AON maintains a coarse topography along the dorsal–ventral axis, suggesting a pre-processing role for olfactory-bulb-derived information before sending to other cortical areas. A lack of apparent spatial organization in the piriform cortex with regard to olfactory bulb input provides an anatomical basis for recent physiological studies[30,31], and suggests that the piriform cortex acts as an association cortex[31,38]. In the cortical amygdala, many neurons seem to receive strongly biased input from the dorsal olfactory bulb. Mice lacking olfactory receptor neurons that project to the dorsal olfactory bulb lose their innate avoidance of odours from predator urine and spoiled food, despite retaining the ability to sense these odours[39]. The cortical amygdala may preferentially process the olfactory information that directs innate behaviours. Our study is in agreement with similar findings using axon tracings from individual glomeruli (D. L. Sosulski and colleagues; manuscript submitted).

Interestingly, axonal arborization patterns of *Drosophila* olfactory projection neurons (equivalent to mitral cells) in higher olfactory centres show a similar organizational principle. Projection neuron axon arborization patterns in the lateral horn—a processing centre directing odour-mediated innate behaviour—are highly stereotyped with respect to projection neuron classes[40,41], and are partitioned according to the biological significance of the odorants[42]. Arborization patterns of axon collaterals of the same projection neurons in the mushroom body, an olfactory memory centre[43], are much less stereotyped[40,42], consistent with a physiological study indicating non-stereotyped connections[44]. Therefore, from insects to mammals, a common theme emerges for the representations of olfactory information: more stereotyped and selective representation of odours is necessary for directing innate behaviours, whereas broader and less stereotyped sampling of the whole olfactory space is better suited for brain regions implicated in associative memories.

The genetically controlled mono-trans-synaptic tracing described here should be widely applicable for mapping neuronal circuitry throughout the mouse brain. It is currently unknown how rabies virus crosses synapses, and whether the efficiency and specificity vary with cell type, connection strength and activity[3,5,45]. Further applications of these trans-synaptic methods to other neurons and circuits[46] will be necessary to address these questions. Nevertheless, the control experiments (Fig. 1 and Supplementary Figs 2–6) confirmed that our strategy labels neurons that are directly presynaptic to starter cells but not neurons whose axons pass through the injection sites without making synapses. Our method will be especially valuable for analysing long-distance connections that are usually refractory to physiological mapping strategies[2]. This method can be further extended to genetic manipulation of starter cells to combine circuit tracing with genetic loss- or gain-of-function experiments. These approaches will facilitate the investigation of not only the organization of information flow within neural circuits, but also the molecular basis of neuronal connections at single-cell resolution *in vivo*.

METHODS SUMMARY

Detailed methods on the generation of *CAG-stop-tTA2* mice, viral preparations, animal surgery, tissue processing, 3D reconstruction and quantitative analyses can be found in Methods.

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

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions K.M. planned and performed all the experiments. F.A. and F.M. developed the computer programs for 3D reconstructions and statistical simulation under the supervision of M.A.H. C.W. and Z.H. performed the initial AAV production. I.W., N.R.W. and E.M.C. provided the modified rabies virus and the construct to make the AAV vector. H.T. and Z.J.H. provided the *GAD2-CreER* mice. B.T. provided DNA constructs. L.L. supervised the project. L.L. and K.M. wrote the manuscript, with contributions from B.T.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to L.L. (lluo@stanford.edu).

METHODS

Generation of *CAG-stop-tTA2* Mice. The *tTA2* transactivator gene[49] was placed after the *CAG* promoter of plasmid *pCA-HZ2* (ref. 50) using a polymerase chain reaction (PCR)-based cloning method. A neomycin resistance (*neo*^{r}) gene and a transcriptional stop signal[51] were flanked by *loxP* sites to create a *loxP-neo*^{r}*-stop-loxP* cassette. This cassette was then introduced between the *CAG* promoter and *tTA2* using PCR-based cloning. An EcoRI fragment obtained from *ETLpA-/LTNL*[13], which contains the *IRES-tau-lacZ* cassette, was introduced after the *tTA2* coding sequence. The resulting cassette (*CAG-stop-tTA2-IRES-tau-lacZ*) was cloned into *pBT264* to flank the cassette with two copies of a ~250-bp β-globin *HS4* insulator sequence[52] on each side. *pBT264* (*pii-TRE-tdTomato-3Myc-ii*) was generated by inserting PCR-amplified copies of ~250-bp-long core insulator fragments (*i*) from the chicken β-globin HS4 insulator on each side of *TRE-tdTomato-3Myc* in *pBT239*. The insulator fragments were amplified from *pJC13-1* (ref. 53). The final construct, *pKM1* (*pii-CAG-stop-tTA2-IRES-tau-lacZii*), was tested by transient co-transfection with *pBT264* into cultured HEK293 cells. When a Cre-encoding plasmid *pBT140* (cytomegalovirus (CMV) promoter driving nuclear localization signal-*Cre*) was further introduced into the same cell, strong tdTomato fluorescence was detected 72 h after transfection. *pKM2* was digested with restriction enzymes SwaI and AscI, the insert was gel-purified using Qiagen gel extraction kit and eluted into 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA. The purified and linearized DNA devoid of plasmid backbone was used for mouse transgenesis via standard pronuclear injection procedure. Founders were screened by PCR primers to detect the *neo*^{r} gene. Four independent transgenic lines were established. They were crossed with mice containing *β-actin-CreER*[7] and *TRE-Bi-SG-T* reporter[53] to screen for functional *CAG-stop-tTA2* transgenes. Mice containing all three transgenes were injected with 1 mg of tamoxifen in corn oil at postnatal day (PD)10, and brains were collected at PD21 for the analysis. Two lines showed broad tdTomato fluorescence throughout the brain. One line (containing 2–3 copies of the transgene based on Southern blotting) was used exclusively in this study.

Virus preparations. All viral procedures followed the Biosafety Guidelines approved by the Stanford University Administrative Panel on Laboratory Animal Care (A-PLAC) and Administrative Panel of Biosafety (APB). To make the AAV containing the *TRE-HTG* cassette, which encodes histone–GFP, TVA and G linked by the 2A 'self-cleaving' peptides, the *HTG* cassette obtained from *pBOB-synP-HTB* (I.W. and E.M.C., unpublished plasmid) was placed after the *TRE-Tight* promoter in *pTRE-Tight* (Clontech), and then the entire construct was subcloned into the pAAV vector (Stratagene). Recombinant AAV serotype 2 was produced using the pAAV helper free kit (Stratagene) according to the manufacturer's instructions. AAV was also produced commercially by the Gene Therapy Center of the University of North Carolina. The AAV titre was estimated to be $\sim 4 \times 10^{12}$ viral particles ml^{-1} based on serial dilution and blot hybridization analysis. Pseudotyped *ΔG* rabies virus was prepared as previously described[3,54]. The pseudotyped rabies virus titre was estimated to be $\sim 5 \times 10^8$ infectious particles per ml based on the infections of cell line 293-TVA800 by serially diluted virus stocks.

Animal surgery. All animal procedures followed animal care guidelines approved by A-PLAC. To activate Cre in animals carrying a *CreER* transgene, we injected intraperitoneally 0.1–1 mg of tamoxifen (Sigma) dissolved in corn oil into mice around PD10. For trans-synaptic labelling, 0.1–0.3 μl of AAV-*TRE-HTG* was injected into brain at PD21 by using a stereotactic apparatus (KOPF). During surgery, animals were anaesthetized with 65 mg kg^{-1} ketamine and 13 mg kg^{-1} xylazine (Ben Venue Laboratories). For motor cortex injections, the needle was placed 1.5 mm anterior and 1.5 mm lateral from the Bregma, and 0.4 mm from the brain surface. For olfactory cortex injections, see Supplementary Fig. 1b for the stereotactic parameters. After recovery, animals were housed in regular 12 h dark/light cycles with food and water *ad libitum*. Two weeks later, 0.3 μl of pseudotyped rabies virus (Δ*G-mCherry*+EnvA) was injected into the same brain location under anaesthesia. After recovery, animals were housed in a biosafety room for 7 days to allow rabies virus to infect, trans-synaptically spread and express sufficient amount of mCherry to label presynaptic cells. All animals were healthy and their brain structures were normal 7 days after rabies virus infection, confirming non-pathogenicity of ΔG mutant rabies virus.

Tissue processing. Brain tissue was processed according to previously described procedures[55]. To set the common coronal plane among different animals, the cerebellum was cut off and the brain was embedded in the Optimum Cutting Temperature (OCT) compound (Tissue-Tek) with the cut surface facing the bottom of the mould. The brain was adjusted to ensure that the left–right axis was parallel to the section plane. Neither mCherry nor histone–GFP required immunostaining for visualization. In some cases, brain sections were immunostained for better signal preservation according to previously published methods[56] using the following antibodies: chicken anti-GFP (1:500; Aves Labs), rabbit anti-DsRed (1:1,000; Clontech), donkey anti-chicken fluorescein isothiocyanate (FITC) and donkey anti-rabbit Cy3 (1:200; Jackson ImmunoResearch). In most trans-synaptic labelling experiments starting from the olfactory cortex, every one of four sections of the olfactory bulb was immunostained by the free-floating method with goat anti-OCAM (1:100; R&D Systems) and donkey anti-goat Alexa488 (Invitrogen) to label OCAM$^+$ olfactory receptor neuron axons. For immunostaining against GABA, 60-μm free-floating coronal sections were treated with rabbit anti-GABA (1:2,000 in PBS with 0.3% Triton-X100; Sigma) for 48 h. GABA$^+$ cells were visualized with donkey anti-rabbit Cy3 (1:200; Jackson ImmunoResearch). Sections were imaged with a Nikon CCD camera by using a 10× objective or by 1-μm optical sectioning using confocal microscopy (Zeiss 510).

3D reconstruction. To compare distribution of labelled glomeruli (olfactory bulb) and starter cells (AON) across different samples, we needed to map them in a common 3D reference frame. To do this, we first saved manual annotations carried out in Adobe Illustrator in a scalable vector graphics (SVG) format. The SVG file saved all the annotations as an extensible markup language (XML) file describing the ellipses and contours (defined later), making it feasible to accurately parse the information by MATLAB scripts. In the olfactory bulb, we represented all glomeruli as ellipses. We used the centre of mass for each ellipse to define a single point, and calculated the centre of mass of all the points to define the centre of each slice. For the AON, we defined the contour as the boundary between layer I and layer II, which can be clearly distinguished by differences in the density of 4′,6-diamidino-2-phenylindole (DAPI) staining. To define the centre of mass for each contour, we replaced it with a dense series of points and used these points to calculate the centre of mass. Now, each slice is represented by a series of points and the centre of mass contained within an SVG file. To assemble the slices represented by SVG files into a 3D shape, we first aligned the centre of mass for each slice to that of the previous slice to form the cylindrical (*z*-)axis. Then, we refined the alignment by sequentially applying the iterative closest points (ICP) algorithm[56], which can identify the local rotation and translation parameters for each slice to maximize the overlap with the previous slice. Once we had aligned all the slices in a sample to generate a 3D shape, we needed to identify an orientation for the polar axis that could be most reliably identified in different 3D reconstructions. As the olfactory bulb is ellipsoidal, the principle component analysis (PCA) can reliably find a plane that contains the *z*-axis and intersects the 3D shape to maximize the surface of the intersection (plane *m*). We then defined the polar axis to be contained within the plane *m*, perpendicular to the *z*-axis, and pointing in the dorsal direction. For the AON, we approximated the contours of the most posterior slide of the AON as a triangle and calculated the rotation around the *z*-axis that minimizes the distance of the three vertices to those of a standard AON sample. We applied the same rotation to the whole 3D shape. To define the orientation of the polar axis, we used the side of the triangle that connects two of its medial vertices and points in the dorsal direction. Then we defined the polar axis as the line that is parallel to it and that intersects the *z*-axis. Finally, we calculated the volume occupied by each shape and applied a uniform scaling factor to account for different sizes of the anatomical structures in different animals.

All the steps explained earlier were implemented in MATLAB, which ran automatically without human intervention to avoid biasing the registration results. Once we had registered each shape, we used a standard algorithm to extract surfaces from two-dimensional (2D) contours[57] to transform the point cloud into a triangulated mesh that could be saved in the visualization toolkit (VTK)[58] format for visualization and analysis purposes.

We used the following landmarks to map starter cells in the amygdala and piriform cortex (Fig. 3): appearance of the olfactory tubercle (Fig. 3, I); end of the olfactory tubercle (Fig. 3, II); appearance of the hippocampus (Fig. 3, III); and appearance of the dentate gyrus of the hippocampus, on the ventral edge of the cortex (Fig. 3, IV).

Quantitative analyses. For each tracing experiment where we analysed the distribution of labelled glomeruli along the dorsal–ventral axis using mean θ'_{OB} (Fig. 3e), we generated a corresponding random distribution of simulated mean θ'_{OB} (mean $^{sim}\theta'_{OB}$) from *M* glomeruli, where *M* is the number of labelled glomeruli in the injection. To generate this random distribution for each experiment, we randomly selected *M* glomeruli from a given 3D reconstruction model (generated from that injection) and calculated the mean $^{sim}\theta'_{OB}$ value for those randomly selected *M* glomeruli to get the mean $^{sim}\theta'_1$. We then repeated the same simulation 50,000 times to obtain mean $^{sim}\theta'_2$,..., mean $^{sim}\theta'_{50,000}$, and therefore to obtain the range of mean $^{sim}\theta'$ for *M* glomeruli that are randomly distributed throughout the olfactory bulb. Once we obtained distributions for mean $^{sim}\theta'_{OB}$ that corresponded to each injection, we compared the mean $^{sim}\theta'_{OB}$ distribution with the experimental mean θ'_{OB}. If the value for the experimental mean θ'_{OB} was outside of the 95% of the mean $^{sim}\theta'_{OB}$ distribution, we considered the glomerular distribution to be non-random for that sample.

Multiple regression analysis (Fig. 4b) was conducted by using Excel (Microsoft). Data from every experiment in Supplementary Table 1 ($n = 8$ for the AON, $n = 10$ for the piriform cortex using *actin-CreER*) was used for the left part of the Fig. 4b. Data from seven experiments obtained from *GAD2-CreER* in the anterior piriform cortex were used in Fig. 4b, right. The number of labelled mitral cells in the olfactory bulb was set as a dependent variable, Y, and the number of starter cells in layer k ($k = \mathrm{I}, \mathrm{II}, \mathrm{III}$) was set as an independent variable, X_k. The constant was set to zero. Excel then calculated the values of coefficients A_k (shown by red crosses in Fig. 4b) and 95% confidence intervals of A_k based on the student's t-test (shown by grey bars in Fig. 4b). R^2 values for these multiple regression assays were: 0.98 for the AON; 0.96 for the piriform cortex (*actin-CreER*); and 0.97 for the piriform cortex (*GAD2-CreER*) data sets.

To estimate the number of dually labelled glomeruli originating from single starter cells (data Ds in Fig. 4c) in our experimental data, we first simulated a hypothetical number of dually labelled glomeruli originating from single starter cells (Ds) and two independent starter cells (Dt) according to the null hypothesis that mitral cells connect randomly with postsynaptic targets. This situation can be modelled by 'balls and bins': there are 2,000 bins (a bin represents a single glomerulus) and N balls (a ball represents a single trans-synaptic labelling event). N balls were randomly thrown into 2,000 bins, and the number of bins that received more than one ball (that is, glomeruli labelled more than once) was counted. To distinguish Ds from Dt, we further introduced n different colours to the balls, where each colour represented an individual starter cell in the cortex. We assumed that an equal number of balls (N/n) were labelled with n different colours. Each ball was randomly thrown into one of 2,000 bins, and the number of bins containing more than one ball was counted. We separately counted the bins with more than one ball of an identical colour (representing Ds) and the bins with more than one ball of different colours (representing Dt). We fixed the number of bins (glomeruli) to be 2,000, while N and n corresponded to the number of labelled mitral cells and the number of starter cells, respectively, in each experiment. We repeated this simulation 100,000 times for each set of N and n to obtain the simulated distribution of Ds and Dt (we call these 'random Ds' and 'random Dt'). To estimate the Ds components in experimental data (data Ds), we assumed that individual starter cells contributed independently to the labelling (random Dt = data Dt). On the basis of the equation: D (number of observed dually labelled glomeruli) = Ds + Dt, we estimated the data Ds distribution by subtracting the random Dt from observed D (Fig. 4c). Then we determined if there was a significant difference in the distribution of data Ds and random Ds. We considered two distributions to be significantly different if the probability of data Ds > random Ds or data Ds < random Ds exceeded 0.95 (shown by asterisks in Fig. 4c). To accurately count dually labelled glomeruli, samples with more than 200 labelled mitral cells were excluded from this analysis.

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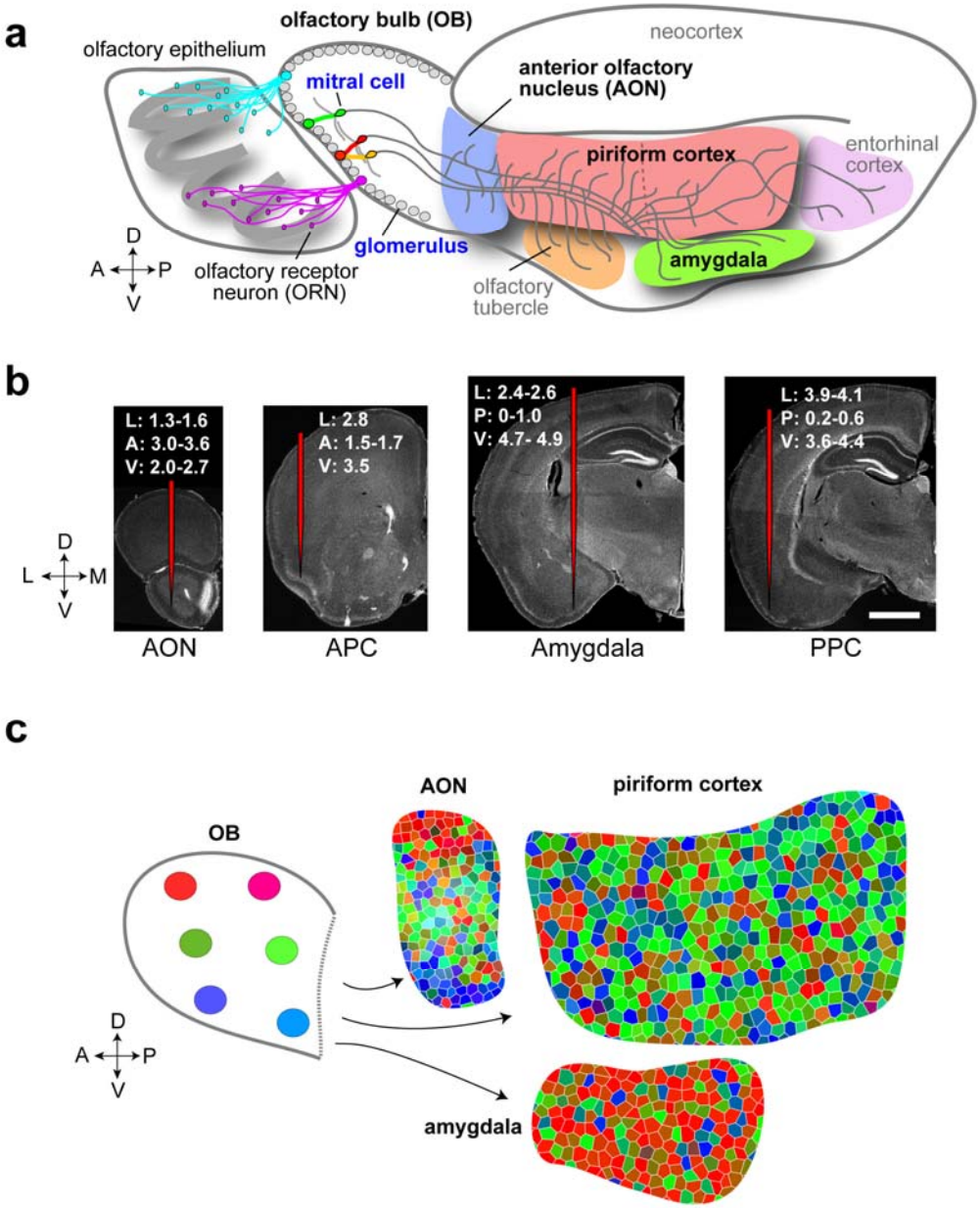
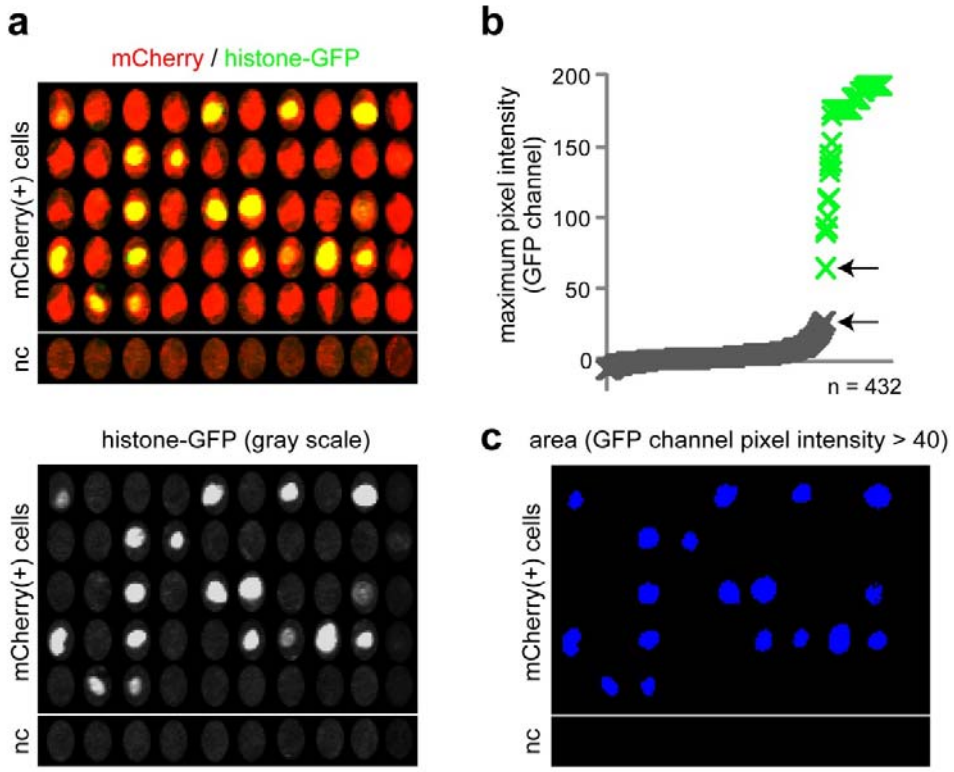
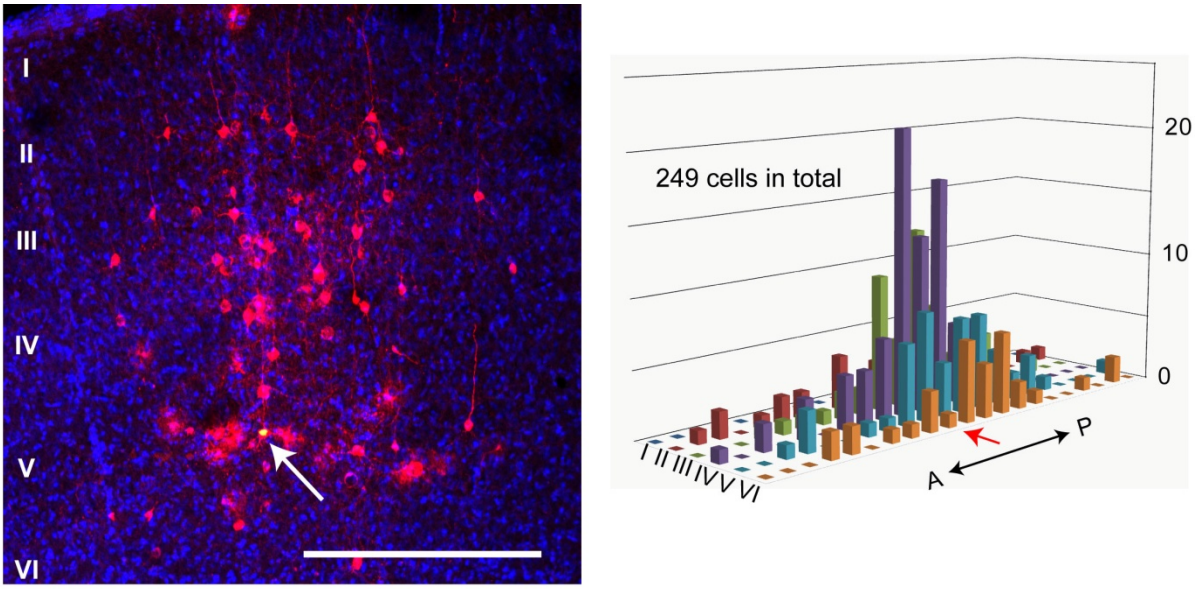


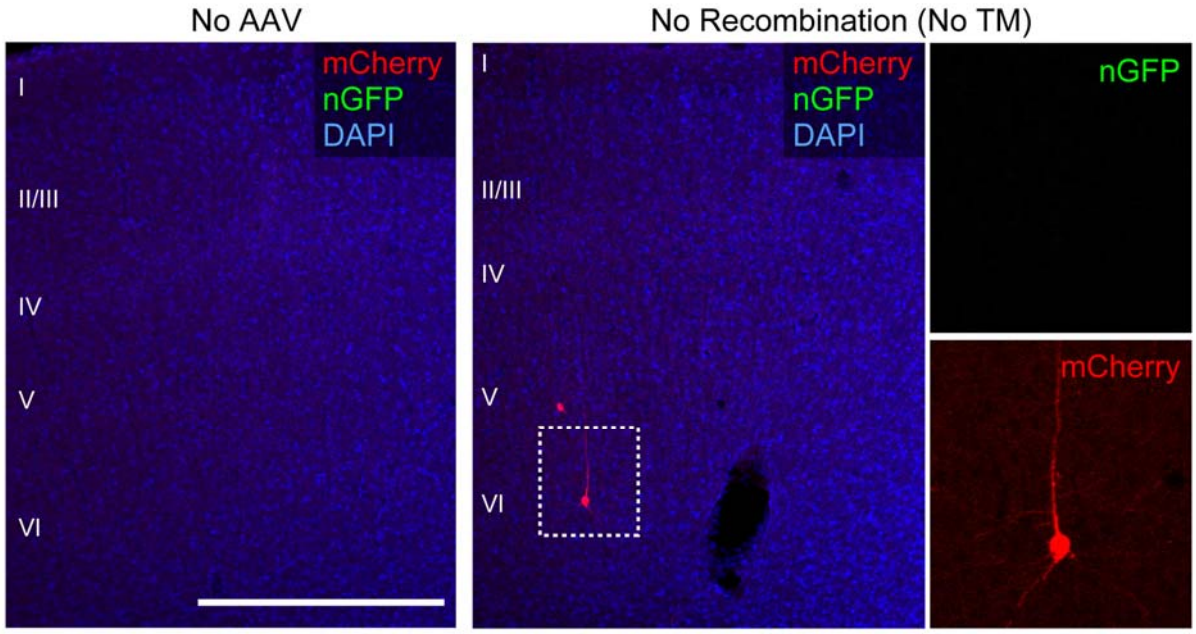
Supplementary Figure 1 | The mouse olfactory system and summary of our results.
a, Schematic of the mouse olfactory system (sagittal view). See text for more details. The red and the orange "sister" mitral cells send apical dendrites to the same glomerulus. The green mitral cell is "non-sister" to the red or orange cell. **b,** Stereotactic conditions for virus injections (mm away from the Bregma for A/P and L, mm from the brain surface for V). A, anterior; P, posterior; D, dorsal; V, ventral; L, lateral; M, medial. Scale bar, 1 mm. **c**, Schematic summary of trans-synaptic labeling data from three different areas in the olfactory cortex. The olfactory bulb input into the AON maintains a coarse topography along the dorsal-ventral axis; the olfactory bulb input into the piriform cortex does not exhibit an apparent spatial organization; the cortical amygdala receives biased input from the dorsal olfactory bulb.



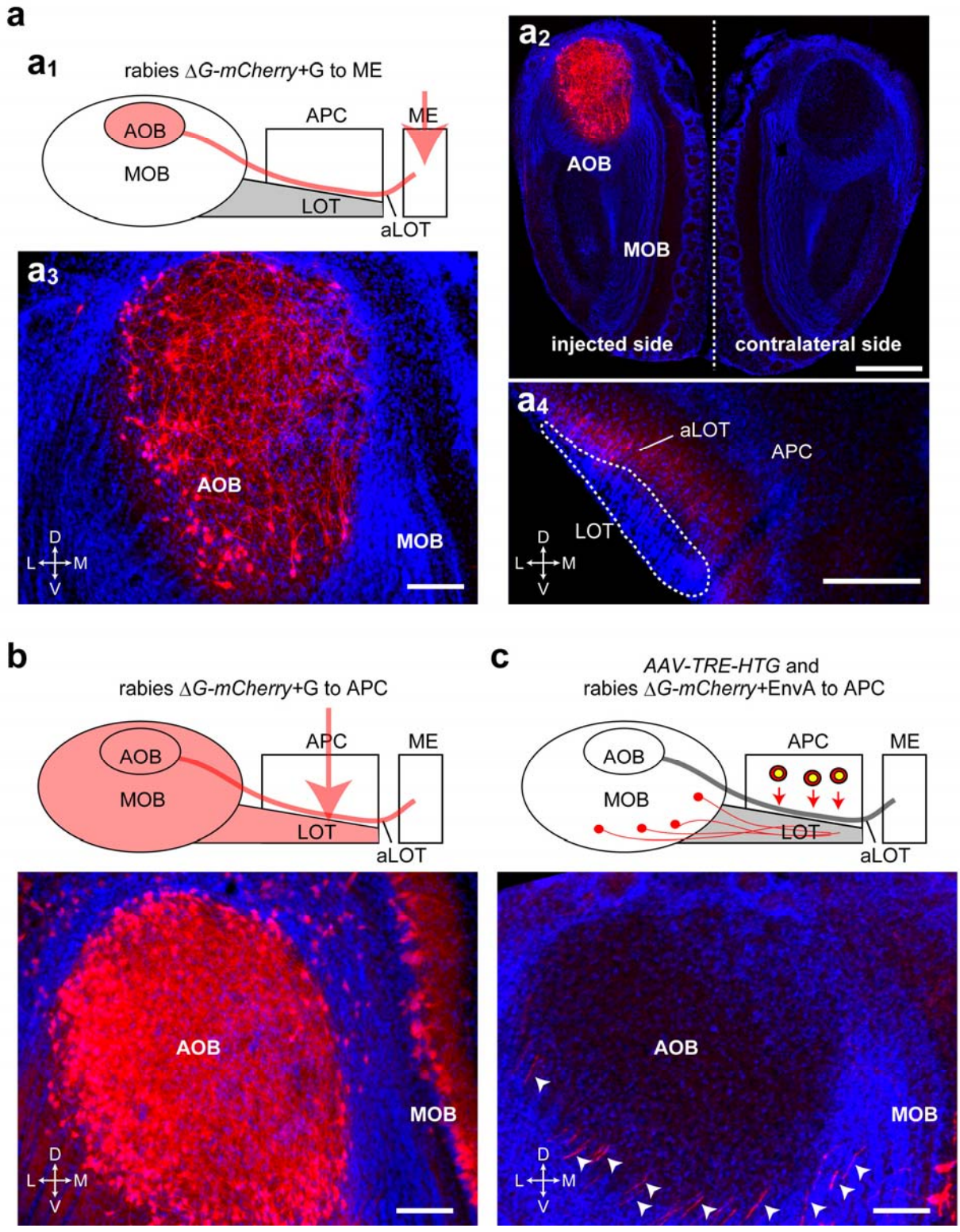
Supplementary Figure 2 | Starter cells can be reliably identified by histone-GFP.
Throughout this study, we identified starter cells as those that are histone-GFP$^+$ and mCherry$^+$ upon visual inspection of fluorescent images. Here, we provide quantitative evidence to support our visual identification of starter cells using neocortical cells as an example. From the confocal images of *actin-CreER;CAG-stop-tTA2* animals that had been subjected to AAV and rabies virus infection in the motor cortex, we randomly selected 432 mCherry$^+$ cells at the injection sites. By visual inspection, we identified 89 cells to be histone-GFP$^+$. **a**, Top, examples of captured images. 50 mCherry$^+$ cells are shown, 18 of which were determined to be histone-GFP$^+$ by visual inspection. For background subtraction, 10 random mCherry$^-$ and histone-GFP$^-$ areas from the same image were captured. Bottom, the GFP channel alone is shown in gray scale for each cell. **b**, Histogram of maximum pixel intensity in the GFP channel for each mCherry$^+$ cell, calculated by ImageJ. To determine background fluorescence for each confocal section, we averaged the maximum pixel intensities in the GFP channel for all negative control (nc) areas from that section. Then, for each cell we analyzed, we determined the maximum pixel intensity and then subtracted the background calculated for the same confocal image. The cells that were characterized as histone-GFP$^+$ based on visual inspection are indicated by green "x" signs. Despite the variability of histone-GFP expression level, maximum pixel intensity can unambiguously identify histone-GFP$^+$ cells from the rest of the cells (see the interval between the two arrows). **c**, In order for any cell to be considered histone-GFP$^+$ in an unbiased fashion, we set the threshold for maximum pixel intensity to 40 based on (**b**). When we extracted the areas with pixel intensity of >40 from the bottom panel in (**a**), all cells that were considered histone-GFP$^+$ by visual inspection corresponded perfectly with cells that contained pixels with intensities of >40 (shown in blue).



Supplementary Figure 3 | An example of a single starter cell in the S2 somatosensory cortex. Left, a single 60-μm coronal section includes the starter cell (arrow; labeled with mCherry and histone-GFP) and its local presynaptic cells (labeled with mCherry alone). Right, cortical layer distribution of a total of 249 mCherry-labeled cells that span 9 anterior and 11 posterior consecutive 60-μm sections from the section that contains the starter cell (arrow). Scale bar, 500 μm. This sample provides an "order-of-magnitude" estimate of the false negative rate of rabies virus tracing (cells presynaptic to a starter cell that are not labeled). A typical neuron in the mouse neocortex receives on the average 2000-8000 presynaptic inputs[1]. Based on anatomical reconstructions of filled cortical neurons after paired recordings[2], a cortical neuron is estimated to connect to each of its presynaptic cortical partner neurons by about 5 synapses. Therefore, each cortical neurons should have 400-1600 presynaptic partners. Thus, about 15-60% of presynaptic partners are labeled in this example.



Supplementary Figure 4 | Negative controls for trans-synaptic labeling.
All neocortical trans-synaptic labeling experiments that followed the protocol presented in Fig. 1b (n=12) resulted in the labeling of hundreds to thousands of trans-synaptically labeled neurons, many of which were located far from the injection sites (see examples in Fig. 1c and Supplementary Fig. 3). As a first negative control, we replaced the AAV with PBS (no AAV, left panel) in the protocol outlined in Fig. 1b. We did not find a single mCherry^{+} cell (n=3 animals), indicating that the infection by pseudotyped rabies virus (*ΔG-mCherry*+EnvA) critically depends on the functional TVA receptor supplied by the AAV. As a second negative control, we replaced tamoxifen (TM) with PBS (no CreER-induced recombination to allow tTA expression), and found no labeled cells at all in one animal, and fewer than 10 mCherry^{+} cells in each of the other three animals. All labeled cells were located at the injection sites (middle panel). In all cases (n=4), no labeled cells were found at a distance >450 μm. Because a very small amount of TVA receptor is sufficient to allow rabies virus infection (E.M.C. unpublished observation), it is likely that leaky expression of the TVA receptor from the AAV, independently of tTA, allows very sparse rabies infection. These cells contained no green labeling in the nucleus (right, top panel) and they did not support trans-synaptic rabies spread, presumably because they did not produce enough histone-GFP and G, respectively. Taken together, these data demonstrate that trans-synaptic rabies virus spread critically depends on the presence of sufficient amounts of tTA2 (from the transgene) and TVA/G (from AAV). Scale bar, 1 mm.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a3
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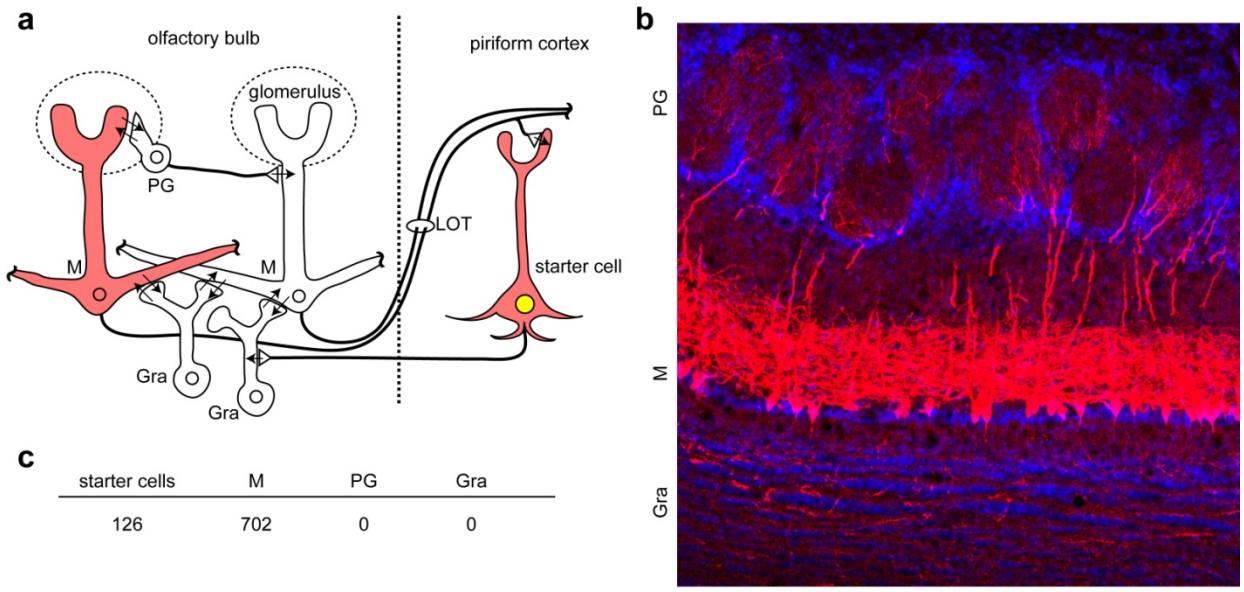
aLOT
APC
LOT
b
rabies ΔG-mCherry+G to APC
c
AAV-TRE-HTG and
rabies ΔG-mCherry+EnvA to APC

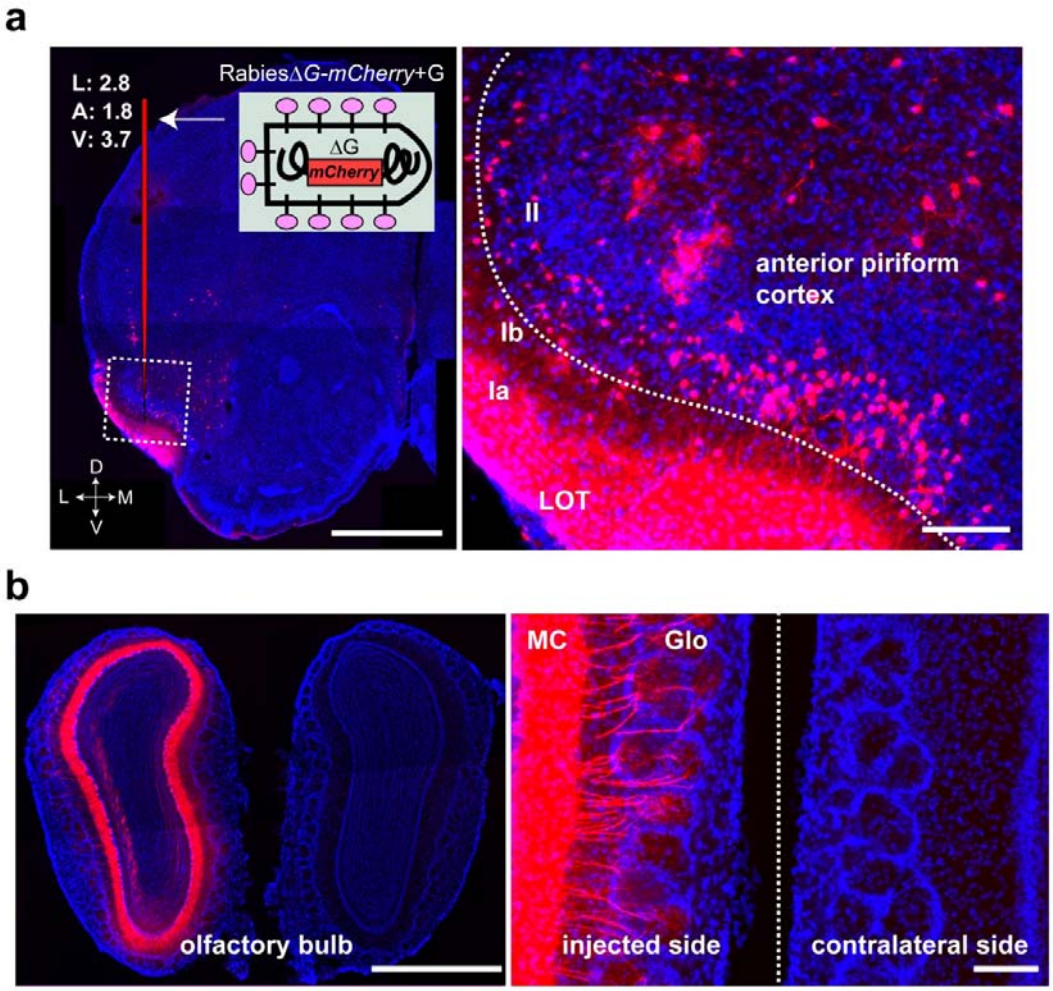
Supplementary Figure 5 | Rabies virus generated *in vivo* does not label axons in passage. To assess if the rabies virus spreads specifically through synapses, we tested if rabies virus particles produced by *in vivo* complementation (Fig. 1a-b) can infect neurons whose axons pass through the injection sites without making synapses (axons in passage). The accessory olfactory bulb (AOB) mitral cells axons form the accessory olfactory tract (aLOT), which passes close to layer Ia of the anterior piriform cortex (APC) without forming synaptic connections with the APC neurons[3,4]. These axons in passage, if infected, would generate false-positive labeling in our experiments. These axons eventually project to the medial amygdala (ME and PMCo, see Fig. 3a), where they make synapses with cortical neurons. **a**, To show that AOB mitral cells can in principle be infected by rabies virus, we injected the *in vitro* complemented rabies virus (Δ*G-mCherry*+G) into ME (**a**$_1$). This mutant rabies virus was produced from a cell line that expresses a complementing *G* transgene, and should infect any cells that can normally be infected by rabies virus, but cannot produce more viral particles for further trans-synaptic infection because the glycoprotein gene was deleted in the viral genome[5]. The AOB mitral cells ipsilateral to the injection site were intensely labeled, whereas the main olfactory bulb (MOB) mitral cells were rarely labeled (**a**$_2$, magnified in **a**$_3$). The labeled axons of AOB mitral cells could also be observed in the sections through the APC where they form the aLOT passing through layer Ia; the lateral olfactory tract (LOT), which contains the axons of mitral cells from the MOB, was not labeled (**a**$_4$). These data not only confirm that axons of AOB mitral cells pass through the APC, but also demonstrate that rabies virus can efficiently infect AOB mitral cells. **b**, To test if rabies virus injected into the extracellular space can infect axons in passing, we injected rabies virus Δ*G-mCherry*+G into the APC. We observed intense labeling of AOB mitral cells as well as MOB mitral cells (see also Supplementary Fig. 7). This observation indicates that *in vitro* complemented rabies virus can infect axons in passage, sharing the same drawbacks with traditional extracellular dye injection methods. **c**, In sharp contrast, rabies particles generated in the APC via *in vivo* complementation (as in Fig. 3b) did not label AOB mitral cells. In a total of 5 individual animals subjected to our trans-synaptic labeling protocol (Fig. 1a-b) using *actin-CreER*, we observed a total of 46 starter cells in the APC, which labeled a total of 381 mitral cells in the MOB. At the same time, we did not observe a single labeled AOB mitral cell. Arrowheads represent passing axons originating from the MOB. The absence of labeled AOB mitral cells cannot be explained by the relative abundance of MOB vs. AOB mitral cells, as there are only about 10-times more MOB mitral cells[6-9]. Thus, this observation indicates that rabies virus particles generated *in vivo* do not infect axons in passage, strongly supporting synapse-dependent spread of the viral particles. Scale bars, 100 μm, except in a$_2$, 500 μm.



c

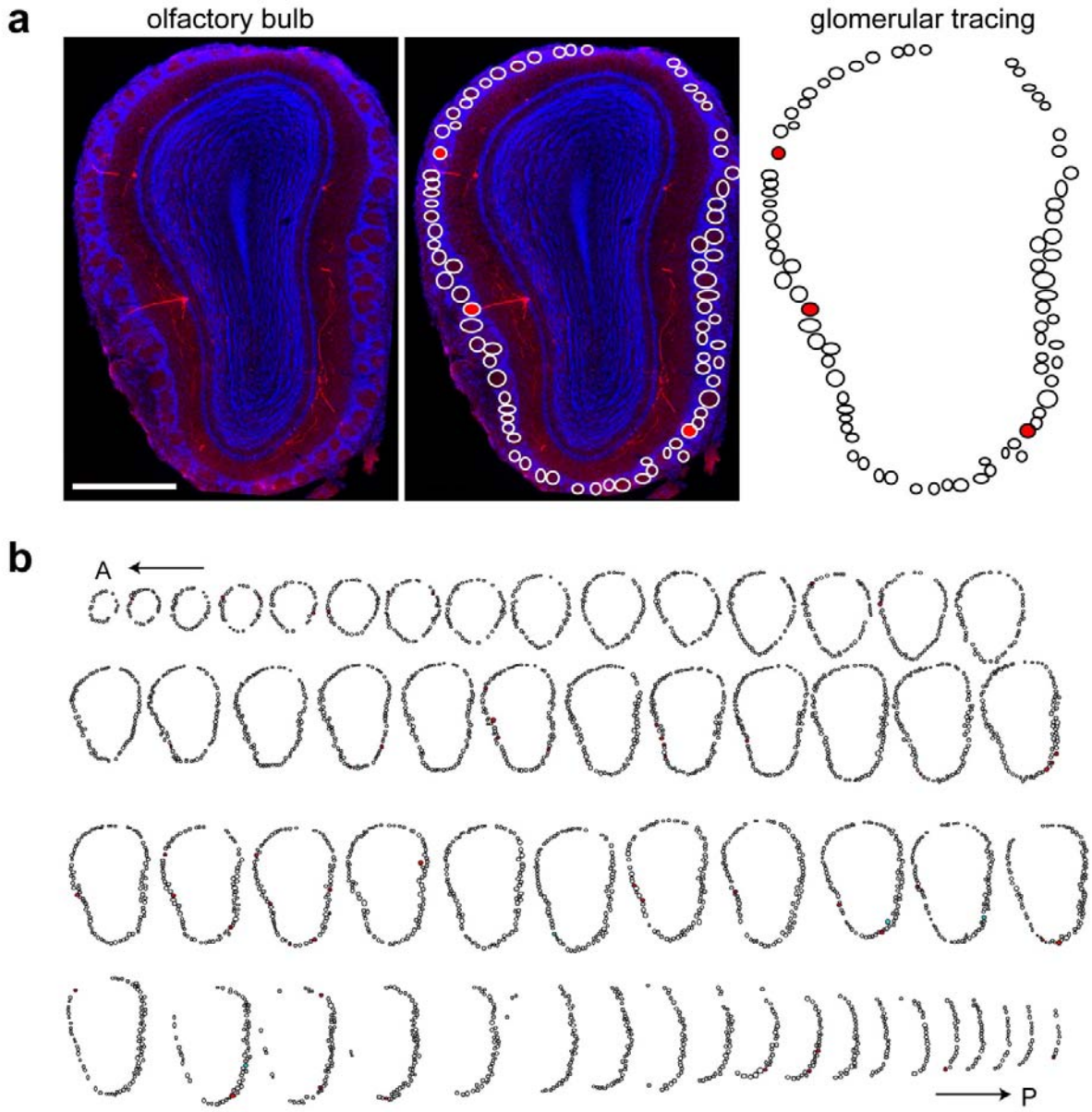
starter cells	M	PG	Gra
126	702	0	0

Supplementary Figure 6 | Rabies virus spreads mono-synaptically and purely retrogradely. To assess if rabies virus spread is restricted to direct connections in a purely retrograde direction, we tested if rabies particles generated in the piriform cortex (PC) *in vivo* can infect the granule (Gra) and periglomerular (PG) cells in the olfactory bulb. **a**, Connection diagrams in the olfactory bulb. Mitral cells (M) send long distance axons via the lateral olfactory tract (LOT) to the PC where they synapse with pyramidal cells. Pyramidal cells in the PC send back projections that synapse with Gra cells and other cell types in the olfactory bulb. Gra and PG cells form dendro-dendritic reciprocal connections with mitral cells[10]. If di-synaptic viral spread can occur, starting from the cortical pyramidal cells, rabies virus should not only label pre-synaptic mitral cells (shown in red), but also Gra and PG cells that are presynaptic to mitral cells. If the rabies virus can spread anterogradely, Gra cells in the olfactory bulb should be labeled by starter cells in the PC. **b**, We did not observe Gra or PG cell labeling in any of our trans-synaptic labeling experiments starting from the PC. This panel shows a single confocal image of the olfactory bulb with densely trans-synaptically labeled mitral cells starting from pyramidal cells in the anterior PC; however, none of the Gra or PG cells are labeled. This sample was not included in our quantitative analyses, as the labeling in the olfactory bulb was too dense to reconstruct. **c**, Summary of all 10 samples with *actin-CreER*$^{+}$ starter cells from the piriform cortex (Supplementary Table 1). In a separate experiment, we confirmed that PG and Gra cells are competent to receive rabies virus particles from mitral cells as starter cells (unpublished data). In addition, AON pyramidal cells send axons to and synapse with neurons in the contralateral olfactory bulb[11]. From a total of 223 starter cells in our AON injection experiments, we detected 1046 labeled mitral cells in the ipsilateral olfactory bulb, but did not find a single labeled cell in the contralateral olfactory bulb as would be predicted if the rabies virus can spread anterogradely. Together, these observations indicate that the rabies virus spread occurs only through direct connections to the starter cells in the purely retrograde direction.

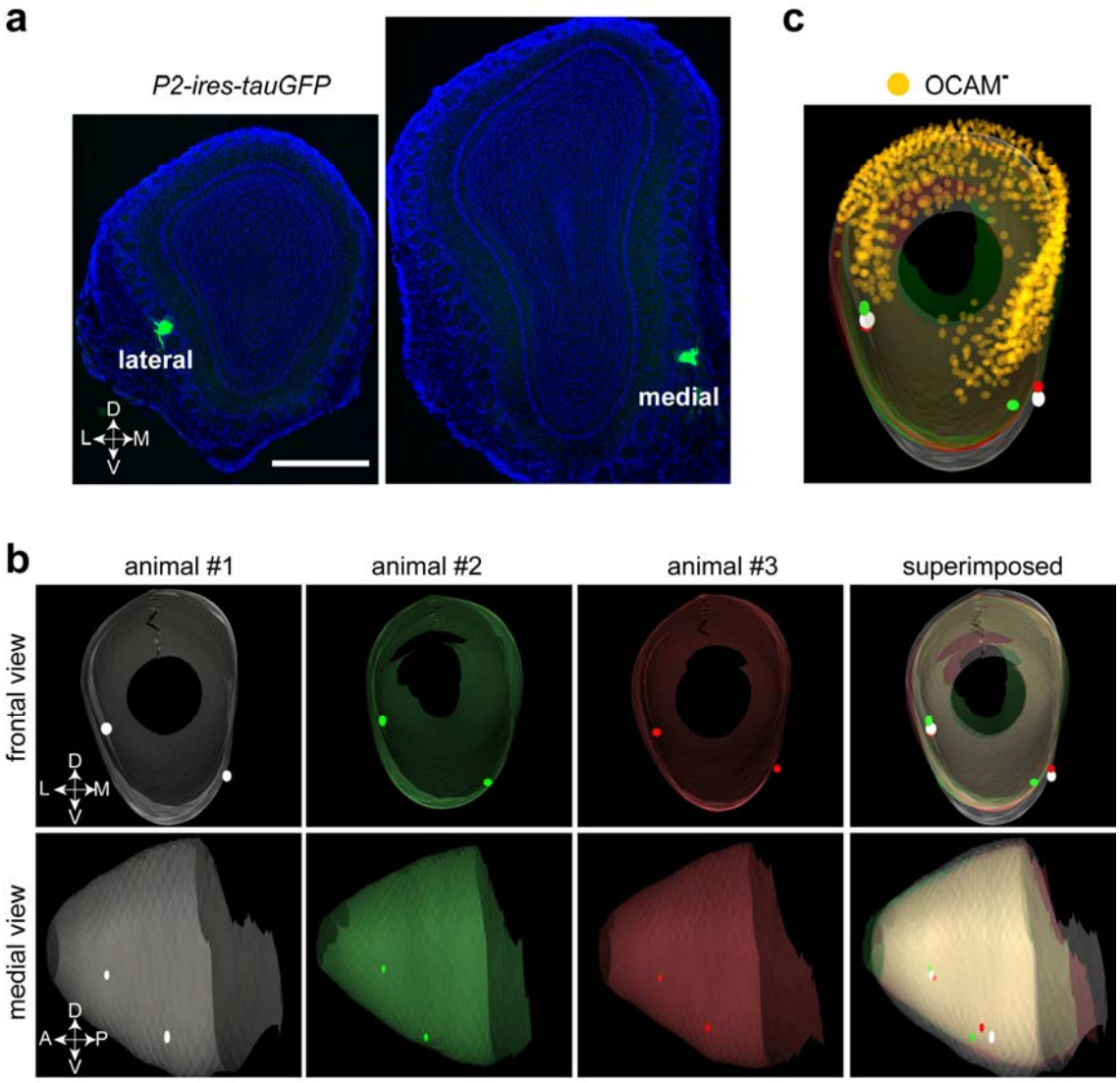


Supplementary Figure 7 | Mitral cells throughout the olfactory bulb can receive rabies virus from their axons.

To test if mitral cells in different olfactory bulb areas are competent to receive rabies virus from their axon termini, we made use of the *in vitro* complemented rabies virus (*ΔG-mCherry*+G). This virus can infect cells that would be infected by the wild-type rabies virus, but cannot produce further viral particles for trans-synaptic transmission (see Supplementary Fig. 5 legend). 0.3 µl of *ΔG-mCherry*+G rabies virus was injected into the anterior piriform cortex of 4-week old wild type mice (n=3). **a,** A typical example of a 60-µm coronal section at the injection site. Near the injection site, many neurons are labeled with the rabies virus (magnified on the right). **b,** In the olfactory bulb, mitral cells ipsilateral to the injection site were intensely labeled in all olfactory bulb regions, whereas no labeled cells could be found in the contralateral side (magnified on the right). This finding is consistent with the unilateral projection of mitral cell axons[12]. Importantly, this experiment validates that mitral cells throughout the entire olfactory bulb can be infected by rabies virus from their axons in the olfactory cortex without obvious bias. Scale bars, 1 mm for low-magnification images, 100 µm for high-magnification images. The stereotactic condition for the injection site of rabies virus is shown in **a**, left panel (mm away from the Bregma for A and L, mm from the brain surface for V). A, anterior; L, lateral; V, ventral; LOT, lateral olfactory tract; MC, mitral cell layer; Glo, glomerular layer.



Supplementary Figure 8 | Glomerular tracing from serial coronal sections of an olfactory bulb.
53 to 60 consecutive coronal sections (60 μm) that cover the entire olfactory bulb were collected for each experiment. **a**, For each coronal section, labeled glomeruli (in red) and unlabeled glomeruli (in white, identified by DAPI staining in blue) were individually traced in Adobe Illustrator. Unlabeled glomeruli that span more than one section might occasionally be traced twice (once in each of the two consecutive coronal sections), but each labeled glomerulus was carefully traced only once in the section where labeled dendrites were densest. **b**, An example of tracing the entire olfactory bulb (corresponding to Fig. 2c, red and Supplementary Movie 1) from 58 consecutive coronal sections of a single olfactory bulb. Red, singly innervated glomeruli; Cyan, dually innervated glomeruli; White, unlabeled glomeruli. A, anterior, P, posterior. Scale bar, 500 μm.



Supplementary Figure 9 | Precision of olfactory bulb 3D reconstruction.
We assessed the precision of our 3D reconstruction for the olfactory bulb glomerular map using *P2-IRES-tauGFP* mice (n=3, obtained from the Jackson laboratory), in which glomerular targets of a single ORN class were labeled with GFP. **a**, A typical example of a single coronal 60-μm section contains lateral (left) and medial (right) major target glomeruli of ORNs that express the P2 odorant receptor. Scale bar, 500 μm. **b**, Independent 3D reconstructions of three olfactory bulbs (in white, green and red outlines) are shown. The surfaces represent the inner boundary of the glomerular layer for each sample. Lateral and medial major target glomeruli of P2 from each sample are shown in corresponding colors. After superimposition (rightmost panels), the corresponding labeled glomeruli are located within a distance equivalent to the diameter of several glomeruli. **c**, The three reconstructed olfactory bulbs shown in **b** were superimposed with an olfactory bulb that had been stained with an anti-OCAM antibody (Fig. 2c, Supplementary Fig. 11). $OCAM^-$ dorsal glomeruli are labeled in yellow. P2 target glomeruli are located in the $OCAM^+$ ventral side, close to the boundary of $OCAM^+$ and $OCAM^-$ glomeruli, consistent with *P2* being a zone-2 OR gene[13].

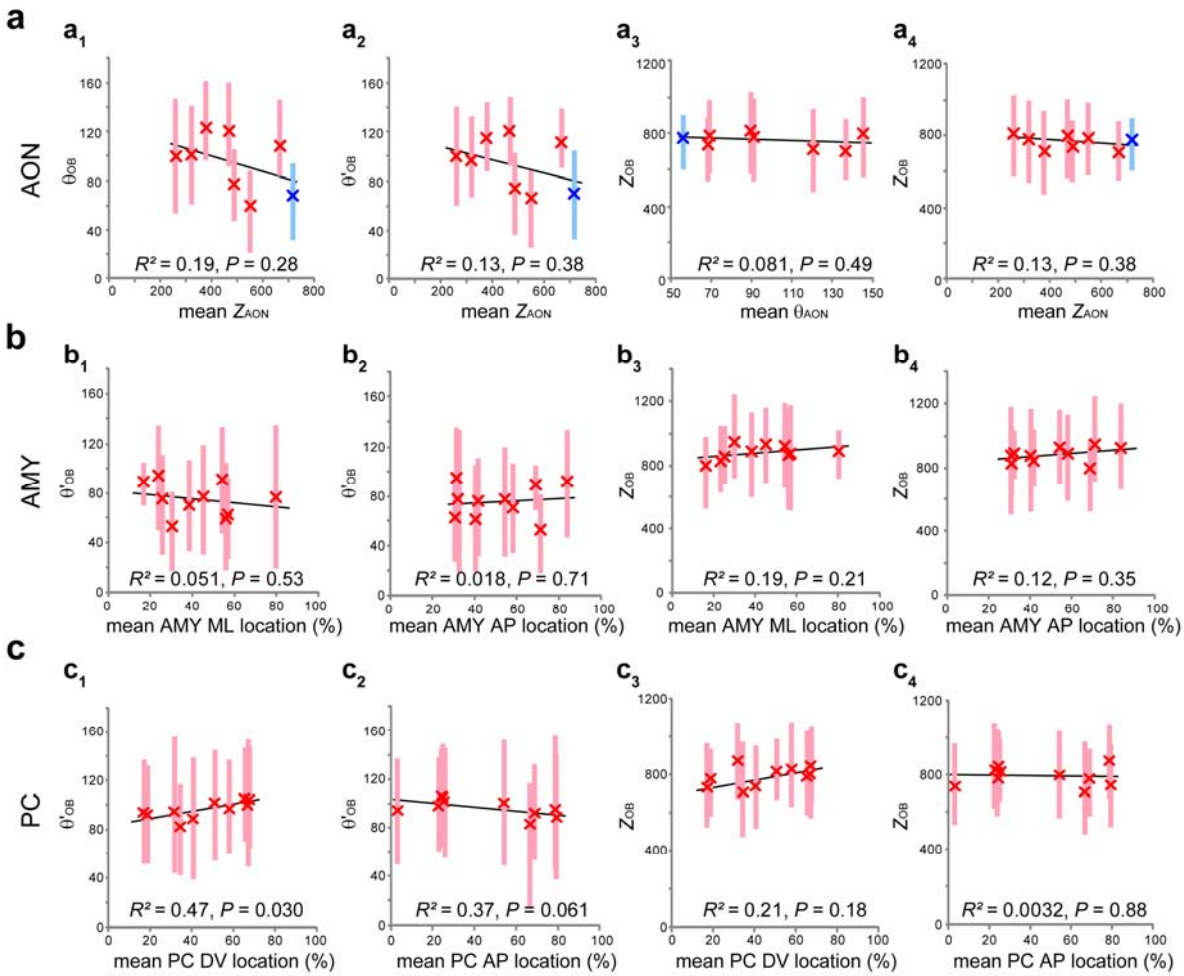
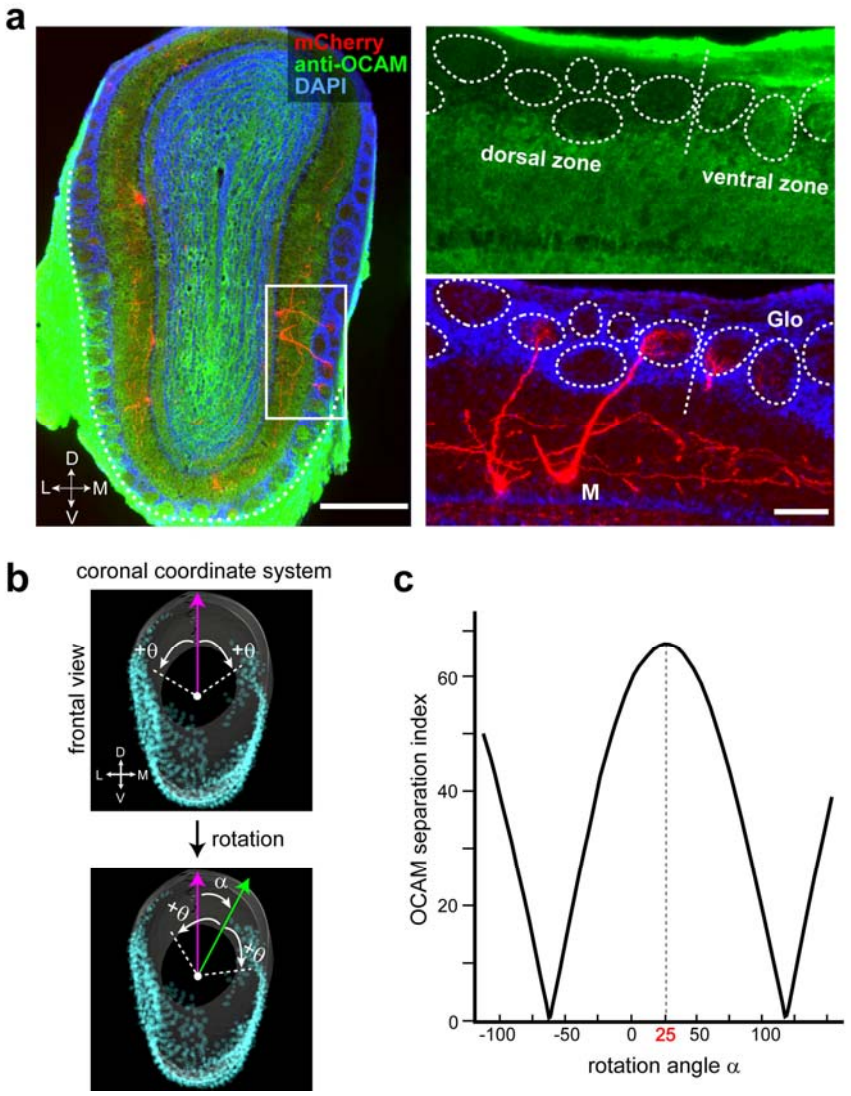


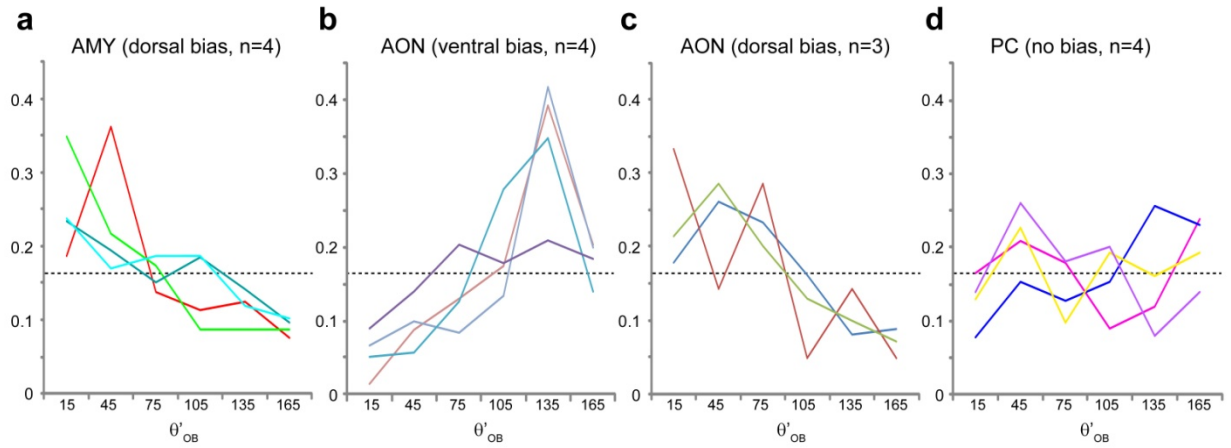
Figure S10 | Additional analyses of spatial correspondence.

Correlation analysis between the location of starter cells in the AON (**a**), amygdala (ACo and PLCo) (**b**) and piriform cortex (**c**), and the location of labeled glomeruli in the olfactory bulb (OB). Bars represent 25%-75% of the distribution for θ_{OB}, θ'_{OB}, or Z_{OB}. All samples were generated using *actin-CreER* except the blue sample, which was generated using *CaMKII-CreER*. In the amygdala samples, we analyzed the correlations along the medial-lateral (ML) instead of dorsal-ventral axis, due to the shape and location of this brain structure (ACo and PLCo can be approximated by a flat surface that lays in the medial-lateral/anterior-posterior plane). R^2, square of Pearson's correlation coefficient; p, statistical significance tested against the null hypothesis assuming R=0. None of these analyses exhibited statistically significant correlation, except $\mathbf{c_1}$, which showed positive correlation between θ'_{OB} and dorsal-ventral location in the PC, although the correlation (R^2=0.47) was weak.



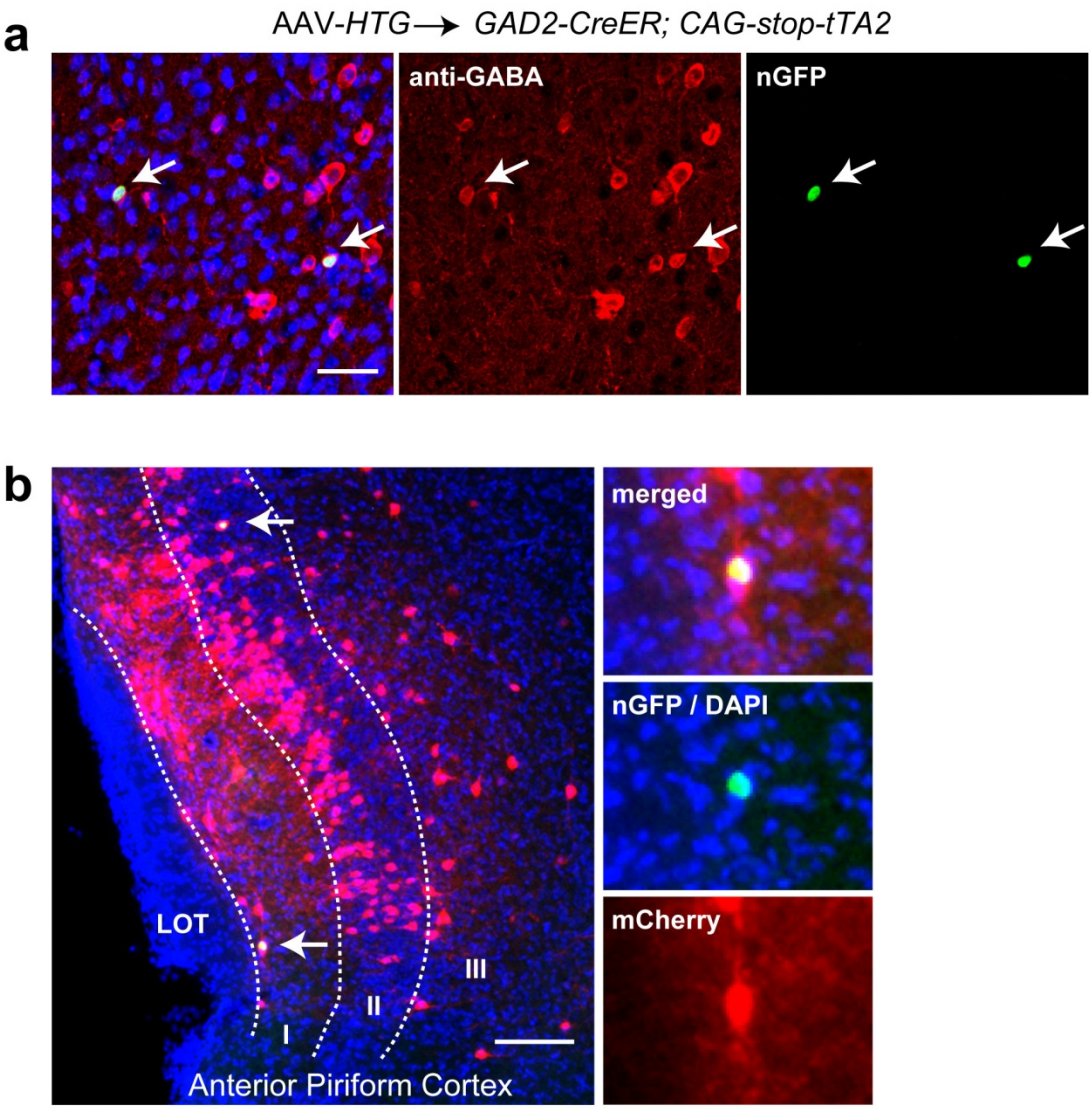
Supplementary Figure 11 | Generation of the OCAM coordinate system for the olfactory bulb.

a, A single coronal section (60 μm) including several trans-synaptically labeled mitral cells (red) stained with an anti-OCAM antibody (green). $OCAM^+$ ORN axons terminate in the ventral area (defined by the white dotted line in the left panel). The area covering $OCAM^+/OCAM^-$ boundary is magnified in the right panels. Glo, glomerular layer, M, mitral cell layer. Scale bars, 500 μm for the low-magnification image and 100 μm for the high-magnification images. **b,** Conversion of the coronal coordinate system to the OCAM coordinate system is based on 3D reconstructions of the olfactory bulb glomerular map with OCAM staining (cyan) from 4 individual brains. The optimal angle (α) that rotates the polar axis (magenta) around the *Z*-axis and transforms the coronal coordinates to the OCAM coordinates (after rotation, the new polar axis is shown in green) was determined by maximizing the OCAM separation index. OCAM separation index was defined as an absolute value of average θ_{OB} for $OCAM^+$ glomeruli subtracted from average θ_{OB} for $OCAM^-$ glomeruli. **c**, The OCAM separation index plotted as a function of the rotation angle α. The angle α that gave the highest OCAM separation index was 25.5°± 2.5° (SEM, n=4).



Supplementary Figure 12 | Distribution of the trans-synaptically labeled glomeruli along the dorsal-ventral axis.

θ'_{OB} values for all labeled glomeruli were classified into one of six 30º-bins. The Y-axis represents the frequency of θ'_{OB} for each bin after normalization for each experiment. For clarity, we show only samples with >20 labeled glomeruli. Samples from the cortical amygdala (**a**) and the piriform cortex (**d**) are color matched with Fig. 3e. The dotted horizontal line shows the frequency of θ'_{OB} if all θ'_{OB} values were evenly represented. **a**, Histograms of θ'_{OB} for 4 amygdala samples that exhibited a dorsal bias. All samples labeled glomeruli in all bins, with preferential labeling in dorsal bins; none of the samples showed exclusive labeling only in dorsal bins. These data exclude the possibility of a sharp boundary in glomerular labeling along the dorsal-ventral axis. **b-c**, Histograms for 4 samples from the AON that showed ventral bias (**b**) and 3 samples from the AON that showed the dorsal bias (**c**). In either case, an obvious sharp boundary in glomerular labeling along the dorsal-ventral axis was not evident. **d**, As a comparison, histograms for 4 samples from the piriform cortex (two each from anterior and posterior piriform cortex) are shown. The distribution of θ'_{OB} resembles the uniform distribution in these samples.



Supplementary Figure 13 | Trans-synaptic tracing from GABAergic neurons.
a, To validate the *GAD2-CreER* transgene, 1 mg of tamoxifen was applied at postnatal day (PD) 6 to mice doubly heterozygous for *GAD2-CreER* and *CAG-stop-tTA2* (n=4). AAV-*TRE-HTG* was injected at PD21 to the motor cortex. 10 days later, animals were sacrificed and brain sections were stained with anti-GABA antibodies. A typical example of a 60-μm coronal section is shown. Although only a small fraction of cells in this section were $GABA^+$, 103 out of 105 cells expressing histone-GFP (i.e., infected with AAV) were co-stained with anti-GABA antibodies (right graph). These data demonstrate that starter cells in our trans-synaptic labeling experiments using *GAD2-CreER* line are almost exclusively GABAergic neurons. **b,** A typical example of a single 60-μm coronal section that includes two starter cells (arrows) located in layers I and II of the piriform cortex, obtained using *GAD2-CreER/wt;CAG-stop-tTA2/wt* mice injected with AAV-*TRE-HTG* and rabies virus *ΔG-mCherry*+EnvA. The starter cell located in layer I is magnified in the panels on the right. Quantitative data from this and other experiments using *GAD2-CreER*, including the number of labeled mitral cells, are summarized in Fig. 4 and Supplementary Table 1. Scale bar, 50 μm.

Supplementary Table 1 | Summary of quantitative data.

		number of starter cells				number of			number of labeled glo		Covergence index
location	CreER	total	I	II	III	M/T cells	D-Glo	S-Glo	L	M	
AON	*Actin*	4	0	4		21	0	21	14	7	5.3
AON	*Actin*	17	0	17		60	2	56	22	38	3.5
AON	*Actin*	11	4	7		69	4	61	33	36	6.3
AON	*CaMKII*	21	0	21		70	2	66	39	31	3.3
AON	*Actin*	17	4	13		149	4	141	66	83	8.8
AON	*Actin*	31	0	31		158	10	138	75	83	5.1
AON	*Actin*	17	5	12		158	9	140	85	73	9.3
AON	*Actin*	105	0	105		361	39	283	163	198	3.4
PLCo	*Actin*	4	0	4		11	0	11	8	3	2.8
PLCo	*Actin*	2	0	2		11	0	11	5	6	5.5
Aco	*Actin*	4	0	4		16	0	16	9	7	4.0
Aco	*Actin*	7	0	7		23	1	21	8	15	3.3
Aco	*Actin*	9	0	9		27	0	27	10	17	3.0
Aco	*Actin*	14	0	14		40	0	40	23	17	2.9
Aco	*Actin*	27	0	27		59	2	55	28	31	2.2
Aco/PLCo	*Actin*	21	0	21		80	2	76	37	43	3.8
PLCo	*Actin*	12	2	10		164	10	144	76	88	13.7
Aco/PLCo	*Actin*	67	1	66		206	14	178	75	131	3.1
APC	*Actin*	6	0	6	0	31	1	29	8	23	5.2
APC	*Actin*	6	0	0	6	39	1	37	16	23	6.5
APC	*Actin*	10	0	6	4	50	0	50	17	33	5
APC	*Actin*	6	2	4	0	53	0	53	22	31	8.8
APC	*Actin*	18	4	9	5	208	3	202	85	123	11.6
PPC	*Actin*	3	0	2	1	29	0	29	15	14	9.7
PPC	*Actin*	15	0	9	6	50	0	50	15	35	3.3
PPC	*Actin*	13	0	9	4	59	2	55	26	33	4.5
PPC	*Actin*	27	0	9	18	67	1	65	27	40	2.5
PPC	*Actin*	22	1	9	12	116	5	106	43	73	5.3
APC	*GAD2*	18	0	4	14	8	0	8	nd	nd	0.4
APC	*GAD2*	11	0	0	11	10	0	10	nd	nd	0.9
APC	*GAD2*	24	1	2	21	29	0	29	nd	nd	1.2
APC	*GAD2*	8	1	1	6	55	0	55	nd	nd	6.9
APC	*GAD2*	6	3	1	2	117	6	105	nd	nd	19.5
APC	*GAD2*	11	3	2	6	128	6	114	nd	nd	11.6
APC	*GAD2*	25	13	2	10	340	36	268	nd	nd	13.6

Abbreviations:
AON, anterior olfactory nucleus; PLCo, posterolateral cortical amygdaloid nucleus; ACo, anterior cortical amygdaloid nucleus; APC, anterior piriform cortex; PPC, posterior piriform cortex; I-III, layer I, II, III; M/T, mitral/tufted; D-glo, dually labeled glomeruli; S-glo, singly labeled glomeruli; L, lateral; M, medial; nd, not determined.

Supplementary Movie 1 | 3D visualization of the distribution of trans-synaptically labeled glomeruli from a typical experiment.
The legends are shown within the movie.

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