Precise Circuitry Links Bilaterally Symmetric Olfactory Maps

Zhiqiang Yan,1,2 Jie Tan,1,2 Chang Qin,2 Yao Lu,2 Cheng Ding,2 and Minmin Luo2,*

1Institute of Biophysics, Chinese Academy of Sciences, Beijing, 100101, China
2National Institute of Biological Sciences, Beijing, 102206, China
*Correspondence: luominmin@nibs.ac.cn
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SUMMARY

Olfactory sensory neurons expressing a common receptor gene converge onto one or a few glomeruli with stereotyped positions within the mouse main olfactory bulb (MOB), producing a map of ~1800 olfactory columns representing ~1000 odorant receptors. Here, we report that this precise olfactory map is maintained over several synapses that ultimately cross MOB hemispheres to link bilateral isofunctional olfactory columns. Focal injection of tracer into genetically identified glomeruli revealed an exquisite topography that involves a bilateral connection via the anterior olfactory nucleus pars externa (AONpE) that links isofunctional olfactory columns in the contralateral MOB. Physiological and behavioral assays revealed an important role for the AONpE in bilateral exchange of odorant-specific information. These results indicate that the interbulbar link through the AONpE integrates bilateral olfactory sensory maps and exchanges olfactory information, positioning it as a unique model system for studying interhemispheric connections.

INTRODUCTION

All sensory modalities utilize topographically organized sensory maps to represent features of sensory stimuli (Luo and Flanagan, 2007; O’Leary et al., 1999). Central topography typically reflects the initial organization of the sensory epithelium, in which receptor neurons are spatially organized based on their types and tuning (Luo and Flanagan, 2007; O’Leary et al., 1999). The olfactory system is unusual in this regard because olfactory sensory neurons (OSNs) expressing a given receptor gene out of a repertoire of ~1000 are scattered in the olfactory epithelium (Buck and Axel, 1991; Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1994; Zhang and Firestein, 2002). This disarray is dramatically transformed to ordered organization at the level of glomerulus in the MOB. Here, OSNs expressing a common receptor gene converge precisely into one or two glomeruli with a stereotyped location in the ipsilateral MOB (Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1994). In mice, each MOB has ~1800 glomeruli, and in each glomerulus OSNs form synapses with a specific set of mitral/tufted cells (Shepherd et al., 2004). Like columns in the cerebral cortex, a glomerulus forms part of a functional unit, or “olfactory column,” consisting of mitral/tufted cells, periglomerular cells, and underlying granule cells (Shepherd et al., 2004; Willhite et al., 2006). Isofunctional olfactory columns are those that are associated with the same odorant receptors. Each mouse MOB can thus be viewed as a spatial map containing ~1800 olfactory columns with parallel inputs and outputs.

The stereotyped position of identified glomeruli is preserved not only between individuals but also between hemispheres within an individual, creating bilaterally symmetric olfactory sensory maps (Mombaerts et al., 1996; Ressler et al., 1994; Rubin and Katz, 1999; Uchida et al., 2000; Vassar et al., 1994; Wachowiak and Cohen, 2001). Although bilateral olfactory bulbs lack direct connections between them, they can interact indirectly via feedback pathways. A cortical area called the anterior olfactory nucleus (AON) is known to contain neurons that project to the contralateral MOB (Davis and Macrides, 1981; Haberly and Price, 1978; Schoenfeld and Macrides, 1984; Scott et al., 1985). Within the AON, the anterior olfactory nucleus pars externa (AONpE) receives input from the ipsilateral MOB and projects exclusively to the granule cell layer of the contralateral MOB, forming the interbulbar association system (Davis and Macrides, 1981; Schoenfeld and Macrides, 1984; Scott et al., 1985). Many important questions remain unaddressed about the organization of this system. In particular, do bilateral olfactory maps interact with any specificity at the level of odorant receptors, and is there a functional role if such interactions exist?

We studied these questions by utilizing genetically engineered mice that express green fluorescence protein (GFP) in a specific set of OSNs that also express the endogenous M71 receptor. This receptor has a characterized ligand; thus, we were able to probe both anatomical and functional organization. By targeting tracers to genetically identified glomeruli, we uncovered an as yet unknown level of organization in the olfactory system, where bilateral isofunctional olfactory columns are precisely linked by neurons in the AONpE. We found that odorant-specific activation of the contralateral MOB required the AONpE. In addition, we developed a behavioral assay to establish the important role of the AONpE in crosslateral transfer of odorant-specific olfactory memory. These results thus demonstrate that the precision of the olfactory sensory map is preserved along interhemispheric circuitry and that these connections contribute to bilateral exchange of specific olfactory information, including memorized olfactory associations.
RESULTS

The Interbulbar Association System Links Bilateral Isofunctional Olfactory Columns

To target tract tracers into a single identified olfactory glomerulus, we first utilized a genetically engineered mouse line in which GFP is coexpressed with the odorant receptor M71 (Bozza et al., 2002). In these mice, the lateral GFP-labeled M71 glomerulus is typically located in the dorsoposterior surface of the MOB. Guided by GFP fluorescence, we made focal iontophoretic injections of fluorescence-conjugated dextran amines into the lateral M71 glomerulus (Figures 1A and 1B; Figure S1A, available online). In all cases, we observed a single patch of intensely labeled axon terminals within layer la of the dorsal AONpE (Figure 1C; n = 7 mice; see Figure S2 for topology of the AONpE). The location of the axonal patches in the AONpE was highly stereotyped across animals and was spatially restricted, spanning only approximately 100 μm at levels approximately 3.2 mm anterior to Bregma. These results indicate specificity in the projections from the MOB to the ipsilateral AONpE. To examine the topographic relationship between the MOB and AONpE further, we made tracer injections into different glomeruli in the MOB (Figure 1; Figure S3). When tracers were injected into the mitral/tufted cells in the ventral MOB, we observed a distinct cluster of anterogradely labeled axonal terminals in the ventral AONpE (Figures 1D–1F; n = 5 mice). Tracer injections into the dorsomedial glomeruli near the medial M71 glomerulus anterogradely labeled a cluster of axonal terminals within the dorsomedial AONpE (Figures S3A and S3B; n = 4 mice). Injections into glomeruli located in the middle level of the medial MOB labeled a small cluster of terminals in ventromedial AONpE (Figures S3C and S3D; n = 3 mice). We next injected two tracers with different fluorophore into two distinct positions along the anterior-posterior axis of the dorsal MOB. Anterogradely labeled terminal fields were largely separated in the dorsal MOB, we observed a distinct cluster of anterogradely labeled axonal terminals in the ventral AONpE (Figures 1D–1F; n = 5 mice). Tracer injections into the dorsomedial glomeruli near the medial M71 glomerulus anterogradely labeled a cluster of axonal terminals within the dorsomedial AONpE (Figures S3A and S3B; n = 4 mice). Injections into glomeruli located in the middle level of the medial MOB labeled a small cluster of terminals in ventromedial AONpE (Figures S3C and S3D; n = 3 mice). We next injected two tracers with different fluorophore into two distinct positions along the anterior-posterior axis of the dorsal MOB. Anterogradely labeled terminal fields were largely separated in the dorsal MOB, we observed a distinct cluster of anterogradely labeled axonal terminals in the ventral AONpE (Figures 1D–1F; n = 5 mice). Tracer injections into the dorsomedial glomeruli near the medial M71 glomerulus anterogradely labeled a cluster of axonal terminals within the dorsomedial AONpE (Figures S3A and S3B; n = 4 mice). Injections into glomeruli located in the middle level of the medial MOB labeled a small cluster of terminals in ventromedial AONpE (Figures S3C and S3D; n = 3 mice). We next injected two tracers with different fluorophore into two distinct positions along the anterior-posterior axis of the dorsal MOB. Anterogradely labeled terminal fields were largely separated in the dorsal
Having established the topography for the projection from the MOB to ipsilateral AONpE, we next examined whether the projection from the AONpE to contralateral MOB was also topographic. The projection from the AONpE terminates in the granule cell layer of the contralateral MOB. We thus made small injections into the granule cell layer and examined the distribution of retrogradely labeled somata in the contralateral AONpE (Figure 2A–2C; n = 6 mice). Across animals, retrograde labeling consistently appeared in the dorsal AONpE and was limited in size to ~100 μm. This area corresponded to the location of anterogradely labeled observed after tracer injection into the ipsilateral M71 glomerulus (Figure 1C; Figure 2C). To test the topography of contralateral AONpE projections to areas distal from the M71 glomerulus, we made tracer injections into the granule cell layer in the ventral MOB and observed a small cluster of retrogradely labeled somata in the ventral AONpE contralateral to the injection site (Figures 2D–2F; n = 3 mice). Focal tracer injection into the granule cell layer in the dorsomedial MOB near the medial M71 glomerulus retrogradely labeled a cluster of somata in the dorsomedial area of the contralateral AONpE (Figure S4; n = 3 mice). This area corresponded to the location of anterogradely labeled after tracer injection into the ipsilateral dorsomedial glomeruli (Figures S3A and S3B). Along the anterior-posterior axis, the topography in the AONpE-to-contralateral MOB projection resembled that of the MOB-to-ipsilateral AONpE projection (Figure 2G; n = 5 mice, r = 0.98, p < 0.001; Pearson’s correlation).

Our data thus suggest that isofunctional olfactory columns in the bilateral olfactory bulbs are linked via their topographic connections with the AONpE. To directly test this possibility, we injected red tracer into one M71 glomerulus and green tracer into the ventral granule cell layer of the contralateral MOB (Figure S4; n = 3 mice). Focal tracer injection into the granule cell layer in the dorsomedial MOB near the medial M71 glomerulus retrogradely labeled a cluster of somata in the dorsomedial area of the contralateral AONpE (Figure S4; n = 3 mice). This area corresponded to the location of anterogradely labeled after tracer injection into the ipsilateral dorsomedial glomeruli (Figures S3A and S3B). Along the anterior-posterior axis, the topography in the AONpE-to-contralateral MOB projection resembled that of the MOB-to-ipsilateral AONpE projection (Figure 2G; n = 5 mice, r = 0.98, p < 0.001; Pearson’s correlation).
Figure 3. Precise Linking of Isofunctional Olfactory Columns of the Bilateral Olfactory Bulbs by the AONpE

(A) Schematic representation of experimental strategy and results. Red tracer was injected into the M71 glomerulus and green tracer was injected into the granule cell layer beneath the contralateral M71 glomerulus. (B) Deposit of red tracer into the dorsal M71 glomerulus of the left hemisphere and green tracer into the granule cell layer beneath the M71 glomerulus of the right hemisphere. Inset in the right-hand panel shows that the injection site (red, arrow) was restricted to the M71 glomerulus (green). (C) Following this injection, retrogradely labeled somata and anterogradely labeled terminals were colocalized in a small region of the dorsal AONpE ipsilateral to the injection site of red tracer. Panel at right shows a high-power view of labeling (arrow at left). (D) Schematic representation of results following focal tracer injection into the AONpE. (E and F) Deposit of tracer into a small region within the AONpE (E) retrogradely labeled mitral/tufted cells within the dorsal MOB of the ipsilateral side and anterogradely labeled terminals in the dorsal bulb of the contralateral side (F). Note the symmetry of labeling (arrows in [F]) within the bilateral bulbs. Insets at right with numbers 1 and 2 show high-power views corresponding to numeric labels shown in left-hand panels. (G) A highly simplified schematic diagram showing the topographic relationship for the projection from the MOB to the ipsilateral AONpE. Small circles of different color in the left-hand panel indicate injection sites made in the MOB, whereas thatched circles with matching color in the right-hand panel indicate the corresponding target areas in the AONpE. A, anterior; DL, dorsolateral; DM, dorsomedial; P, posterior; VL, ventrolateral; VM, ventromedial.
Precise Linking of Bilateral Olfactory Maps

(Figures 3A and 3B; n = 4 mice). In all four cases, we observed colocalization of anterogradely labeled terminals and retrogradely labeled somata within a highly restricted region of the AONpE ipsilateral to the injected M71 glomerulus (Figure 3C). The dendrites of retrogradely labeled somata intermingled with ramifying axon terminals from the ipsilateral glomerulus injection (Figure 3C, right-hand panel). To further test whether this homotopic linking is specific to the M71 glomerulus alone or applies more generally to other glomeruli, we carried out tracing experiments targeting lateral mI7 → M71 glomeruli in mI7 → M71-ires-tauGFP mice (Bozza et al., 2002). In these mice, the gene encoding the M71 receptor is replaced with the gene for the mouse I7 receptor, and the lateral mI7 → M71 glomeruli are ectopically located in a position anterior to the endogenous lateral M71 glomeruli in the dorsal MOB. Injections into the lateral mI7 → M71 glomerulus anterogradely labeled a cluster of axon terminals in the dorsal AONpE that was more anterior to the AONpE area targeted by the lateral M71 glomerulus (Figures S5A–S5C; n = 4 mice). Small injections into the granule cell layer below the lateral mI7 → M71 glomerulus retrogradely labeled an area in the contralateral AONpE apparently corresponding to the area targeted by ipsilateral mI7 → M71 glomerulus (Figures S5E and S5F; n = 4 mice).

Further demonstrating the homotopic linking by the AONpE, tracer deposit into small regions of the dorsal AONpE labeled bilaterally symmetric regions in the MOB (Figures 3D–3F; n = 6 mice). Retrogradely labeled somata of mitral/tufted cells within the ipsilateral bulb and anterogradely labeled axon terminals in the contralateral bulb occupied highly restricted regions of the dorsal bulb that were bilaterally symmetric (arrows in Figure 3F). Confirming previous studies indicating that the AONpE projects exclusively to the contralateral MOB (Davis and Macrides, 1981; Schoenfeld and Macrides, 1984), we did not observe any anterograde labeling in cortical regions after tracer injection into the AONpE.

OSNs expressing common receptors tend to project to just two glomeruli, one medial and one lateral in the olfactory bulb, forming two mirror-symmetric isofunctional glomeruli within each MOB (Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1994). The two isofunctional glomeruli form precise reciprocal connections (Belluscio et al., 2002; Lodovichi et al., 2003), suggesting tight intrabulbar connections between the medial and lateral maps. Confirming the precision of the intrabulbar connections, we observed small axonal patches in the granule cell layer underlying the medial isofunctional glomeruli in the ipsilateral MOB when tracers were injected into the lateral M71 or mI7 → M71 glomeruli (Figures S1B and S5D). We next tested whether the medial and lateral maps were represented by same maps in the AONpE. If the isofunctional glomeruli within the MOB converge into same areas within the AONpE, then small tracer injection into the AONpE will retrogradely label two mirror-symmetric clusters of mitral/tufted cells in the ipsilateral MOB. Small injections into the AONpE retrogradely labeled only a single cluster of mitral/tufted cells instead of two mirror-symmetric clusters in the ipsilateral MOB (Figures 3D–3F; Figure S6; n = 9 mice). Consistently, tracer injections into the medial and lateral glomeruli labeled distinct clusters of axonal terminals in the AONpE (Figure 1; Figure S3). These results strongly suggest that the two mirror-symmetric olfactory maps within the MOB are represented by spatially distinct areas of the AONpE.

Our data indicate that mitral/tufted cells of a defined glomerulus project topographically to a small region within the ipsilateral AONpE. In turn, neurons in this AONpE region project to the contralateral granule cell layer directly beneath the bilaterally symmetric glomerulus associated with the same odorant receptor. The topographic relationship between the MOB and the AONpE is illustrated in Figure 3G. Linking of bilateral isofunctional olfactory columns by the AONpE is depicted schematically in Figure 3H.

Contralateral Relay of Specific Olfactory Sensory Information Requires the AONpE

The linking of isofunctional olfactory columns in bilateral olfactory bulbs suggested that odorant-specific information might be relayed across bilateral bulbs by the AONpE. We therefore tested whether odor-evoked activity within one MOB could be transferred to the contralateral bulb, and if so, whether such contralateral activation required the AONpE. Following an established stimulation protocol (Schaefer et al., 2001), we used c-Fos as a marker of odor-evoked neuronal activity within the MOB. We found that acetophenone (PMK), a known ligand for M71 receptor (Bozza et al., 2002), evoked c-Fos expression in both bulbs when presented to mice with both nares open. PMK robustly activated periglomerular cells and granule cells associated with each M71 glomerulus and adjacent glomeruli (Figures 4A–4D; n = 6). When PMK was applied while a single nostril was occluded, c-Fos expression was absent from the glomerular layer of the occluded side (Figures 4E–4H). However, c-Fos labeling was observed in a substantial number of cells within the granule cell layer of the occluded side, although the overall density of c-Fos immunopositive cells was significantly reduced (Figures 4E and 4F; 60.5 ± 10.0 cells/0.04 mm² for both nares open versus 17.8 ± 3.1 cells/0.04 mm² for ipsilateral naris occluded; mean ± SD; n = 6 mice, p < 0.001, between-group t test). Activated granule cells on the occluded side were clustered into areas beneath the M71 glomerulus and adjacent glomeruli, and formed an activation map symmetric to that seen in the bulb ipsilateral to the open naris (Figure 4E). The activation of granule cells within bilaterally symmetric regions in contralateral MOB strongly suggested odorant-driven activation of contralateral granule cells instead of nonselective activation by enhanced mechanosensory input following naris occlusion (Grosmaire et al., 2007). We confirmed that granule cells can be activated by contralateral glomerular activity by electrically stimulating the lateral M71 glomerulus. Consistent with odorant-driven c-Fos expression, electrical stimulation activated granule cells below the lateral M71 glomerulus in the contralateral bulb in addition to periglomerular
Physiological recordings were carried out to further examine the effect of neuronal activation by olfactory responses of the contralateral MOB. Bulbar neurons were recorded extracellularly from urethane-anesthetized mice. Neuronal responses to odorant pulses were recorded and compared before and after contralateral naris occlusion. Naris occlusion was monitored by local field potentials recorded from the glomerular layer in the bulb ipsilateral to the occluded naris (Figure 5A, top traces). Putative granule cells were selected based on low spontaneous activity, a bursting firing pattern, and recording sites 100–500 μm ventral to the dorsal mitral cell layer. In contrast, mitral/tufted cells were selected based on high spontaneous firing coupled to respiratory rhythm and recording sites within a thin layer 200–300 μm ventral to the dorsal surface. Among 29 granule cells that showed strong olfactory responses to odorants, a substantial number of them (n = 14/29 or ~48%) exhibited significant change in odorant-evoked responses following contralateral naris occlusion (p < 0.05, t test). Of the 14 cells with significant changes, a vast majority showed reversible reduction in odor-evoked responses following contralateral naris occlusion (Figure 5A; change of normalized response intensity = −54.7% ± 20.8%; n = 12/14 cells), although most of these cells remained responsive to ipsilateral odorant application. A substantial number of putative mitral/tufted cells (n = 12/25) showed significant changes in their olfactory responses following contralateral naris occlusion as well. Five of these twelve mitral/tufted cells with significant changes exhibited a reversible increase in response strength (Figure 5B; change in response intensity = 70.0% ± 48.9%), whereas the remaining seven cells exhibited reversible decreases (Figure 5C; change in response intensity = −81.3% ± 45.9%; n = 7/12 cells). Interestingly, we also observed significant changes of spontaneous firing rates from a substantial number of mitral/tufted cells (Figure 5C; n = 8/25 cells). Most of these cells (n = 7/8) showed a reversible increase of spontaneous firing following contralateral naris occlusion (8.0 ± 5.3 Hz versus 16.7 ± 4.0 Hz, before and after naris occlusion, respectively; p < 0.001, within group t test), suggesting that the contralateral input may regulate both olfactory responses and spontaneous activity of mitral/tufted cells.

We then tested if the AONpE played a role in activating granule cells in the contralateral MOB. Bipolar stimulating electrodes were targeted to the soma layer of the dorsal AONpE of anaesthetized mice (Figure 6A). Following local electrical stimulation of the dorsal AONpE, significantly more granule cells were
c-Fos+ in the dorsal contralateral MOB compared with those of unstimulated controls (Figures 6B–6D; 12.3 ± 3.0 cells/0.04 mm² versus 0.6 ± 1.0 cells/0.04 mm², with versus without stimulation, respectively; n = 4 mice, p < 0.001, t test, n = 4 mice). To test whether the AONpE is necessary for the contralateral activation, we made small unilateral lesions of the AONpE by pressure-injecting ibotenic acid (Figure 6E), which kills somata in an area largely restricted to the AONpE but leaves passing fibers intact (Figure S7). We then occluded the naris contralateral to the AONpE lesions and examined c-Fos expression patterns in the bulbs following the presentation of PMK (n = 6 mice). Virtually no c-Fos expression was observed in the granule cell layer of the MOB ipsilateral to the occluded naris and contralateral to the lesioned AONpE (Figures 6F–6I; 1.2 ± 1.8 cells/0.04 mm² for occluded side following AONpE lesion; n = 6 mice, p < 0.001, between-group t test for ipsilateral naris occlusion with and without AONpE lesion), but the pattern of c-Fos expression on the side of the open naris appeared normal (Figures 6F and 6I). Thus, activation of the contralateral MOB requires the AONpE. In accordance with the role of AONpE in contralateral transfer of olfactory information, application of PMK activated ipsilateral AONpE regions comparable to the target area of M71-associated mitral/tufted cells from mice with contralateral naris occluded (Figures 6J and 6K).

The AONpE Contributes to Interhemispheric Transfer of Olfactory Memory

Our tracing results showed that the AONpE links bilateral iso-functional olfactory columns. Our results of c-Fos mapping and physiological recordings indicated that odorant-specific information is relayed between the MOB hemispheres by the AONpE. Since studies have shown that olfactory memory stored in one hemisphere can be transferred to the contralateral hemisphere via the anterior commissure in adult rats (Kucharski and Hall, 1987, 1988), we asked whether the intrabulbar association system could contribute to transferring olfactory information that guides behavioral learning. An adult mouse was allowed to explore freely in two opposing arms of a T-maze and one of two odorants (PMK and amyl acetate [AA], 0.5% saturated vapor) was randomly infused into the arm where the mouse
settled (Figure 7A). If the odorant was AA, the animal received a mild footshock in the same arm after a 10 s delay, whereas PMK was associated with voltage application to a bottom plate in the opposite arm. After 100 trials, mice learned to avoid footshock by rapidly moving to the opposite arm following AA application but staying in the same arm following PMK, with a performance asymptote over 95% correct (Figure 7B). Their responses to odorant cues were tested without footshock the following day.

After establishing this behavioral paradigm of olfactory learning, we trained mice with one naris open and the other occluded. Mice were divided into four groups based on whether their AONpE was intact or bilaterally lesioned before training and whether they were tested with the training naris or the previously occluded naris (Figure 7C; n = 4, 4, 5, and 5 mice for Groups 1–4, respectively). By mixing the drug ibotenic acid with dextran tracers, we confirmed that most of the AONpE neurons were killed, although in some cases the toxin appeared to spread to small areas adjacent to the AONpE (Figure 7E). Mice with AONpE lesions could be trained to associate odorants with footshock, indicating that the AONpE lesion did not result in general anosmia. When tested with the training naris, mice performed equally well with the AONpE either intact or lesioned (Figure 7D; 97.5% ± 2.5% and 95.0% ± 3.9% at asymptote for Groups 1 and 3, respectively; mean ± SEM for all behavioral assays; p = 0.63, t test), suggesting that olfactory learning within the ipsilateral hemisphere was independent of the AONpE. When tested with the previously occluded naris, mice performed similarly well with a sham-lesioned AONpE (Figure 7D; correct ratio = 91.0% ± 5.1%; p = 0.58, between-group t test for Groups 1 and 2). However, their performance dropped to near-chance level when tested with the previously occluded naris and lesions of the AONpE (Figure 7D; correct ratio = 60.8% ± 5.0%, p < 0.01, t test for Groups 2 and 4). Thus, these results are consistent with the concept that the AONpE contributes to bilateral exchange of information about odorant identity.

**DISCUSSION**

In this study, we have integrated tract tracing in transgenic and wild-type mice, c-Fos labeling, electrophysiology, and behavioral assays to examine the connectivity and functional role of the interbulbar association system. Our use of transgenic mouse...
lines with GFP-labeled glomeruli permitted a level of mapping that has been impossible in previous studies. By depositing tract tracers focally into single identified glomeruli, we find that mitral/tufted cells associated with a specific glomerulus project topographically to the AONpE, where the AONpE neurons in turn project to granule cells underlying the isofunctional glomerulus in the contralateral MOB. Previous studies observed no topography in the longitudinal direction and quadrant-to-quadrant topography in other directions for the interbulbar association system (Davis and Macrides, 1981; Haberly and Price, 1978; Schoenfeld and Macrides, 1984; Scott et al., 1985). This conflict is likely a result of large tracer injection sites in previous studies. Furthermore, these early studies were carried out prior to the discovery of odorant receptors and their symmetric representation in the bilateral olfactory bulbs. Because of the lack of receptor identity for the bilaterally isofunctional glomeruli, it had remained unclear what the interbulbar association system connected exactly. By targeted tract tracing, we have uncovered a link between isofunctional olfactory columns in the bilateral olfactory bulbs by the interbulbar association system. More importantly, as suggested by the exquisite anatomical linkage, we find that the AONpE mediates bilateral exchange of odorant-specific signals.

Consistent with the precision of integration within the AONpE, morphological studies have shown that AONpE pyramidal neurons possess small dendritic trees (Reyher et al., 1988; Scott et al., 1985), a specialization that could permit maintenance of odorant selectivity originating in the MOB. Future studies on the synaptic distribution between bulbar neurons and the dendritic tree of AONpE neurons will allow precise quantification of the topography and convergence ratio. Nevertheless, the highly topographic and stereotypical representation of each glomerulus in the AONpE is reminiscent of the projection neurons in Drosophila antennal lobes, an area comparable to the mammalian olfactory bulb. These projection neurons possess stereotyped patterns of axonal distribution in the protocerebrum (Jeffries et al., 2007; Marin et al., 2002; Wong et al., 2002). Genetic studies have contributed substantially to our understanding of the molecular mechanisms underlying the wiring in the MOB (Wang et al., 1998; Yoshihara et al., 1997; Yu and Bargmann, 2001) and the Drosophila antennal lobe (Komiyama et al., 2007). It will be interesting to examine the developmental mechanisms underlying the precise linking of ~1800 pairs of glomeruli by the interbulbar association system. The topographic precision of the interbulbar association system is comparable to that of the intrabulbar association system, which precisely connects the two mirror-symmetric olfactory maps within the MOB (Belluscio et al., 2002; Liu and Shipley, 1994; Lodovichi et al., 2003; Schoenfeld et al., 1985). These two intrabulbar mirror-symmetric maps appear to be represented by distinct areas in the AONpE, suggesting that both receptor identity and glomerular spatial location interact to establish the linking of bilateral olfactory bulbs. The refinement of the intrabulbar connections...
is activity dependent (Marks et al., 2006), a subject that remains to be explored in the interbulbar association system.

Olfactory memory formed in one hemisphere can be transferred to the contralateral hemisphere in rats and humans (Kucharski and Hall, 1987, 1988; Mainland et al., 2002). The specific neural circuitry underlying this contralateral transfer of olfactory memory was unknown. Our c-Fos mapping and physiological recordings show that the activity of specific glomeruli within one hemisphere of the MOB is transferred to the granule cells underlying the isofunctional glomeruli in the contralateral hemisphere. In behavioral assays, mice showed clear deficits in contralateral transfer of olfactory memory when their AONpE was lesioned with ibotenic acid. Thus, our experiments suggest that the AONpE is a critical locus for bilateral relay of olfactory signals, although cortical areas other than the AONpE can also contribute to this process. We hypothesize the following scenario in olfactory signal propagation between hemispheres: the activation of a specific glomerulus activates its associated mitral/tufted cells, which in turn activate their postsynaptic target neurons in the AONpE, followed in turn by their targets: the granule cells underlying the isofunctional glomeruli in the contralateral MOB. The activity of these granule cells may in turn enhance the activity of mitral/tufted cells associated with the isofunctional glomeruli by mechanisms such as lateral inhibition (Luo and Katz, 2001; Yokoi et al., 1995). This scenario may be directly tested by targeted recordings from mitral/tufted cells associated with a specific glomerulus and optical stimulation of the contralateral isofunctional glomerulus using a recently developed transgenic mouse line expressing Channelrhodopsin-2 in mitral/tufted cells (Arenkiel et al., 2007).

The interbulbar association system may be important for other behaviors as well. Rats can localize olfactory pulses in space as arriving from either the left or the right side (Rajan et al., 2000). The linking of isofunctional columns between the bulbs may contribute to “stereo” olfactory perception by allowing the system to calculate the bilateral differences of odor-evoked activity patterns between the isofunctional columns. In addition, human and animal nasal passages exhibit spontaneous change in unilateral airflow resistance (nasal-cycle congestion) (Stoksted, 1953). The AONpE may balance the activity of the bilateral olfactory bulbs when the same odorant generates sensory signals of distinct intensities in the bilateral olfactory epithelia during different phases of nasal cycles (Sobel et al., 1999).

The two symmetric hemispheres of mammalian forebrain are connected by dense fibers coursing within the corpus callosum and anterior commissure. The studies on “split-brains” illustrate the essential role of interhemispheric communication in perception and sensory learning (Gazzaniga, 1995). For example, animals with severed corpus callosum have bilaterally separate “learning centers,” with one hemisphere failing to access the learned information in another hemisphere (Myers and Sperry, 1958). Our use of transgenic mice with a specific glomerulus expressing GFP permitted unprecedented mapping of bilateral connections for specific olfactory glomeruli. These data indicate a highly structured organization of anatomical pathways carrying specific olfactory information between the bilateral bulbs, suggesting the interbulbar association system as an advantageous model for studying the cellular circuits, function, and development of interhemispheric connections.

EXPERIMENTAL PROCEDURES

Adult M71-ires-tauGFP and M71/iRES-tauGFP mice (7–12 weeks old) were used in all experiments except for T-maze assays, in which C57BL/6 mice were used.

Tract Tracing and Ibotenic Acid Lesion

Mice were anesthetized with a mixture of ketamine (200 mg/kg) and xylazine (25 mg/kg, i.p.). Guided by green fluorescence of the M71 or M71 glo- merulus, biotinylated dextran amines (10K MW, BDA, 10%, Invitrogen) mixed with Texas red dextran amines (10K MW, TRDA, 1%) or fluorescently-conjugated dextran amines (10K MW, FDA, 10%) were iontophoretically injected (1 µA, 1–20 s) into target areas through a micropipette. For lesion of the AONpE, 0.1–0.5 µl of 0.5% ibotenic acid in saline was pressure injected into the AONpE. Ibotenic acid was mixed with 1% tetramethylrhodamine dextran amine (TMR-DA) to visualize the lesion sites. Sham lesions were carried out by pressure injection of vehicle (1% TMR-DA in saline).

Histology

Mice were overdosed with pentobarbital (300 mg/kg, i.p.) and perfused transcardially with 0.1M phosphate buffered saline (PBS) and then 4% paraformaldehyde in PBS. Following postfixation and cryoprotection, coronal sections (60 µm) were prepared on a freezing microtome (Leica CR 1900). BDA tracers were visualized by fluorescence-conjugated streptavidin. Sections were mounted in 50% glycerol with DAPI.

Mapping Neuronal Activity with c-Fos

For odorant exposure, mice were placed overnight in a chamber (volume = 1.8 liters) with continuous airflow (4 l/min) filtered with active charcoal. PMK pulses (~1% saturated vapor) were applied for 2 min at 5 min intervals over a 15 min period. For electrical stimulation, mice were first anesthetized with urethane (1.64 g/kg injected as 20% solution i.p.). After 4 h of exposure to purified air, bipolar tungsten electrodes (1 MΩ, Microprobes) were placed within the M71 glomeruli or the AONpE to generate 240 trains of electric pulses over 20 min, with each train comprising 100 pulses at 30–50 µA (100 Hz; pulse duration = 0.1 ms). Mice were transcardially perfused 90 min after either odorant or electrical stimulation and brains were later processed for c-Fos immunostaining. The primary antibody was a rabbit polyclonal antibody against c-Fos (1:500, Santa Cruz) and the secondary antibody was Cy3-conjugated goat anti-rabbit (1:500, Jackson ImmunoResearch).

Confocal Imaging and Analysis

Sections were imaged with a laser scanning confocal microscope (Zeiss LSM 510). Optical stacks (Z-interval 1.0 µm) were projected into single frames. Whole-view images were constructed by assembling multiple adjacent frames of 10× images. Borders and cell layers were identified by DAPI labeling and autofluorescence. To define c-Fos+ cells, we first measured the mean and SD of background optical signals. Cells with optical intensities >[mean + 3*SD] were considered c-Fos+. The density of c-Fos+ neurons was calculated by counting the number of c-Fos+ neurons within a square with side length equal to 200 µm, which is roughly the size of large glomeruli in the olfactory bulb.

Electrophysiological Recordings

Animals were anesthetized with urethane. After the dorsal M71 glomerulus was identified, a small piece of bone above the glomerulus was removed and tungsten electrodes were lowered below the M71 glomerulus. Odorants (1% saturated vapor, 2 s pulse duration) were applied with a custom-made 64-channel robotic olfactometer. Data were band-pass filtered at 0.5–3.0 KHz, digitized at 6 KHz, and analyzed with a custom-written Matlab program. For a neuron to be considered responsive to an odorant, its mean firing rate during odorant application needed to be significantly different (p < 0.05, paired t test) from its mean spontaneous rate within 2 s preceding stimulus.
onset. Its response intensity to an odorant was calculated by subtracting the spontaneous rate from the firing rate during the odorant application. Change of normalized response intensity was calculated as \([\frac{\text{response intensity after naris occlusion}}{\text{response intensity before naris occlusion}}]\) – 100%.

**Behavioral Assay**

A detailed description of this method can be found in **Supplementary Online Material**. Briefly, adult C57BL/6 mice were trained in a customized T-maze to associate AA with footshock in the same arm and PMK with footshock in the opposite arm. Mice learned to avoid footshock by moving to the opposite arm following AA or staying within the same arm following PMK. After learning (100% correct for ten trials), they were tested without actual application of voltage. A nostril was occluded by inserting a soft rod (0.8 mm in diameter, 4 mm in length) and then sealing with a small drop of Vetbond tissue adhesive. Animal movement was monitored with an infrared CCD camera. Training and testing were controlled by a custom-written Matlab program.

**SUPPLEMENTAL DATA**

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/58/4/613/DC1/.

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