A Developmental Switch of Axon Targeting in the Continuously Regenerating Mouse Olfactory System

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The mammalian olfactory system has the natural capacity to regenerate throughout the animal’s life span. Despite constant neurogenesis, olfactory sensory neurons project to precise, stereotypical positions in the brain. Here, we identify a critical period of olfactory sensory axon targeting during postnatal development in mouse. Perturbing axon projection beyond postnatal day 7 permanently disrupts targeting specificity of the sensory neurons. In addition, we find that the establishment of the convergence map requires perinatal sensory neurons. Late-born neurons appear to connect with prospective glomeruli based on homotypic interactions among neurons expressing the same odorant receptor. Our results reveal a developmental switch in axon guidance and a mechanism of circuit integration of adult-born neurons.

Neural regeneration holds the potential to repair dysfunctional or damaged nervous systems. A key step that must be achieved for an effective cure is to allow new neurons to make precise and functional connections. Adult neurogenesis in mammals occurs in the olfactory epithelium, dentate gyrus of the hippocampus, and subventricular zone of the lateral ventricle (1–3). In olfactory epithelium, neurogenesis peaks around postnatal day 14 (P14), gradually subsides, and continues at a steady rate after weaning at P21 (2). The newly generated sensory neurons project into existing glomeruli to maintain constant innervation of the bulb (13). Although some evidence supports lifelong plasticity of the olfactory map (3, 14), other evidence suggests that the sensory neuron projection may not be properly

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Fig. 1. Silencing spontaneous activity of olfactory sensory neurons during postnatal development permanently alters axonal projection. (A) Representative images of P2-GFP glomeruli in Kir2.1 mice showing divergent projection patterns in postnatal day 2 (P2) and adult (8-week-old, 8wks) mice. (B) In situ hybridization using antisense riboprobe against Kir2.1 (i and ii, purple) and immunofluorescent staining of Kir2.1 protein (iii and iv, red) in the olfactory epithelium of Kir2.1 mice treated with or without doxycycline (DOX) diet. Duration of treatment is indicated. (C) Representative images of P2-GFP neuron projection in the olfactory bulb from (i) controls, Kir2.1 mice treated with doxycycline (ii) at P0 for 8 weeks, (iii) at P21 for 5 weeks, and (iv) at P21 for 12 weeks. Green: GFP; blue: 4′,6′-diamidino-2-phenylindole (DAPI). Scale bars, 100 μm.
reestablished after damage to the olfactory epithelium or large-scale axotomy (5, 15).

In mice carrying OMP-IREs-tTA:tetO-Kir2.1-IREs-tauLacZ alleles (Kir2.1 mice)—in which OMP is the olfactory marker protein; IRES, the internal ribosome entry site; tTA, tetracycline-controlled transcription activator; and tetO, a promoter that drives expression of the fusion protein tau-β-galactosidase—ectopically expressed Kir2.1 channels in the olfactory sensory neurons suppressed neural activity and led to erroneous innervation of multiple glomeruli by neurons expressing the same OR (Fig. 1A and fig. S1A) (16). Kir2.1 transgene expression could be effectively turned off by feeding mice a doxycycline diet, so that Kir2.1 transcript and protein levels were drastically reduced within 48 hours of treatment (Fig. 1B). After doxycycline treatment, odor-evoked responses imaged from the dorsal bulb (17, 18) were restored (fig. S1B).

We were curious about whether doxycycline treatment would restore the stereotypic convergence pattern. In mice fed with doxycycline from P21 onward, axons expressing the same OR gene continued to project to multiple glomeruli throughout the course of treatment (Fig. 1C, iii and iv). Thus, suppressing neural activity till P21 led to persistent changes in projection pattern (fig. S1C). In contrast, when Kir2.1 mice were fed doxycycline from birth, neurons expressing the same OR projected into a single glomerulus (Fig. 1C, ii). It appeared that a time window existed between birth and P21 (weaning) during which developing olfactory sensory neurons could reestablish a convergence map.

In a time-course experiment, we quantified the eventual number of glomeruli innervated by a single type of sensory neurons in mice treated with doxycycline at different postnatal ages (fig. S2A). We examined sensory neurons expressing ORs projecting to different regions of the olfactory bulb, including M72 (M72-IREs-tauGFP), P2 (P2-IREs-GFP), and MOR28 (MOR28-IREs-GFP), all tagged with the gene for green fluorescent protein (GFP) (fig. S2B) (16, 19, 20). As demonstrated with the M72 receptor (Fig. 2A), the number of glomeruli increased significantly when mice were treated with doxycycline later than P5 (Fig. 2B and fig. S2C). Although the degree of divergence differs for each OR and the olfactory bulb follows a dorsal-ventral progression in the expression of guidance cues (21, 22), we did not find an obvious difference in the transition period for the three ORs (Fig. 2B and fig. S2C). The projection patterns were most sensitive to deprivation of neural activity after P5. Because Kir2.1 expression was suppressed within 48 hours, we reasoned that the plasticity in restoring the map began to diminish after P7. Before P7, olfactory axons had the capacity to reestablish converging projection patterns when activity was restored.

During the first postnatal week, mitral cells in the olfactory bulb, which receive input from the sensory neurons, prune their dendrites from innervating multiple glomeruli to a single one (23). We examined mitral cell dendritic morphology in Kir2.1 mice by retrograde lipophilic dye labeling and found that mitral cells completed the pruning process by P7 in both control and mutant mice (Fig. 2C). Thus, the divergent sensory axon projection had little impact on postsynaptic cell dendritic development. The regulation of sensory axon projection appears independent from mitral cell development.

Is the permanent change in sensory neuron projection the result of electrical silencing or does it reflect an intrinsic developmental program revealed by this perturbation? To address this question, we examined OMP-IREs-tTA:tetO-LBR (LBR) mice, in which ectopic expression of lamin B receptor in the sensory neurons led to deregulated OR gene expression and multiglomerular projection (24). In these mice, axon projection began to diminish at P0 (Fig. 3A). In contrast, we observed multiglomerular projection patterns in mice fed with doxycycline at later stages (Fig. 3A). The number of P2 glomeruli in LBR mice followed a similar time course to that found in Kir2.1 animals (Fig. 3B). Thus, using independent methods to perturb sensory axon projection, we observed the same critical period of olfactory map formation.

It is not known whether mistergated axons reorganize to restore convergence, or the sensory neurons are replaced by newly generated neurons.

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**Fig. 2. Critical period of olfactory axon convergence.** (A) Representative images of M72-GFP neuron projection in coronal olfactory bulb sections from control and Kir2.1 mice treated with doxycycline starting at different postnatal dates. Green: GFP; blue: DAPI. Arrowheads indicate the glomeruli receiving the M72-GFP axon inputs. (B) Quantification of the M72, P2, and MOR28 glomeruli in Kir2.1 mice and control littermates treated with doxycycline at different postnatal stages. Two-sample, one-way Student’s t test is applied for statistical analysis. \( P < 0.05 \), \( **P < 0.01 \), \( ***P < 0.001 \) (t test). Error bars, SEM. (C) Olfactory axon convergence is independently regulated from the mitral cells. Representative image of retrograde Dil labeling of mitral cells in Kir2.1 and control mice during perinatal period (P0 to P1) and P7. Arrows indicate the branches of the primary dendrite of the labeled mitral cells. Scale bars, 100 μm.

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that form the single glomerulus convergence. We tested whether neurons generated after birth were able to reestablish the convergence patterns by ablating early neurons using transgenically expressed diphtheria toxin (3, 25) in OMP-ires-tTA:tetO-DTA (DTA) mice. Doxycycline feeding beginning at P0 resulted in normal projection of olfactory axons (Fig. 3C). However, treatment after P3 resulted in projections to multiple glomeruli (Fig. 3C). This observation suggested that sensory neurons present at birth were required to establish the converging projection pattern. Late-born neurons were not able to restore convergence.

In a previous study, neurons expressing the P2 receptor were ablated, and the newly generated neurons continued to converge their axons onto single glomerular locations (3). In this case, a small percentage of P2 axons remained intact. It is possible that existing axonal tracts acted to guide the new axons via a presorting mechanism (26) mediated by homotypic interactions (19, 27). We hypothesized that perturbing the projection of sensory neurons expressing one OR type would affect only neurons of the same type. We tested this hypothesis using the OMP-ires-tTA:tetO-M71-ires-taulacZ mice (28), in which a large population of neurons ectopically expressed the M71 OR (fig. S3, A and B).

In these animals, sensory neurons expressing endogenous M71 (M71-ires-tauGFP) were found to project to multiple glomeruli in the dorsal region of the bulb (Fig. 4A). In animals fed doxycycline after P7, neurons expressing endogenous M71 receptor continued to project to multiple glomeruli (Fig. 4B, bottom). In contrast, doxycycline feeding before P3 rescued the single-glomerulus projection pattern (Fig. 4B, top). Note that neurons expressing M72 or MOR28 receptor maintained single-glomerulus convergence patterns in the M71 transgenic mice (Fig. 4C). Thus, perturbation of axons by ectopic M71 expression only affected endogenous M71 neurons (fig. S3C). This result demonstrated that homotypic interaction was sufficient to redirect axon routing before, but not after, P7.

The regenerative capacity of the olfactory system makes it an unlikely system to be regulated by a developmental critical period. Our study demonstrates that, during the perinatal stage, sensory axons have the reorganizational capacity to ensure the segregation of axons expressing different ORs. Early axons appear to have the ability to correct erroneous projections (9, 11) and provide the track for late-born neurons to follow. Late-born neurons appear to interact with existing paths through homotypic affinity, possibly by using guidance molecules, such as Kirrels and ephrins, which provide identity code for the olfactory sensory neurons expressing different ORs (27, 29). Ectopic tracks, formed as a result of suppressing neural activity or lamin B receptor overexpression, divert the axons to innervate ectopic glomeruli persistently. These results imply that different mechanisms are used for establishment of the olfactory map than are used for maintenance of correct erroneous projections (11), but not after, P7.

Fig. 3. Establishment of axon convergence requires perinatal olfactory sensory neurons. (A) Representative images of P2-tauLacZ axon projection in olfactory bulb sections from OMP-tTA:tetO-LBR and control mice treated with doxycycline beginning at P0, P7, or P21. Analyses are performed 8 to 12 weeks after doxycycline treatment. Magenta: LacZ; blue: DAPI. (B) Quantification of the P2-tauLacZ glomeruli in the LBR mice treated with doxycycline at different postnatal stages. The average number of P2-GFP glomeruli in Kir2.1 mice is shown by dotted line (Fig. 2B). Two-sample, one-way Student’s t test is applied for statistical analysis. *P < 0.05, **P < 0.01, and ***P < 0.001 with t test. (C) Representative images of M72-tauGFP neuron projection in olfactory bulb sections from OMP-tTA:tetO-DTA and control mice. Doxycycline diet starts at P0, P3, or P21 as marked. Green: GFP; blue: DAPI. Arrowheads indicate the glomeruli receiving the P2 (A) or M72 (C). Scale bars, 100 μm.

Fig. 4. Homotypic interaction among olfactory sensory neurons expressing the same OR. (A) Ectopic expression of M71 receptor gene perturbs projection of olfactory sensory neurons expressing endogenous M71 receptor (M71-ires-tauGFP). Coronal olfactory bulb section of OMP-ires-tTA:tetO-M71 mice without doxycycline treatment is shown. Magenta: LacZ; green: GFP; blue: DAPI. (B) Coronal bulb sections from tetO-M71 mice treated with doxycycline beginning at P0, P3, and P7. Arrowheads indicate the glomeruli receiving endogenous M71-ires-tauGFP axons. (C) Ectopic expression of M71 does not alter projection of neurons expressing other OR genes. Representative images of M72-ires-tauGFP and MOR28-ires-GFP neuron projection in coronal bulb sections from tetO-M71 and control mice treated with doxycycline beginning at P14 and P21, respectively. Scale bars, 100 μm.
the map. Although transient ectopic glomeruli may be generated during normal postnatal development, they are pruned in adult animals (30,31). The pruning process may reflect a refinement process at a later postnatal stage.

Our study puts the olfactory system in line with other sensory systems in that it undergoes a change in circuit plasticity during the critical period of development (32). Although, traditionally, the discussions of critical period have focused on how sensory deprivation affects the development of neural circuits, our study reveals an intrinsic developmental program that unfolds whether or not neural activity is perturbed. Even though the olfactory system is regulated by a critical period, late-generated neurons adopt a different strategy for axon projection. Thus, a developmental critical period may function to restrict the reorganization of the neural circuit and to maintain an established map.

References and Notes

A Critical Period Defined by Axon-Targeting Mechanisms in the Murine Olfactory Bulb

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The olfactory system remains plastic throughout life because of continuous neurogenesis of sensory neurons in the nose and inhibitory interneurons in the olfactory bulb. Here, we reveal that transgenic expression of an odorant receptor has non–cell autonomous effects on axons expressing this receptor from the endogenous gene. Perinatal expression of transgenic odorant receptor causes rerouting of like axons to new glomeruli, whereas expression after the sensory map is maintained throughout adult life (1). This prolonged plasticity is achieved by the continuous generation of the inhibitory granule cells that migrate into the olfactory bulb and integrate into the circuits and by the generation of olfactory sensory neurons (OSNs) that incorporate into the circuits throughout life (2, 3). Although we know that plasticity is retained in the mature olfactory system, does a critical period exist in the formation of the sensory map in the olfactory bulb?

In mice, each OSN expresses only one of the ~1300 odorant receptor (OR) genes (4–7) from only one allele (8). The OSNs that express the same OR are randomly dispersed within a broad zone in the main olfactory epithelium in the nose (9, 10). In the olfactory bulb, the first olfactory center in the brain, the axons of OSNs expressing the same OR converge on spatially fixed neuropil structures called glomeruli (9–11). Further, ORs actively participate in the axon guidance of OSNs to particular glomeruli (12, 13). In the glomeruli, the axons synapse with the dendrites of mitral and tufted cells, the projection neurons in the bulb. Each projection neuron receives input from a single glomerulus and sends its axon to the olfactory cortex. Thus, an olfactory sensory map is formed in the bulb. In this map, the identity of each odor is encoded by the combination of glomeruli that it activates (3). In contrast to the somatosensory, auditory, and visual maps, neighboring relations between peripheral sensory neurons are not maintained in the olfactory sensory map. Because OSNs continue to integrate into the circuits throughout life, the challenge of axon guidance persists in adulthood (3).

We devised a strategy for ectopic expression of a specific OR, MOR28, in a temporally controlled manner using the tetracycline response element (tetO) to drive its expression. The tetO promoter is activated by the tetracycline-controlled transcription activator tTA, which is inhibited by the antibiotic doxycycline. When doxycycline is removed, expression from the tetO promoter is induced within days (14–16). A similar approach for inducing ectopic expression of ORs was previously used (17–19). Our strategy involved the use of three alleles (fig. S1A). In the first, designated OMP-ires-tTA, the olfactory marker protein (OMP) drives expression of tTA in all OSNs (16). In the second, designated tetO::MOR28-ires-tau-LacZ (TO28), tetO drives the expression of MOR28 and the fusion protein tau–β-galactosidase (β-gal). To distinguish between the OSNs that express MOR28 from its endogenous genomic locus (endogenous MOR28 OSNs) versus OSNs that express MOR28 from the transgene (transgenic MOR28 OSNs), we introduced a third allele, designated MOR28-ires-gfp. OSNs that express MOR28 from this allele also express green fluorescent protein (GFP) (20). Thus, GFP expression marks OSNs expressing MOR28 from its endogenous locus. Because β-gal and GFP are exogenous to mice, staining for each identifies transgenic or endogenous MOR28 OSNs, respectively (fig. S1B).