

Olfactory neurons are interdependent in maintaining axonal projections

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In mice, individual olfactory neurons express one of the thousand distinct olfactory receptor genes [1–3]. Neurons that express a given receptor converge on distinct loci in the olfactory bulb to form structures called glomeruli [4–6]. The olfactory receptor is involved in an instructive manner in this axonal convergence [6,7] but little is known about the mechanisms involved in maintaining convergence. We have previously created a transgenic olfactory receptor locus that functions independently of the endogenous loci [8]. Here, we show that, although the projections of neurons expressing this ectopic transgenic olfactory receptor always converge in newborn mice, surprisingly, in adult mice, convergence is not always maintained. Moreover, in adult mice there is a positive correlation between the number of neurons expressing the transgenic receptor and the probability of maintaining convergence. These observations, taken together with the variability observed in wild-type [4,6] and genetically manipulated mice ([6] and our unpublished observations), suggest that olfactory neurons require the presence of other similar axons to maintain a glomerulus. We call this phenomenon interdependence.

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Received: 25 November 1999
Accepted: 17 December 1999

Published: 11 February 2000

Current Biology 2000, 10:219–222

0960-9822/00/\$ – see front matter
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Results and discussion

Each of the continuously regenerated olfactory neurons [9] appears to express only one olfactory receptor gene [1–3] and, indeed, only one of the two alleles is expressed (allelic inactivation [10]). Neurons expressing a given olfactory receptor gene are restricted to one of four broad but circumscribed zones in the olfactory epithelium [1,2]. Within each zone, the neurons expressing a given receptor are randomly distributed among neurons expressing other receptors [1,2]. Axonal projections from neurons expressing a given receptor generally converge on a small number of topographically fixed glomeruli in each olfactory bulb [4–6].

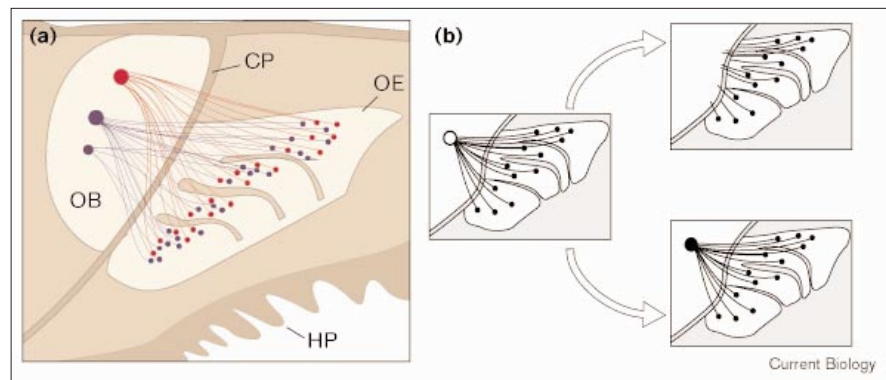
In the context of a relatively fixed map, there is in fact some animal-to-animal variability in the number, size and shape of glomeruli receiving the projections of neurons expressing a given olfactory receptor gene [4,6]. This variability has been observed in wild-type mice and, perhaps most clearly, in mice in which the P2 olfactory receptor gene has been marked such that neurons expressing the marked P2 allele also express a tau-lacZ fusion protein. In the case of the P2-marked mice, a small fraction of animals have an extra glomerulus that receives a smaller number of axons in addition to a consistently observed single medial and single lateral glomeruli (see Figure 1a for schematic; [6] and F.A.W.E. and A.C., unpublished observations). Moreover, in our analyses of newborn P2-marked mice, we observed an extra glomerulus on the medial surface of the olfactory bulb in over half the observations, suggesting that more of these glomeruli initially begin to form than ultimately persist (F.A.W.E. and A.C., unpublished observations). The presence of extra glomeruli, taken together with their decline over time, raises interesting questions about the mechanisms involved in establishing and maintaining individual glomeruli.

We propose a model in which interdependent interactions between olfactory neurons are critical for the stabilization of glomeruli. In our model, neurons expressing a given receptor have a variety of differentially attractive targets in the medial and lateral aspects of the olfactory bulb. The site at which an extra glomerulus sometimes forms would represent a less attractive site than the primary site where convergence always occurs. The central aspect of our model is that neurons, after they arrive at a particular glomerulus, require the presence of the axons of other neurons expressing the same olfactory receptor to maintain the glomerulus. The stabilization has a stochastic component to it, such that with an increasing number of inputs, there is an increasing probability of stabilization. We call this phenomenon interdependence (Figure 1b). Interdependence, together with the presence of multiple differentially attractive targets, can explain the observation that a subset of wild-type animals have additional medial or lateral glomeruli receiving the axons of neurons expressing a given receptor. Although the primary glomerulus always has a sufficient number of inputs to guarantee stabilization, extra glomeruli would represent less efficacious target sites that reach the threshold to maintain a glomerulus only a fraction of the time.

A prediction of interdependence is that, if there are only a small number of neurons expressing a given receptor, then convergence will not always be maintained in adult mice.

Figure 1

Schematic diagrams of the mature olfactory system and its formation. **(a)** Schematic diagram of the left half of the olfactory system (half-nose). The nasal cavity and olfactory bulb (OB) are viewed from the perspective of the midline. The olfactory epithelium (OE), the site of olfactory receptor expression, covers the turbinate bones. The olfactory bulb is separated from the turbinate bones by the cribriform plate (CP). The cortex is immediately posterior (to the left) of the olfactory bulb, the snout is anterior (to the right), and the hard palate (HP) is ventral (below). Representative projection patterns of neurons expressing two distinct olfactory receptors are colored red and blue, respectively. Although the neurons expressing the 'blue' and 'red' receptors are present in the same zone of the olfactory epithelium, they project to distinct glomeruli in the olfactory bulb. (There are a total of ~1800 glomeruli in the olfactory bulb.) The small blue glomerulus is representative of an



extra glomerulus, which is present only in a fraction of mice. **(b)** A model explaining the presence of extra glomeruli. The olfactory system of a newborn animal is depicted in the panel on the left. Convergence is depicted as an empty circle to indicate that, although the glomerulus has been formed, stabilization has

not occurred. Two different paths can be taken from this starting point. Either the requirement for interdependence is met and stabilization occurs (indicated by the black glomerulus, lower right panel), or it does not (upper right panel), and the unstabilized glomerulus disappears.

When these neurons do exhibit convergence, the glomerulus to which they converge (because it receives a relatively low number of inputs) would be analogous to the extra glomerulus in wild-type mice. To test this prediction, we have taken advantage of a model system in which a 300 kb transgene functions as an independent olfactory receptor locus [8]. Within the transgene, an olfactory receptor gene (M12) is marked by inserting an internal ribosomal entry site followed by tau-lacZ to allow visualization of axonal projections as well as cell bodies. The transgene is expressed in a relatively small number of neurons; these neurons are distinct from the neurons expressing the endogenous M12 gene and project to distinct glomeruli.

In both of the transgenic lines we generated, Y11 and Y1 [8], expression of the transgenic M12 gene is restricted to olfactory neurons. These neurons are clearly distinct from the neurons expressing the endogenous M12 gene as they are located in an area (encompassing more than one zone) adjacent to the endogenous M12 zone [8]. In heterozygous mice of the Y11 line, an average of 100 transgene-expressing neurons were visible in a characteristic punctate pattern when viewed from the perspective of the nasal septum (Figure 2a,b). (Only around one-tenth of the olfactory epithelium is visible from this perspective.) The number of transgene-expressing neurons was approximately one-quarter to one-third the number of neurons expressing the endogenous M12 gene. Taken together with the observed allelic inactivation of the transgene [8], these observations suggest that the transgene functions as a 'new', independent olfactory receptor locus in the mouse genome.

The transgene-expressing neurons project to glomeruli that are distinct from the glomeruli to which neurons

expressing the endogenous M12 gene project [6–8]. It is important to note that the distinct site to which transgene-expressing neurons project allows us to analyze these projections without the interference of neurons expressing the endogenous alleles. Thus, mice expressing the transgene provide an ideal model system in which to study the effect of the number of neurons expressing a given receptor on the formation and maintenance of their axonal projections.

In adult Y11 transgenic mice, we observed a striking phenomenon — in some half-noses there was convergence, usually to a single glomerulus, whereas in other half-noses, there was no convergence. This all-or-none difference was sometimes observed in the left and right halves of a single animal with equal numbers of transgene-expressing neurons (Figures 2a,b). When there was no convergence, we occasionally observed labeled axons that extended only into the part of the olfactory bulb adjacent to the cribriform plate but, the majority of the time, we detected no labeled axons in the olfactory bulb.

To determine whether there is a positive correlation between the number of transgene-expressing neurons and the probability of convergence, we analyzed 65 half-noses of Y11 transgenic mice. A bivariate scattergram (Figure 2c) displays the number of neurons in a given half-nose along with whether or not convergence was observed. When there were very few transgene-expressing neurons, convergence was not observed and when there were many transgene-expressing neurons, convergence was always observed. In the intermediate range — 90–155 neurons — convergence was present 69% of the time (Figure 2d). Thus, there is a clear correlation between

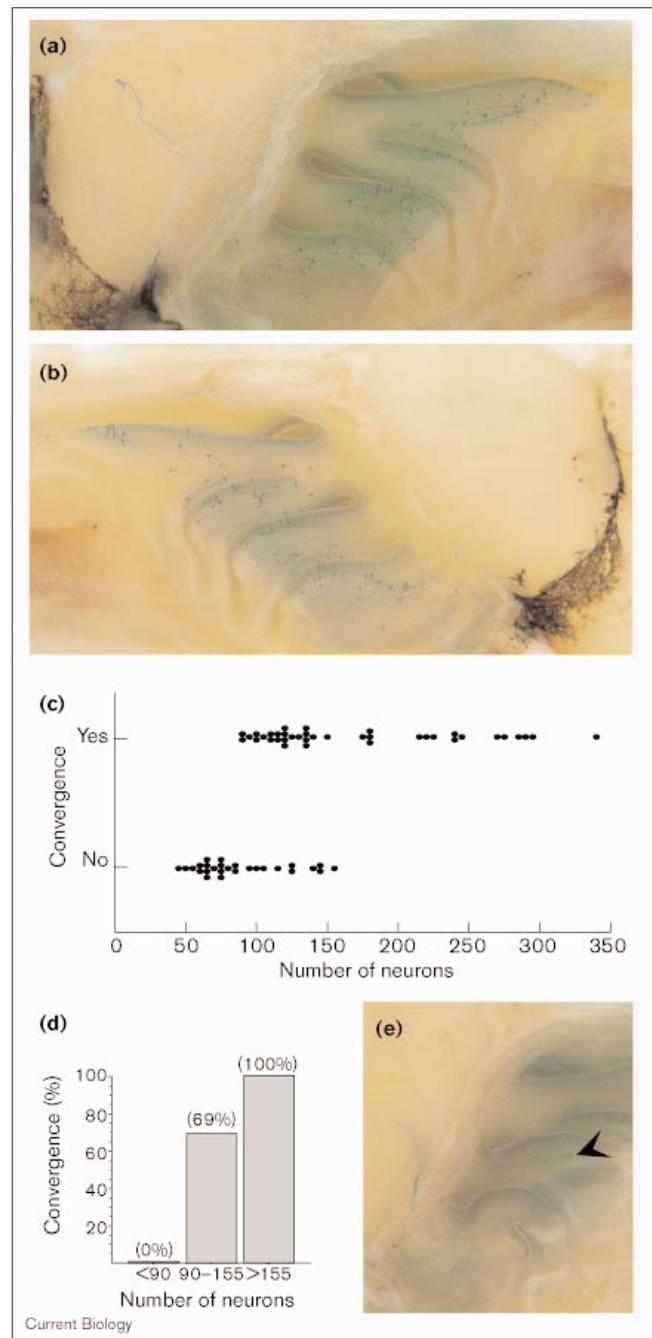
Figure 2

Glomerular convergence in adult mice. **(a,b)** Whole-mount views of the left and right half-noses of an X-gal-stained heterozygous Y11 mouse. Although the (a) left and (b) right half-noses have similar numbers of transgene-expressing neurons visible from the perspective shown (124 and 129, respectively), convergence is only present on the left side. We also dissected a few transgenic animals so that the lateral portion of the olfactory bulb was visible. X-gal staining of these half-noses revealed that a medial glomerulus was sometimes labeled without a lateral glomerulus being labeled; in other half-noses a lateral glomerulus was labeled in the absence of a labeled medial glomerulus. **(c)** Bivariate scattergram plotting the number of neurons in a given half-nose together with whether or not convergence was observed. Neuron counts were rounded to the nearest five neurons. Thus both the medial and lateral sides of a given olfactory bulb as well as the left and right olfactory bulbs function independently in maintaining axon projections. **(d)** Cell bar chart showing the percentage convergence as a function of the number of transgene-expressing neurons (Chi-square analysis, $p < 0.0001$, $n = 16$, 32 and 17 for the three categories). The data in (c,d) include analyses of 5–12 month old heterozygous and homozygous animals on a 129 background. It is important to note that we found no relationship between age and the number of neurons expressing the transgene or the probability of convergence. We also looked at a larger data set, which also included mice of a mixed 129 \times C57Bl/6 background, and found the same result. **(e)** Whole-mount view of a half-nose from an X-gal-stained mouse chimeric for the marked endogenous P2 gene. Only a small fraction of the P2-expressing olfactory neurons are marked, demonstrating that convergence can consistently be visualized even though only a few neurons are marked, in this example only nine. The glomerulus is not sharply defined as only a small fraction of axons innervating it are labeled. These chimeric mice were generated by injecting wild-type ES cells into blastocysts heterozygous for the marked endogenous P2 allele. Mice were analyzed at 5–6 months to control for any effect of age on X-gal staining. All whole-mount X-gal stainings were carried out for 12–24 h. In (e), the 24 h staining used to visualize axons in the olfactory bulb led to overstaining of the olfactory epithelium, but individual neurons are still visible (arrowhead). Analysis of the time of onset of X-gal staining in transgenic mice and the P2-marked mice indicates that the level of receptor expression per cell is indistinguishable, suggesting the transgenic receptors are expressed at a wild-type level.

the number of transgene-expressing neurons and the likelihood of convergence (Chi-square analysis, $p < 0.0001$).

To ensure that these results were not due to an inability of X-gal staining to consistently detect convergence when there are few transgene-expressing neurons, we performed control experiments with mice chimeric for the marked endogenous P2 gene [6]. Although all such chimeras contain the wild-type number of P2-expressing neurons, we analyzed chimeras in which only a small fraction of these neurons were marked. As expected, even though only a small number of neurons were marked, these neurons always converged in the olfactory bulb (Figure 2e). This control experiment demonstrates that convergence, if present, can be consistently visualized by X-gal staining even when only a small number of neurons are marked.

In order to determine whether our observations result from a primary inability to converge or from a secondary



loss of convergence, we examined newborn transgenic mice. In all twelve newborn Y11 mice examined, transgene-expressing neurons converged in the olfactory bulb. Most newborns examined had two glomeruli visualized by X-gal staining, suggesting that more glomeruli are initially formed than will ultimately persist into adulthood. The glomeruli in newborns (Figure 3) are not as tightly formed as adult glomeruli (Figure 2a). These differences suggest that, although convergence is observable at birth, the formation of the mature olfactory bulb is not complete. We also analyzed transgenic mice from a second line (Y1 [8]) at birth and at the adult stage. Similar to the observations

Figure 3



Convergence is always present at birth. A whole-mount view of an X-gal-stained Y11 newborn olfactory bulb reveals two labeled medial glomeruli.

with the Y11 line, transgene-expressing neurons always converged at birth, whereas in adults, convergence was not always observed (data not shown).

Several mechanisms could explain the phenomenon of interdependence. Interactions between incoming axons of primary neurons might be involved and/or there might be stabilization involving second order neurons in the target site glomerulus. As for the nature of the interdependent interactions, activity dependence, which has been observed in a number of other neural systems (for example ocular dominance columns and whisker barrels, reviewed in [11]), might underlie interdependence. Given this possibility, it would be interesting to know the convergence patterns of adult mice harboring deletions of either G_{olf} or the downstream cyclic-nucleotide-gated channel; these mice have normal convergence at birth [12,13]. Alternatively, interdependence could result from the requirement for many axons to act simultaneously in forming the glomerulus (without the simultaneous firing required for activity dependence). Whatever the precise mechanisms, the requirement for coordination might allow the olfactory system to refine its topographic map beyond the original projection pattern. Thus, interdependence might maintain order in the olfactory bulb in the face of the expansion of the olfactory receptor gene family during evolution; interdependence could ensure that there are never many more glomeruli than expressed olfactory receptor genes.

Our data lead us to hypothesize that olfactory neurons are interdependent in maintaining glomeruli in the olfactory bulb. Our analyses of transgenic mice indicate that,

although newborn mice always exhibit convergence, the probability of having maintained convergence in an adult mouse is dependent on the number of transgene-expressing neurons. Moreover, there appears to be a stochastic component to the maintenance process. Thus, the olfactory system, like other areas of the nervous system, appears to employ mechanisms involving interactions between similar neurons to establish complex patterns of wiring from an initially less defined projection pattern.

Acknowledgements

We thank Peimin Qi for generating chimeras, Amy Harry for assistance in preparing the manuscript, Jen Cook-Chryso for assistance in creating schematics and Richard Axel for providing the P2-marked mice. We also thank Brian Bates, Martha Constantine-Paton, Doug Fambrough, Paul Garrity, Pamela Sklar and members of the lab for discussions and critical comments. During a portion of this work, F.A.W.E. was a NSF predoctoral fellow. A.C. is a Rita Allen Foundation Scholar. This work was supported by the NIH-NIDCD: R01DC03263 (A.C.) and R03DC04035 (F.A.W.E.).

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