Genetic tracing reveals a stereotyped sensory map in the olfactory cortex

Zhihua Zou*†, Lisa F. Horowitz*†, Jean-Pierre Montmayeur*, Scott Snapper‡ & Linda B. Buck*

* *Howard Hughes Medical Institute, Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115, USA*
‡ *Gastrointestinal Unit (Medical Services), Massachusetts General Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114, USA*
† *These authors contributed equally to this work*

The olfactory system translates myriad chemical structures into diverse odour perceptions. To gain insight into how this is accomplished, we prepared mice that coexpressed a transneuronal tracer with only one of about 1,000 different odorant receptors. The tracer travelled from nasal neurons expressing that receptor to the olfactory bulb and then to the olfactory cortex, allowing visualization of cortical neurons that receive input from a particular odorant receptor. These studies revealed a stereotyped sensory map in the olfactory cortex in which signals from a particular receptor are targeted to specific clusters of neurons. Inputs from different receptors overlap spatially and could be combined in single neurons, potentially allowing for an integration of the components of an odorant's combinatorial receptor code. Signals from the same receptor are targeted to multiple olfactory cortical areas, permitting the parallel, and perhaps differential, processing of inputs from a single receptor before delivery to the neocortex and limbic system.

Mammals perceive an immense variety of volatile chemicals as having different odours[1–3]. The initial event in olfactory perception is the detection of odorants by odorant receptors (ORs) on olfactory sensory neurons in the nose[4,5]. Signals generated by these neurons in response to odorants are relayed through the olfactory bulb of the brain, and then the olfactory cortex, ultimately reaching higher cortical areas that are thought to be important in odour discrimination, as well as limbic areas believed to mediate the emotional and physiological effects of odours[1,2,6–9].

In the olfactory epithelium of the mouse nose there are about 5,000,000 olfactory sensory neurons[10], each of which expresses only one of about 1,000 different OR genes[11]. Each OR recognizes multiple odorants (refs 11, 46–48), but different odorants are recognized, and thereby encoded, by different combinations of ORs[11]. The olfactory epithelium has four spatial zones that express non-overlapping sets of ORs and project axons to four corresponding zones in the olfactory bulb[10,12–15]. In the nose, neurons expressing the same OR are scattered throughout one zone[10,12]; however, in the olfactory bulb their axons converge at two fixed locations, where they form synapses with bulb mitral and tufted relay neurons in only a few of the bulb's 2,000 glomeruli[13–15]. The result is a stereotyped spatial map in which inputs from different ORs are targeted to different glomeruli and bulb neurons. Consistent with patterns of odour-induced activity[16–22], an odorant's receptor code is represented in the nose by a dispersed ensemble of neurons and in the bulb by a specific combination of glomeruli.

How is olfactory information organized in the olfactory cortex to ultimately yield the perception of different odours? Anatomical studies indicate that the bulb map is not recapitulated in the olfactory cortex[6–9]. Individual bulb relay neurons, whose axons form synapses in the olfactory cortex, are retrogradely labelled by tracers that are deposited in more than one cortical region[23], and neurons in different parts of the bulb are retrogradely labelled from a single cortical region[24,25]. These findings have prompted speculation that there may be little or no topographical organization of sensory information in the olfactory cortex. However, a few studies suggest that neighbouring bulb neurons may form synapses at the same sites, hinting that cortical inputs may not be organized diffusely, but in some manner not yet discerned[26,27].

We were interested in three questions. First, is input from a given OR targeted to discrete sites in the olfactory cortex, as in the bulb; scattered, as in the nose; or arranged in yet another way? Second, do different areas of the olfactory cortex, which may have different functions, receive inputs from different subsets of ORs, or does each area receive input from the entire OR repertoire? And finally, given that each odorant is recognized by multiple ORs, are inputs from different receptors, which are segregated in the nose and bulb, combined in individual cortical neurons?

Preparation of mice expressing a tracer with one OR gene

To address these three questions, we used a transneuronal tracer—barley lectin (BL)—that we previously found could cross multiple synapses if expressed from a transgene in mice[28]. When expressed in all olfactory sensory neurons, BL (or wheat germ agglutinin[29]) is transneuronally transferred to second-order bulb neurons and then from those neurons to third-order neurons in the olfactory cortex[28,29].

To determine how inputs from a single OR are organized in the cortex, we prepared mice in which BL was coexpressed with only one OR gene. Using gene targeting in embryonic stem cells, we modified a single OR gene by inserting—just after its coding region—an internal ribosome entry site (IRES) sequence[15,30] followed by a BL complementary DNA (Fig. 1). The altered stem cells were then used to generate mice that produce a bicistronic messenger RNA from which the OR and BL are independently translated. We did this for two OR genes: *M5*, which is expressed in the most dorsal nasal zone (zone 1), and *M50*, which is expressed in the most ventrolateral zone (zone 4)[10,31]. We then used immunostaining of serial nose and brain sections from these 'M5BL' and 'M50BL' knock-in mice to visualize neurons that contained BL.

In transgenic mice expressing BL under the control of the olfactory marker protein promoter (OmpBL mice), BL is expressed in all olfactory sensory neurons[28]. In those mice, BL labelling is seen in neurons throughout the olfactory epithelium and in the axons of those neurons in all of the glomeruli of the olfactory bulb. In contrast, in M5BL and M50BL knock-in mice, BL was detected in only a small fraction of olfactory sensory neurons in the nose and in only a few glomeruli in the olfactory bulb (Fig. 2). No BL labelling was seen in the nose or bulb of wild-type control mice.

In the nose, labelled neurons were located in zone 1 in M5BL mice ($n = 10$) and in zone 4 in M50BL mice ($n = 10$) (Fig. 2a, b). This patterning resembled that seen in wild-type mice by *in situ* hybridization with M5 and M50 antisense RNA probes (Fig. 2c, d)[10,28]. The BL^+ glomeruli of the knock-in mice ($n = 10$ per knock-in strain)

were also similar in both number and position to glomeruli that hybridize to M5 and M50 probes in wild-type animals (Fig. 2e, f, h, i)[13,28]. Moreover, in alternate sections of heterozygous M50BL bulb, the immunostained glomeruli were the same as those labelled by *in situ* hybridization with the M50 probe (Fig. 2f, g, i, j). In each knock-in strain, one or two BL^+ glomeruli were seen at one medial and one lateral site in the bulb. These findings were consistent with our previous observations that BL expression in OmpBL mice does not alter OR gene expression in the nose or the targeting of axons to specific glomeruli in the bulb[28].

In the glomeruli of the bulb, the axons of olfactory sensory neurons form synapses with the dendrites of bulb mitral and tufted relay neurons[32]. In M5BL and M50BL mice, BL was detected in approximately 35–40 mitral and tufted neurons near each BL^+ glomerulus. This is similar to the number of these neurons that innervate individual rat glomeruli[33,34]. The BL^+ neurons were invariably located near labelled glomeruli (Fig. 2k, l). In contrast, in OmpBL mice, BL^+ mitral and tufted neurons are seen throughout the bulb[28]. The patterns of labelling observed in the knock-in mice indicate that BL was transferred from olfactory sensory neurons expressing M5 or M50 ORs to the mitral and tufted relay neurons with which they form synapses in the olfactory bulb.

OR inputs are clustered in the olfactory cortex

Mitral and tufted relay neurons in the bulb transmit signals to the olfactory cortex. The olfactory cortex is composed of several anatomically distinct areas, including the piriform cortex (the largest area), olfactory tubercle, anterior olfactory nucleus, and specific parts of the amygdala and entorhinal cortex[6–9]. The anterior and posterior halves of the piriform cortex differ morphologically and may be functionally distinct. Mitral cells project axons to the entire olfactory cortex, but tufted cells project only to the most anterior areas, such as the anterior olfactory nucleus and olfactory tubercle.

In the piriform cortex, mitral cell axons travel in the lateral olfactory tract and then fan out over the cortical surface, branching to form synapses with the dendrites of cortical pyramidal neurons[6–9]. The synapses are formed in cortical layer Ia, whereas pyramidal neuron cell bodies are located in layers II and III (refs 6–9). In OmpBL mice, BL-labelled cells are seen in layers II and III throughout the entire olfactory cortex, consistent with the transfer of BL from mitral cells to the pyramidal neurons with which they form synapses (Fig. 3d)[28].

In M5BL and M50BL knock-in mice, BL-labelled neurons were

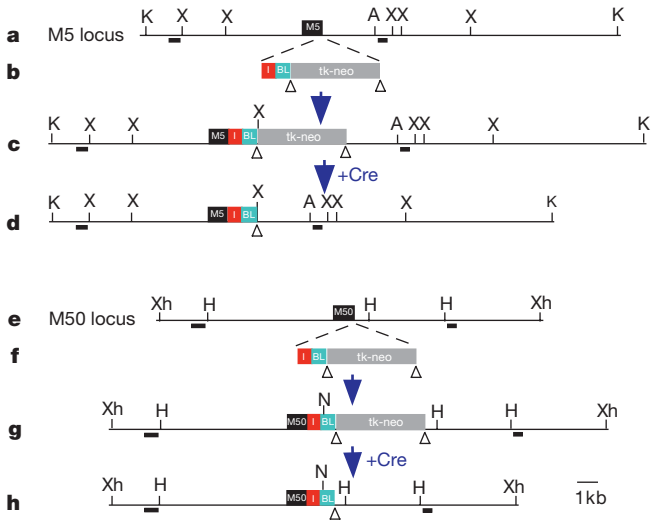

Figure 1 Gene targeting strategy used to coexpress barley lectin (BL) with the *M5* or *M50* odorant receptor gene. Using homologous recombination in embryonic stem cells, an IRES/BL/loxP/tk-neo/loxP sequence (**b**, **f**) was inserted immediately 3′ to the M5 or M50 odorant receptor coding region (**a**, **c**, **e**, **g**). The tk-neo cassette was removed by transient transfection with a Cre recombinase expression plasmid, leaving a single loxP site (**d**, **h**). The M5 or M50 coding region is represented by a black box; the IRES sequence by a red box; the BL coding region by a blue box; the tk-neo cassette by a grey box; and loxP sites by triangles. Thick black horizontal bars show regions used as probes in Southern blot screening for homologous recombinants. Restriction enzyme sites: A, *Afl*II; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; X, *Xba*I; Xh, *Xho*I.

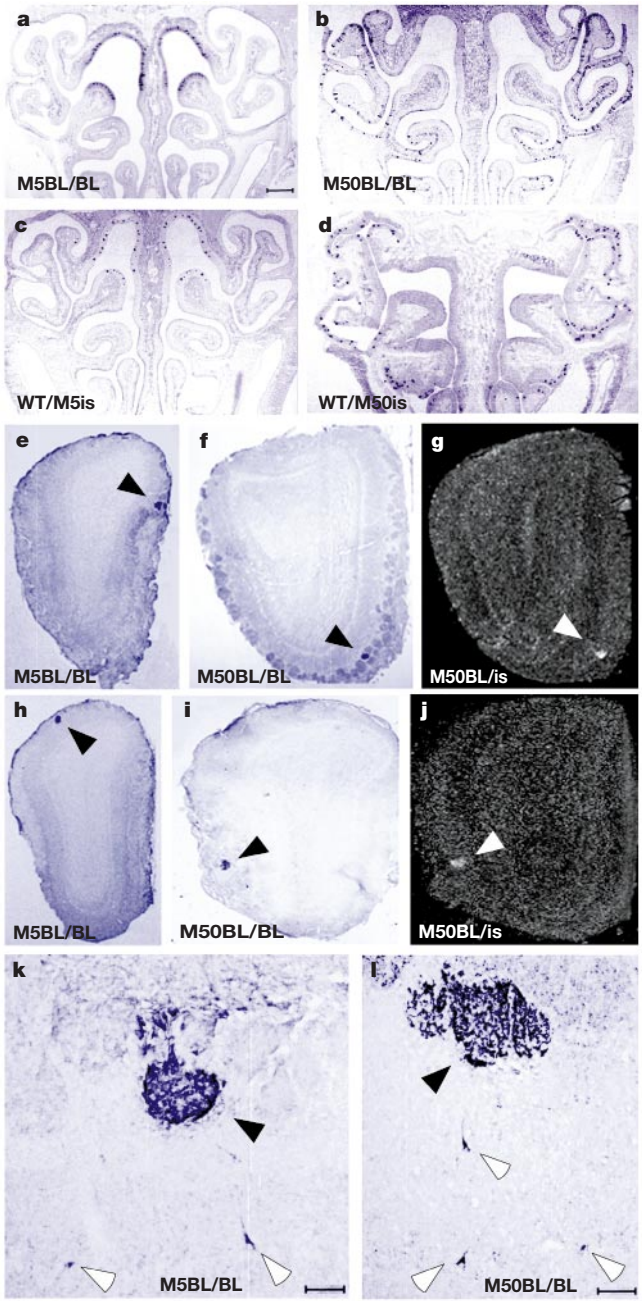

Figure 2 Detection of BL in the nose and olfactory bulb. Patterns of BL labelling in the nose of M5BL (**a**) or M50BL (**b**) knock-in mice resembled those seen in wild-type (WT) mice by *in situ* hybridization (is) with an M5 (**c**) or M50 (**d**) cRNA probe. A few glomeruli (black arrowheads) were BL-labelled at characteristic locations on the medial (**e**, **f**) and lateral (**h**, **i**) sides of the olfactory bulb in M5BL (**e**, **h**) and M50BL (**f**, **i**) mice. The M50 probe hybridized to the same glomeruli seen in **f** and **i** in adjacent sections (**g**, **j**), but not to other glomeruli. BL-labelled mitral and tufted cells (white arrowheads) were seen underlying the labelled glomeruli (black arrowheads) (**k**, **l**). Scale bars: **a**, 320 μm; **k**, **l**, 40 μm. Panels **a**–**j** are to the same scale.

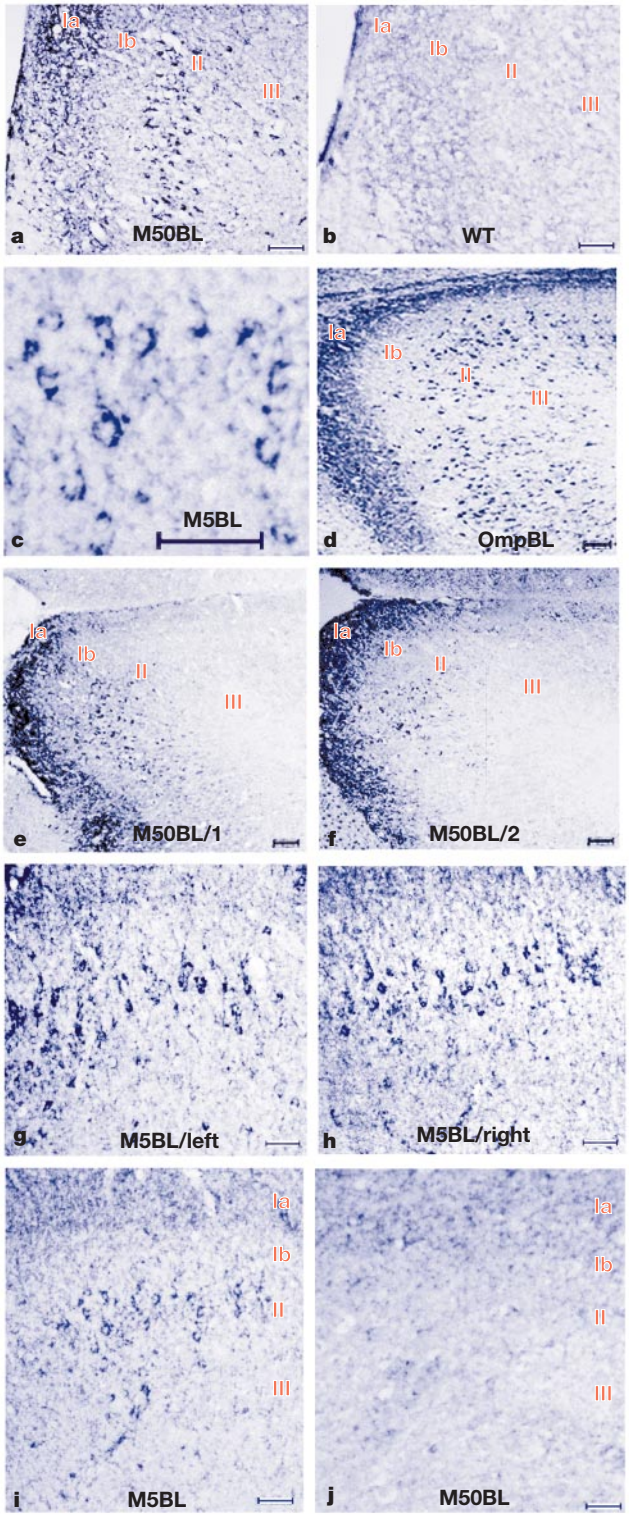

Figure 3 BL-labelled neurons in the anterior piriform cortex of M5BL and M50BL mice. BL^+ cells were arranged in clusters in layers II and III of the anterior piriform cortex of M5BL (**c**, **g**, **h**, **i**) and M50BL (**a**, **e**, **f**) mice. No BL-labelling was seen in wild-type (WT) mice (**b**), whereas in OmpBL mice, BL^+ cells were distributed throughout the anterior piriform cortex in layers II and III (**d**). The BL-labelled clusters had the same locations in different individuals of the same knock-in strain (**e**, **f**) and most were bilaterally symmetrical in the left and right hemisphere of the brain (**g**, **h**). The locations of clusters differed in the two strains (**i** (M5BL) compared with **j** (M50BL)). Scale bars: **a**–**c**, **g**–**j**, 40 μm; **d**–**f**, 80 μm.

also seen in layers II and III of the piriform cortex and other olfactory cortical areas (Figs 3–5). However, there were far fewer BL^+ cells in the knock-in mice than in OmpBL mice (Fig. 3d–f; see also below). Labelled cells were not observed in wild-type animals (Fig. 3b) nor were any cells expressing M5 or M50 detected by *in situ* hybridization in the brains of the knock-in animals (data not shown).

Notably, in the knock-in mice, the BL^+ neurons were organized in discrete clusters in most olfactory cortical areas. In M5BL mice, there were two clusters of BL^+ neurons in the anterior piriform cortex (Figs 3g–i and 4b, c), one in the olfactory tubercle (Fig. 5a), one in the anterior olfactory nucleus and one in the entorhinal cortex (Fig. 5e). M50BL animals had three labelled clusters in the anterior piriform cortex (Figs 3a, e, f and 4d–f), one in the olfactory tubercle (Fig. 5b) and two in the entorhinal cortex (Fig. 5f). In the posterior piriform cortex of both strains, there were numerous labelled neurons. Although there appeared to be a cluster of more densely labelled neurons here in each strain, there were also many less densely labelled neurons scattered over much of this area (Fig. 5c, d). The patterns of labelled neurons seen in olfactory cortex are depicted in Fig. 6. In both strains, a few labelled neurons were also detected in the horizontal limb of the diagonal band, which innervates the bulb. In addition, weakly labelled cells were sometimes seen in the neocortex in M5BL animals and, in M50BL mice, BL^+ neurons were detected in the paraventricular and arcuate nuclei of the hypothalamus (data not shown). Given the lack of evidence for a direct bulb–hypothalamus projection[35], this might reflect a polysynaptic transport of the tracer.

Although some olfactory cortical neurons project axons to the bulb[6–9,32], it is unlikely that BL was retrogradely transported to the cortex through these axons, as they terminate deep in the bulb, distant from BL^+ glomeruli and mitral/tufted neurons[32]. Moreover, the amygdala, which sends axons to the bulb, lacked BL^+ cells in the knock-in mice. In OmpBL mice, BL that was retrogradely transported from the bulb to neuromodulatory brain centres appeared to be relayed to many brain structures[28], but no evidence of this was seen in the knock-in mice, perhaps because there was much less BL in the bulb.

The olfactory cortex has a stereotyped sensory map

We next compared the locations of the BL^+ neuronal clusters in different animals ($n = 5$ per strain). We focused first on the piriform cortex, the principal olfactory cortical area. Locations along the anterior–posterior axis were calculated from the number of tissue sections between the centre of a cluster and the end of the lateral olfactory tract (eLOT), a landmark that divides the anterior and posterior piriform cortex (Table 1). An atlas of the mouse brain was used to further examine the anterior–posterior locations of the clusters and to determine their positions along the dorsal–ventral and medial–lateral axes of individual olfactory cortical areas[36].

These comparisons revealed an unexpected level of organization in the olfactory cortex. Detailed analyses of the clusters seen in the anterior piriform cortex indicated that the BL^+ neuronal clusters in this area had nearly identical locations in individuals of the same knock-in strain (Fig. 3e, f; see also Table 1). In addition, with the exception of one cluster that was invariably seen in only the left hemisphere of the brain of M50BL mice, the clusters had bilaterally symmetrical locations in the left and right hemispheres (Figs 3g, h and 4; see also Table 1). For example, in one M5BL mouse, the centre of the first (most anterior) M5BL cluster was 2.45 and 2.48 mm from eLOT in the left and right hemisphere, respectively, and 2.40 and 2.42 mm from this landmark in the left and right hemispheres of a different M5BL mouse (Table 1). The number of labelled neurons in each cluster was also similar among individuals and in the two hemispheres. For example, the numbers of BL^+ neurons in the first cluster in the left and right hemispheres of two M5BL mice were 1,926, 1,800, 1,566 and 1,776.

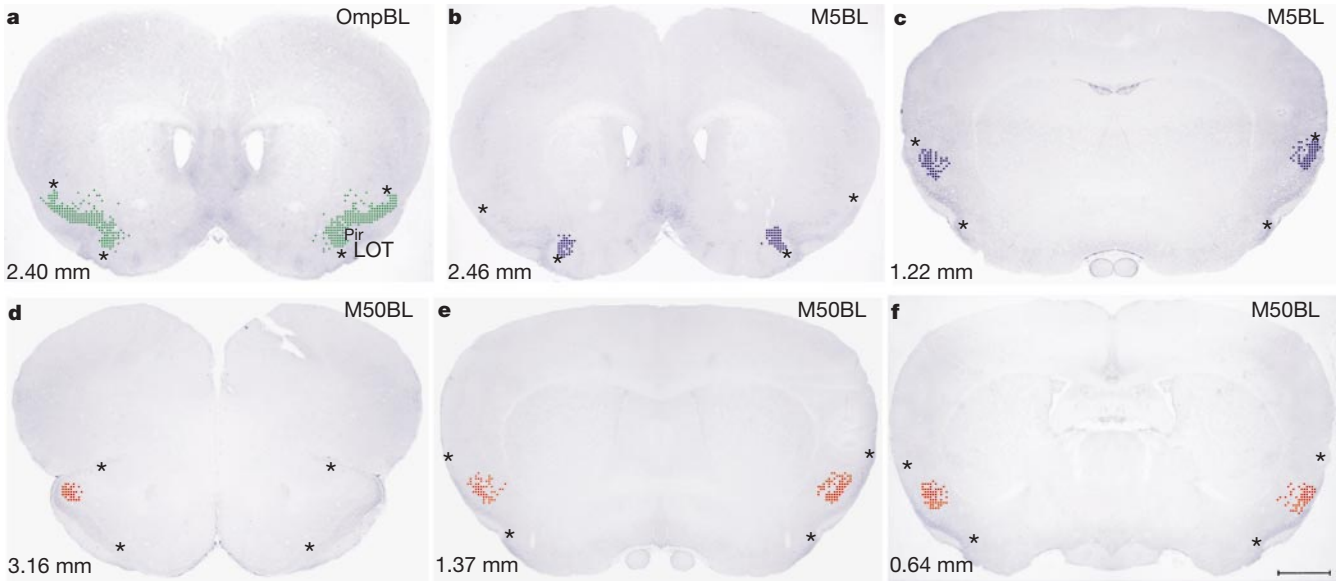

Figure 4 Locations of labelled neurons in the anterior piriform cortex. The locations of BL-labelled neurons in high-magnification photographs of brain sections were marked by coloured dots, scaled, and then transferred to low-magnification photographs of the same sections. **a**–**f**, Coronal sections from OmpBL (**a**), M5BL (**b**, **c**) and M50BL (**d**–**f**) mice. The dorsal surface is at the top. The anterior–posterior distance of each section from eLOT is shown in millimetres. The dorsal and ventral limits of the piriform cortex (Pir) are indicated by asterisks. LOT, lateral olfactory tract. Scale bar, 640 μm.

The positions of labelled neuronal clusters in the posterior piriform cortex, olfactory tubercle, anterior olfactory nucleus and entorhinal cortex were also similar in different individuals, and most were bilaterally symmetrical in the two hemispheres (Fig. 5). The only exception was in the entorhinal cortex, where labelled clusters appeared to have slightly different locations in the left and right hemispheres. Notably, the centres of the BL^+ clusters had different locations in the two knock-in strains. For example, in the anterior piriform cortex, the centres of clusters in M5BL mice were 2.44 ± 0.03 and 1.16 ± 0.06 mm from eLOT, whereas, in M50BL mice, they were 3.15 ± 0.06, 1.34 ± 0.07 and 0.59 ± 0.06 mm from eLOT (Table 1). These clusters were also centred at different locations along the dorsal–ventral and medial–lateral axes in the two strains (Figs 4–6).

These results indicate that there is a stereotyped map of sensory inputs in the olfactory cortex. In this map, inputs from individual

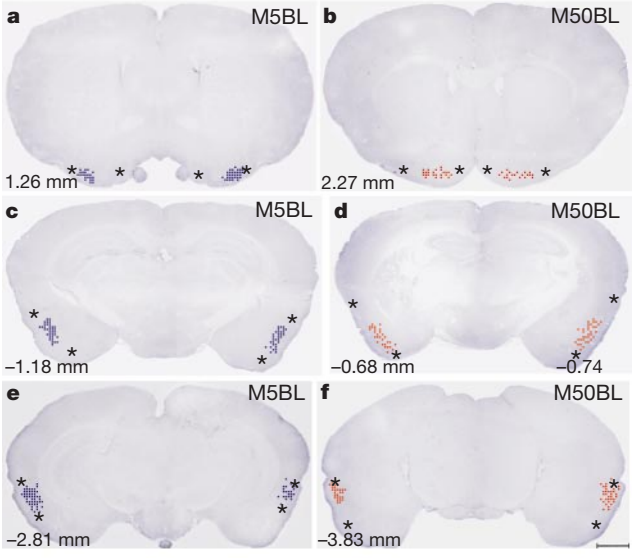

Figure 5 BL-labelled neurons in other olfactory cortical areas. **a**–**f**, The locations of labelled neurons, determined as in Fig. 4, are shown as coloured dots in photographs of coronal sections through the olfactory tubercle (**a**, **b**), posterior piriform cortex (**c**, **d**) and entorhinal cortex (**e**, **f**) of M5BL (**a**, **c**, **e**) and M50BL (**b**, **d**, **f**) mice. The dorsal–ventral or medial–lateral limits of each of these areas are indicated by asterisks. The dorsal surface is at the top. Scale bar, 640 μm.

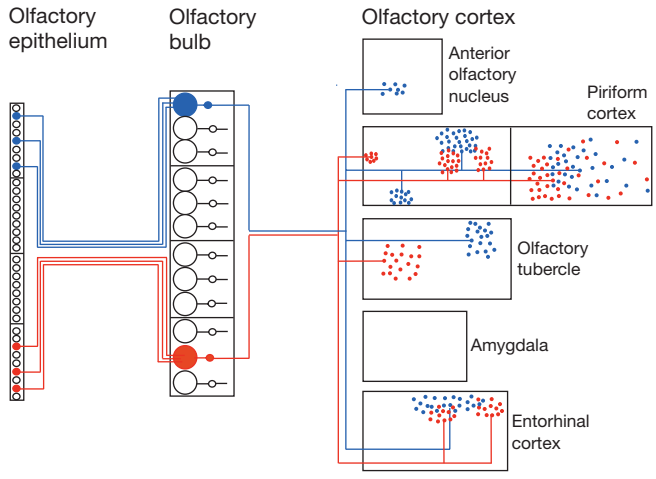

Figure 6 Transformations of odorant receptor inputs in the nervous system. The locations of BL labelling in the olfactory system of M5BL (blue) and M50BL (red) mice are shown. In the olfactory epithelium, sensory neurons expressing the M5 or M50 OR are scattered in one zone; however, in the olfactory bulb, their axons all form synapses with mitral cells (small circles) in the same few glomeruli (large circles), creating a stereotyped map of OR inputs. In the nose and bulb, inputs from different ORs are segregated. Mitral cells carrying input from M5 or M50 form synapses with clusters of neurons (coloured dots) at specific sites in multiple olfactory cortical areas (boxes), creating another stereotyped map of sensory information. In this map, inputs from different ORs overlap spatially and single neurons may receive combinatorial input from multiple ORs. Inputs from the same ORs reach different olfactory cortical areas, allowing for parallel and perhaps differential processing of the same sensory inputs.

ORs are targeted to multiple, discrete clusters of cortical neurons, whose locations are similar or identical in different individuals.

Combinatorial OR inputs in the olfactory cortex

While inputs from different ORs are spatially segregated in different glomeruli in the olfactory bulb, they appear to overlap, at least partially, in the olfactory cortex. Although the centres of labelled neuronal clusters differed in the two knock-in strains, partial overlap seems probable in many cases. For example, in the anterior piriform cortex, the centres of the second clusters in M5BL and M50BL mice were 1.16 ± 0.06 and 1.34 ± 0.07 mm from eLOT, respectively (Table 1). However, the clusters appeared to be partially overlapping in the dorsal–ventral dimension (Figs 4 and 6) and the M5BL cluster extended for 0.86 ± 0.02 mm and the M50BL cluster for 0.39 ± 0.06 mm (Table 1) along the anterior–posterior axis, suggesting that the two partially overlap along this axis as well (Fig. 6). Evidence for partially overlapping clusters in the two strains was also seen in the posterior piriform cortex and the entorhinal cortex, but not in the olfactory tubercle or the most anterior piriform cortex (Figs 5 and 6).

The proportion of the anterior piriform cortex occupied by neuronal clusters in the knock-in strains is consistent with overlapping inputs from different ORs. If this region received input from all of the approximately 1,000 ORs, and those inputs were spatially segregated, the labelled clusters could theoretically occupy approximately 0.1% of its total area of about 10.5 mm^2 (see Methods)—about 0.01 mm^2. However, together the labelled clusters in this region in M5BL and M50BL mice occupied about 0.59 (5.7%) and 0.44 mm^2 (4.2%), respectively. Similarly, in the olfactory tubercle, labelled clusters in the knock-in strains occupied roughly 1.7% (M5BL) and 7.5% (M50BL) of the total area.

Do individual cortical neurons receive inputs from multiple ORs? We determined that there were $179,570 \pm 3,935$ labelled neurons in the anterior piriform cortex of OmpBL mice ($n = 2$), in which BL is expressed in all olfactory sensory neurons. In the same area, there were $6,570 \pm 217$ labelled neurons in M5BL mice ($n = 2$) and $4,390 \pm 179$ in M50BL animals ($n = 2$). This is markedly different from the number of BL^+ neurons that one might expect to see in knock-in mice (about 180) if each cortical neuron received input from only one of 1,000 ORs. Although BL released from mitral cell axon terminals in layer Ia could conceivably be taken up by the intermingled dendrites of both postsynaptic and non-postsynaptic cortical neurons, a more probable explanation is that individual cortical neurons receive input from a combination of different ORs. Further studies will be needed to clarify this point.

Table 1 Locations of BL^+ clusters in the anterior piriform cortex

M5BL	Distance to eLOT (μm)*	Length (μm)†	M50BL	Distance to eLOT (μm)*	Length (μm)†
Cluster 1			Cluster 1		
1-L	2,450	630	1-L	3,178	420
1-R	2,478	628	2-L	3,136	490
2-L	2,401	462	3-L	3,208	336
2-R	2,415	465	4-L	3,045	385
3-L	2,485	455	5-L	3,180	350
3-R	2,485	455	Average	3,149	396
4-L	2,415	455	±s.d.	64	62
4-R	2,413	449	Cluster 2		
5-L	2,457	458	1-L	1,351	432
5-R	2,415	455	1-R	1,379	490
Average	2,441	491	2-L	1,281	420
±s.d.	33	73	2-R	1,295	478
Cluster 2			3-L	1,463	343
1-L	1,211	868	3-R	1,428	350
1-R	1,225	860	4-L	1,260	350
2-L	1,085	826	4-R	1,251	329
2-R	1,099	834	5-L	1,374	336
3-L	1,085	867	5-R	1,325	385
3-R	1,090	871	Average	1,341	391
4-L	1,187	865	±s.d.	71	60
4-R	1,192	868	Cluster 3		
5-L	1,205	871	I-L	637	283
5-R	1,213	874	1-R	651	319
Average	1,159	860	2-L	539	280
±s.d.	61	17	2-R	553	322
			3-L	638	210
			3-R	648	210
			4-L	490	210
			4-R	516	224
			5-L	620	280
			5-R	613	315
			Average	591	265
			±s.d.	60	47

Data obtained from the left (L) and right (R) hemispheres of the brains of five mice (3 female and 2 male) of each knock-in strain. Clusters are numbered from anterior to posterior.
* Distance from the centre of each cluster of BL^+ neurons to the posterior end of the lateral olfactory tract (eLOT).
† Anterior–posterior length of each cluster.

Do all cortical areas receive inputs from the same ORs?

Does each olfactory cortical area receive information from the entire OR repertoire, or from only a subset of ORs? The patterns of labelled neurons seen in the knock-in animals clearly indicate that input from a single OR can be routed to multiple different areas of the olfactory cortex (Fig. 6). Both M5BL and M50BL mice had clusters of BL^+ neurons in the piriform cortex, olfactory tubercle and entorhinal cortex. However, the anterior olfactory nucleus contained a small cluster of labelled neurons in M5BL but not M50BL animals. In addition, the amygdala was devoid of BL^+ neurons in both knock-in strains, even though numerous labelled neurons are seen in amygdaloid nuclei that receive input from the main olfactory bulb in OmpBL mice[28,29]. It may be that there were BL-containing neurons in these areas that were not detected, because they contained only a small amount of tracer and/or were too few in number or too scattered to be noticed. However, another possibility that cannot be excluded is that some (or all) olfactory cortical areas receive input from only a subset of ORs.

Discussion

Here, we used a genetic tracing system to investigate how sensory information is organized in the olfactory cortex. In knock-in mice that coexpressed a transneuronal tracer (BL) with one of about 1,000 different odorant receptor genes, the tracer travelled from sensory neurons in the nose that express the OR gene to second-order neurons in the olfactory bulb, and from the bulb neurons to third-order neurons in the olfactory cortex. This allowed visualization of neurons in the olfactory cortex that receive input from a particular OR.

These studies indicate that there is a stereotyped sensory map in the olfactory cortex. In this map, input from one OR is mapped onto clusters of neurons at a limited number of sites in the olfactory cortex. Sites that receive input from a given OR have similar or identical locations in different individuals, and most are bilaterally symmetrical in the two hemispheres of the brain. The existence of a stereotyped map in the cortex suggests a mechanism by which odorants could elicit similar perceptions, and perhaps emotional and physiological responses, in different individuals.

In marked contrast to the olfactory bulb, where inputs from different ORs are segregated in different glomeruli and relay neurons[3,13–15,22], it seems that inputs from different ORs are mapped onto partially overlapping clusters of neurons in the olfactory cortex. In addition, it is possible that individual cortical neurons receive input from many different ORs. Given that different odorants are recognized by different combinations of ORs[11], such an arrangement could permit an integration of the components of each odorant's unique receptor code that is important to the generation of diverse odour perceptions.

These studies also showed that input from one OR can be routed to multiple olfactory cortical areas as well as to several sites within one area. Considering that different olfactory cortical areas can transmit information to different target regions in the brain, this presumably allows input from the same OR to ultimately reach multiple brain regions that may serve different functions. Indeed,

although information from most olfactory cortical areas is relayed to the frontal cortex and lateral hypothalamus[6–9], the anterior and posterior piriform cortex transmit signals to different frontal cortex regions[37], and only the olfactory tubercle relays information to the nuclei gemini of the hypothalamus[38]. Divergent inputs to different olfactory cortical areas should, in addition, permit a parallel—and possibly differential—processing of OR inputs in which those inputs may be combined and modulated in different ways for transmission to either the same or different areas of the neocortex and limbic system.

It cannot be excluded, however, that some (or all) cortical areas receive input from only a subset of ORs. This is one possible explanation for the absence of labelled neurons in the amygdala in the knock-in strains. Given the importance of the amygdala in emotional states[1,2,39] and its projection to parts of the hypothalamus that do not receive input from other olfactory cortical areas, it may be that this area receives input only from ORs that convey information relevant to particular emotional states or to instinctive behaviours or physiological states that are governed by the hypothalamus[1,2,40,41].

The generation of diverse olfactory perceptions appears to arise from a series of transformations of sensory inputs that involve the convergence, divergence and parallel processing of signals derived from the OR family (Fig. 6). In the nose, thousands of neurons that receive input from the same OR are scattered in one zone. In the olfactory bulb the axons of those neurons converge in a few specific glomeruli to form synapses with 50–100 relay neurons, each dedicated to input from one OR. In the olfactory cortex, inputs from a particular OR diverge to thousands of neurons located in stereotypical clusters in multiple olfactory cortical areas. Here, inputs from different ORs overlap spatially and might also be combined in single cortical neurons. Information from the same OR is processed in parallel in different olfactory cortical areas, potentially allowing OR inputs to be integrated or refined in different ways before delivery to the neocortex and limbic system. □

Methods

Generation of M5BL and M50BL mice

Clones containing *M5* or *M50* odorant receptor genes were isolated from a mouse (129/SvJ) genomic library (Stratagene) screened with a M5 or M50 cDNA probe[10,31]. An *Asc*I restriction enzyme site was created just 3′ to the stop codon of the *M5* or *M50* gene using PCR[42]. We used the plasmid pETLpA/LTNL[15] to generate an IRES/BL/loxP/tk-neo/loxP (IBL/LTNL) construct. Briefly, the tau-lacZ portion of pETLpA/LTNL was replaced with a truncated BL cDNA[28], generating plasmid pIBL/LTNL. The IBL/LTNL fragment was excised from the plasmid with *Asc*I and inserted into the unique *Asc*I site 3′ to the M5 or M50 coding region to give the targeting vector pM5BL or pM50BL. Embryonic stem cells were electroporated with the constructs, cultured with G418, and individual clones were isolated and analysed by Southern blotting to identify homologous recombinants[43]. To remove the floxed tk-neo cassette, cells were electroporated with a Cre expression plasmid (GIBCO), cultured with Gancyclovir (Syntex Chemicals), and then analysed by Southern blotting. Transgenic mouse lines (M5BL and M50BL) were generated[43] to give mice in a mixed (129/SvJ × C57BL/6) background.

Immunohistochemistry

Mice were perfused with 4% paraformaldehyde, the brain and nose were isolated, fixed in 4% paraformaldehyde for an additional 2 h, soaked in 30% sucrose for 48 h, and then frozen in OCT (Sakura)[10]. Coronal cryostat sections (14 μm) were treated with goat anti-wheat germ agglutinin (WGA) antibodies (0.8 μg ml^{-1}) (Vector Laboratories) (at room temperature for 2 h) and then with biotinylated anti-goat IgG (0.8 μg ml^{-1}; Vector Laboratories) for 1 h (ref. 44). Sections were then treated according to manufacturers' instructions with components of the TSA (NEN Life Science), ABC and DAB (Vector Laboratories) kits, and examined microscopically.

In situ hybridization

Fresh frozen sections (14 μm) were hybridized to fluorescein-labelled cRNA probes at 58 °C as described[45]. The M5 probe was a 926-nucleotide coding region fragment whereas the M50 probe included the coding region plus 700 nucleotides of the 5′ and 300 nucleotides of the 3′ untranslated region. After hybridization, sections were incubated with peroxidase-anti-fluorescein antibody (Roche; 1:750 dilution) at room temperature for 1 h, followed by TSA, ABC and DAB treatment as described above. *In situ* hybridization with ^{33}P-labelled cRNA probes was performed on 10 μm paraffin sections of olfactory bulb from wild-type 129/SvJ and heterozygous knock-in mice as described[10,13].

Data analyses

Each brain was sectioned from the anterior tip of the olfactory bulb to the brain stem, and whenever possible all the sections were collected. Positions of a few missing sections were carefully recorded to accurately estimate anterior–posterior distances from the section numbers. Initially, two out of every five consecutive sections of each knock-in mouse brain were stained. If positive cells were detected, all sections around the positive cells were then immunostained. Knock-in mice from one week to four months of age were analysed. Some adult mice were caged with one member of the opposite sex, and some were exposed periodically to a mixture of odorants. Wild-type 129/SvJ mice, wild-type littermates of the knock-in mice and OmpBL transgenic mice of the same age and sex were treated the same way as the knock-in mice, and were used as controls throughout the studies. One out of every twenty sections was analysed in OmpBL mice. Counting of labelled cells was carried out blind, at least three times on different days.

We identified brain structures microscopically by reference to a mouse brain atlas[36]. The anterior–posterior lengths of brain structures and BL-labelled clusters were calculated from the number of sections multiplied by 14 μm. Dorsal–ventral and medial–lateral distances were measured on computer images of sections photographed with a size bar. To obtain the total area of the anterior piriform cortex or olfactory tubercle, we first used the brain atlas to measure the dorsal–ventral and medial–lateral extent of the surface of the structure at different points along the anterior–posterior axis and then determined the average. We then compared the anterior–posterior length of the structure in the atlas with that determined in the knock-in mice, and normalized the dorsal–ventral/medial–lateral extent of the structure accordingly.

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Correspondence and requests for material should be addressed to L.B.B. (e-mail: lbuck@hms.harvard.edu).