

# Spontaneous Neural Activity Is Required for the Establishment and Maintenance of the Olfactory Sensory Map

C. Ron Yu,<sup>1,2</sup> Jennifer Power,<sup>2</sup> Gilad Barnea,<sup>2</sup>  
Sean O'Donnell,<sup>3</sup> Hannah E.V. Brown,<sup>4</sup>  
Joseph Osborne,<sup>3</sup> Richard Axel,<sup>2,3,4,\*</sup>  
and Joseph A. Gogos<sup>2,5,\*</sup>

<sup>1</sup>Department of Anatomy and Cell Biology

<sup>2</sup>Center for Neurobiology and Behavior

<sup>3</sup>Department of Biochemistry  
and Molecular Biophysics

<sup>4</sup>Howard Hughes Medical Institute

<sup>5</sup>Department of Physiology and Cellular Biophysics  
College of Physicians and Surgeons  
Columbia University  
New York, New York 10032

## Summary

We have developed a genetic approach to examine the role of spontaneous activity and synaptic release in the establishment and maintenance of an olfactory sensory map. Conditional expression of tetanus toxin light chain, a molecule that inhibits synaptic release, does not perturb targeting during development, but neurons that express this molecule in a competitive environment fail to maintain appropriate synaptic connections and disappear. Overexpression of the inward rectifying potassium channel, *Kir2.1*, diminishes the excitability of sensory neurons and more severely disrupts the formation of an olfactory map. These studies suggest that spontaneous neural activity is required for the establishment and maintenance of the precise connectivity inherent in an olfactory sensory map.

## Introduction

Neurons connect to one another with remarkable precision. The specificity of connections in the nervous system is essential for the translation of electrical activity into meaningful neural codes. In sensory systems, peripheral neurons in the receptor sheet project axons to precise loci in the CNS to create an internal representation of the sensory world. The spatial segregation of afferent input from primary sensory neurons provides a topographic map that can define the location of the sensory stimulus in the environment as well as the quality of the stimulus itself. The development and maintenance of sensory maps require the participation of both spatially and temporally regulated guidance molecules elaborated by central targets that are recognized by receptors on sensory axons. Once the map is established, it forms a substrate for plastic changes that allow the organism to respond to a changing environment. This plasticity is likely to result from activity-dependent changes in neural connectivity.

In the visual system, a visual image is represented by at least 32 retinotopic maps in the primate cortex (Van Essen et al., 1992). The retinotopic map, in both thala-

mus and cortex, results from complementary gradients of ephrin receptors on retinal afferents and ephrins expressed by the multiple targets in the brain (Wilkinson, 2001). These retinotopic representations deconstruct the visual scene into submaps that refine the image, including ocular dominance columns and orientation columns, as well as cortical blobs that uniquely respond to color, motion, and spatial frequency (Van Essen et al., 1992). These refinements are sensitive to visual activity, and this has led to efforts to distinguish the relative contributions of innate determinants (timing of axon arrival and molecular cues) and experiential or activity-dependent mechanisms in the generation of the visual circuitry (Katz and Shatz, 1996). The problem is perhaps best illustrated by early recordings by Hubel and Wiesel that demonstrated that cortical neurons respond preferentially to one or the other eye and segregate into ocular dominance columns (LeVay et al., 1978; Wiesel and Hubel, 1963). Recent experiments indicate that these columns form even before the eyes open (Horton and Hocking, 1996) and are also present if the eyes are removed at birth (Crowley and Katz, 1999). Despite caveats, the simplest interpretation of these observations argues that the sorting of axons into eye-specific columns is an innate process independent of visual activity. However, Hubel and Wiesel also showed a dramatic effect of visual activity on the ocular dominance map most marked during a "critical period" of postnatal development. The temporary closure of one eye results in sharp decreases in the number of cortical neurons responding to the once-deprived eye and a coordinate expansion in the response to the unperturbed eye (LeVay et al., 1980). Thus, an innately determined map, once formed, is exquisitely sensitive to visual experience by virtue of an activity-dependent competition between axons from the two eyes for cortical synaptic targets.

What is the role of activity in the establishment of precise synaptic connections in the olfactory system? In mammals, olfactory sensory neurons express only one of 1000 odorant receptor genes (Buck and Axel, 1991; Chess et al., 1994). Neurons expressing a given receptor are randomly dispersed within one of four broad zones in the olfactory epithelium, but their axons converge upon one medial and one lateral, spatially conserved glomerulus within the olfactory bulb (Mombaerts et al., 1996; Ressler et al., 1993; Vassar et al., 1993). The olfactory bulb therefore provides a two-dimensional map of receptor activation that may provide a neural code to allow for the discrimination of olfactory sensory information. This observation raises the question as to how topographically dispersed cells in the periphery converge to establish a sensory map in the bulb. What is the contribution of innate determinants of guidance and activity-dependent processes in specifying the invariant pattern of connections of olfactory sensory neurons? Genetic experiments demonstrate that the odorant receptors themselves (Wang et al., 1998), along with the ephrin-A molecules on sensory axons (Cutforth et al., 2003), are essential determinants in the establishment of the patterns of olfactory neuron convergence in the bulb.

\*Correspondence: ra27@columbia.edu (R.A.); jag90@columbia.edu (J.A.G.)

The identification of genetic determinants of guidance, however, does not preclude the contribution of activity-dependent processes in the formation of a topographic map. In one model, the correlated firing of olfactory neurons could play an instructive role in the establishment of a sensory map. The excitation of like axons producing a correlated pattern of neural activity requires odorant-evoked excitation of sensory neurons. Mice lacking functional olfactory cyclic nucleotide-gated channels fail to exhibit odorant-evoked responses to a wide range of odorant stimuli (Brunet et al., 1996). In these mice, the convergence of like axons to the olfactory bulb onto spatially invariant glomeruli is only subtly perturbed, suggesting that correlated presynaptic neural activity is not required for the establishment of the highly ordered synaptic connections that define an olfactory sensory map (Lin et al., 2000; Zheng et al., 2000). These data do not address the possibility that spontaneous, noncorrelated activity on neurotransmitter release may be required for the establishment or maintenance of the spatial map. For example, the spontaneous firing of sensory neurons may be required to allow a genetically encoded targeting process to unfold. Alternatively, spontaneous activity might play a role in the survival, strengthening, or stabilization of synapses, once formed within appropriate glomeruli.

We have examined the role of spontaneous activity and synaptic release in the establishment and maintenance of the olfactory sensory map. We have developed a genetic approach that allows for the conditional cell-specific expression of molecules that alter the activity of olfactory sensory neurons. Conditional expression of tetanus toxin light chain, a molecule that inhibits synaptic release in the majority of olfactory sensory neurons, does not perturb the development or maintenance of the sensory map. In contrast, inhibition of synaptic release in a small subpopulation of neurons expressing the P2 receptor results in correct targeting during development, but the P2 glomerulus is not maintained, and P2 neurons ultimately diminish. Thus, in a competitive scenario, neurons that fail to release neurotransmitter target correctly but fail to maintain stable synaptic contact and gradually disappear. Overexpression of the inward rectifying K<sup>+</sup> channel, Kir2.1, diminishes excitability by hyperpolarizing the sensory neuron, and this disrupts the formation of the olfactory map. These observations reveal a role for spontaneous release of neurotransmitter and spontaneous firing in the generation and stability of specific synaptic connections in the olfactory bulb.

## Results

Spontaneous firing of action potentials that exhibit a bursting behavior is observed in olfactory neurons (Rosparis et al., 1994), raising the possibility that spontaneous activity or neurotransmitter release may participate in the establishment or maintenance of a spatial map. We have therefore developed gene-targeting strategies that permit either the conditional inhibition of synaptic release or conditional hyperpolarization of olfactory sensory neurons, allowing an examination of the contribution of presynaptic neural activity to the establishment of a topographic map.

We have used gene targeting in ES cells to generate strains of mice in which the entire population of sensory neurons express a variant of the transcriptional activator, tTA (Gossen and Bujard, 1992). We have introduced the tTA gene in 3' untranslated DNA flanking the *OMP* gene (*OMP-IRES-tTA*) such that its translation is directed by an internal ribosome entry site (IRES) (Gogos et al., 2000). *OMP* is specifically expressed in all sensory neurons in the main olfactory epithelium (Farbman and Margolis, 1980). Cells that activate *OMP* gene transcription will now express a bicistronic RNA encoding both *OMP* and tTA (Figure 1A).

We also generated transgenic mouse lines that express either tetanus toxin light chain (TeTxLC) (Dubnau et al., 2001; McGuire et al., 2001; Sweeney et al., 1995; White et al., 2001) or the inward rectifying potassium channel, Kir2.1 (Johns et al., 1999; Kubo et al., 1993), under the control of the tTA-responsive promoter tet<sub>o</sub>. Tetanus toxin light chain proteolytically cleaves the synaptic vesicle protein VAMP2, which plays an essential role in synaptic vesicle release (Schoch et al., 2001; Sudhof, 1995). The overexpression of Kir2.1 will hyperpolarize the neuron and inhibit the firing of action potentials (Johns et al., 1999). Transgenic mice were generated containing the transgenes tet<sub>o</sub>-TeTxLC-IRES-tau-lacZ and tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ. Transcription of these transgenes is dependent on the presence of tTA and results in the combined expression of a neural silencer and tau-lacZ, allowing the visualization of cell bodies and processes of neurons expressing the transgene.

### Inhibition of Synaptic Release in Olfactory Sensory Neurons

In initial experiments, we analyzed the consequences of TeTxLC expression in compound heterozygotes bearing the *OMP-IRES-tTA* and tet<sub>o</sub>-TeTxLC-IRES-tau-lacZ alleles. One transgenic line generated compound heterozygotes that exhibit robust expression of both TeTxLC and tau-lacZ in 70% of the olfactory neurons (Figure 1B). In situ hybridization with antisense RNA probes to tetanus toxin light chain revealed expression only in neurons in the olfactory epithelium (Figure 1B), with no hybridization observed in any cells in the olfactory bulb, ensuring that the effect of TeTxLC is solely the consequence of expression in presynaptic sensory neurons. Coordinate expression of tau-lacZ allows us to visualize the sensory axons as they project to the bulb and ultimately converge to form the multiple glomeruli. Axons from cells expressing TeTxLC and tau-lacZ reveal a temporal and spatial pattern of glomerular formation indistinguishable from that observed in control animals bearing the *OMP-IRES-tTA* and tet<sub>o</sub>-IRES-tau-lacZ allele.

Tetanus toxin light chain inhibits synaptic transmission by cleaving VAMP2 between residues Gln-76 and Phe-77 within the cytoplasmic domain, releasing the N-terminal 76 amino acids from the vesicle membrane (Link et al., 1992; Schiavo et al., 1992). We have used a monoclonal antibody against an amino-terminal epitope of VAMP2 to demonstrate the efficacy of TeTxLC-mediated cleavage (Figure 1B). In control olfactory bulbs, VAMP2 immunoreactivity is observed in glomerular structures as well as in the internal plexiform layers and granule cell layers, consistent with its localization within

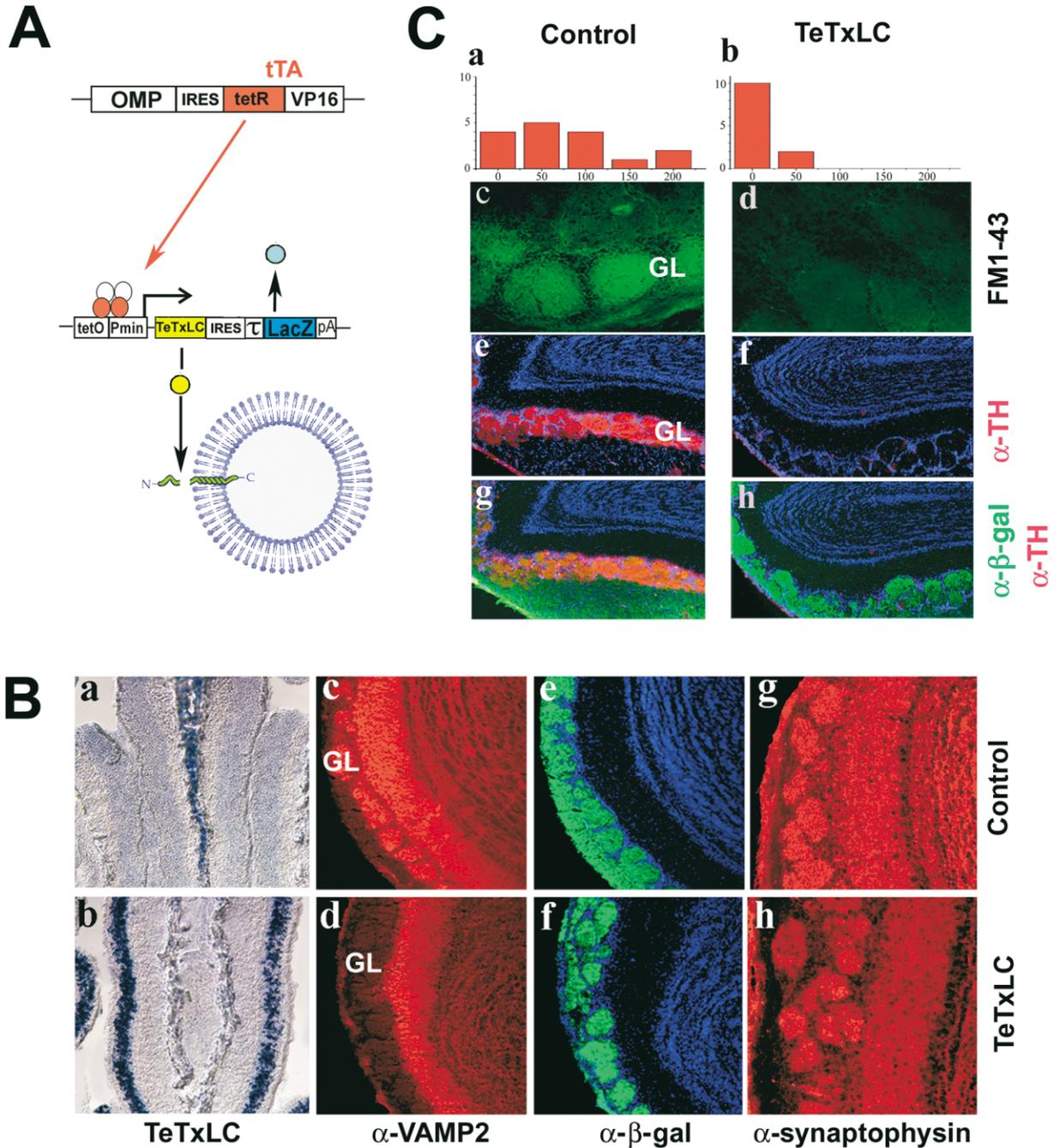


Figure 1. Olfactory Neurons Expressing Tetanus Toxin Are Inactive

(A) Schematic illustration of the induced expression of tetanus toxin light chain in olfactory sensory neurons. Compound heterozygotes containing *OMP-IRES-tTA* and *tet<sub>o</sub>-TeTxLC-IRES-tau-lacZ* alleles will express tetanus toxin and tau-lacZ in the absence of doxycycline.

(B) Coronal sections through the olfactory epithelium of control (*OMP-IRES-tTA/tet<sub>o</sub>-IRES-tau-lacZ*) (Ba) and TeTxLC-expressing mice (*OMP-IRES-tTA/tet<sub>o</sub>-TeTxLC-IRES-tau-lacZ*) (Bb), after in situ hybridization with an antisense riboprobe to TeTxLC. Coronal sections through the olfactory bulbs of control (top panels) and TeTxLC-expressing mice (bottom panels) were stained with antibodies against VAMP2 (Bc and Bd),  $\beta$ -galactosidase ( $\alpha$ - $\beta$ -gal) (Be and Bf), and synaptophysin (Bg and Bh).

(C) Patch-clamp recordings measuring evoked EPSCs from mitral cells in adult control mice ( $n = 16$  cells) (Ca) and mice expressing tetanus toxin ( $n = 12$  cells) (Cb) in response to olfactory nerve stimulation. EPSCs were grouped according to response amplitude in 50 pA increments. x axis values represent EPSC amplitude increments, and y axis values correspond to the number of cells with a given amplitude. FM1-43 staining of olfactory bulb slices from control (Cc) and TeTxLC-expressing (Cd) mice shows a reduced staining in (Cd). Analysis of tyrosine hydroxylase (TH) expression in coronal sections through olfactory bulbs in control (Ce and Cg) and TeTxLC (Cf and Ch) mice shows a diminution of TH level in (Cf) and (Ch). Sections are stained with  $\alpha$ -TH (red),  $\alpha$ - $\beta$ -gal (green), and the nuclear marker TOTO-3 (blue). GL, glomerular layer.

axon termini. Only weak staining is observed in the outer nerve layer, which consists largely of coursing axons. In mice expressing TeTxLC, we observe a striking diminution in VAMP2 immunoreactivity in glomeruli, with little change in the VAMP2 levels in the other synaptic layers of the olfactory bulb. The absence of VAMP2 expression is not due to a decrease in the accumulation of synaptic vesicles at the presynaptic terminal, since control immunohistochemistry for the synaptic vesicle protein synaptophysin reveals intense staining within the glomeruli of control and TeTxLC-expressing mice. Similar results were obtained for synapsin-1, a vesicle protein thought to be associated with both axodendritic and dendrodendritic synapses (Kim and Greer, 2000). These data demonstrate that olfactory sensory neurons expressing TeTxLC maintain synaptic vesicles at their axon termini, but these vesicles are deficient in intact VAMP2.

We next examined whether depletion of VAMP2 in olfactory sensory neurons blocks synaptic transmission using three different experimental approaches (Figure 1C). First, we performed patch-clamp recording from the mitral cells in slice preparations from control and TeTxLC-expressing animals. Synaptic currents were evoked by stimulating the outer nerve layer with bipolar electrodes and excitatory postsynaptic currents (EPSCs) and were recorded with conventional whole-cell configuration. In control animals, 12 out of 16 neurons that were recorded showed evoked currents, with amplitudes ranging from 20 to 200 pA. These EPSCs were dramatically reduced in amplitude and frequency in TeTxLC-expressing animals depleted in VAMP2. Weakly evoked currents (<50 pA) were observed in only 2 of the 12 neurons, and the evoked currents exhibited a slow time course likely to result from dendritic excitation from neighboring mitral cells rather than from sensory termini (Figures 1Ca and 1Cb).

We also used FM1-43, a fluorescent indicator of vesicle reuptake (Pyle et al., 1999), to examine the effect of TeTxLC on synaptic release (Figures 1Cc and 1Cd). High concentration of external potassium induces massive release of vesicles, which in turn stimulates vesicle reuptake by endocytosis. When the lipophilic dye FM1-43 is present during evoked transmitter release, subsequent endocytosis will result in the labeling of nerve terminals by fluorescent FM1-43 trapped in recycled vesicles. In bulb slices from control animals, potassium stimulation results in vesicle release and reuptake, resulting in robust fluorescent staining of the glomeruli. In contrast, little FM1-43 staining is observed after potassium stimulation of sensory terminals in animals expressing TeTxLC.

In a third experimental approach, we exploited previous observations demonstrating that TH is a sensitive indicator of sensory afferent activity (Baker et al., 1993; Stone et al., 1990). In control mice, strong TH activity is observed in periglomerular cells and in fibers within the glomeruli. In mice expressing TeTxLC, the glomeruli reveal a profound reduction in TH immunoreactivity (Figures 1Ce–1Ch). These data demonstrate that TeTxLC expression suppresses synaptic vesicle release from olfactory sensory neurons, inhibiting the activation of synaptic partners in the olfactory bulb. The expression of TeTxLC, however, did not noticeably alter the morphology of the sensory epithelium or the olfactory bulb. Moreover, quantitative *in situ* hybridization experiments

with RNA probes to individual olfactory receptors reveal that the patterns and frequency of specific receptor expression are unaltered in TeTxLC-expressing animals.

### Global Inhibition of Synaptic Release Does Not Alter Glomerular Targeting

We next examined the pattern of glomerular targeting of subpopulations of neurons expressing a specific receptor in mice in which synaptic release is inhibited in olfactory sensory neurons. Compound heterozygotes expressing TeTxLC under the control of the *OMP* promoter were crossed with mice homozygous for the *P2-IRES-GFP* allele. In these mice, neurons that express P2 will also express GFP, allowing the visualization of P2 neurons along with their axonal projections. The pattern of GFP-expression in the epithelium, as well as the pattern of axon convergence to one lateral and one medial glomerulus in the ventral aspect of the bulb, is indistinguishable in control and TeTxLC-expressing mice (Figures 2B and 2C). The number of P2-expressing cells, the timing of axon extension and entry into the bulb, and the position of the individual P2 glomeruli are unaltered by the global inhibition of synaptic release. We have also examined the glomerulus formed by the convergence of fibers from neurons expressing the MOR28 receptor in mice carrying the *MOR28-IRES-GFP* allele. The convergence of GFP-expressing fibers in MOR28 glomeruli is indistinguishable in wild-type mice and transgenic animals expressing TeTxLC (Figures 2E and 2F).

### Inhibition of Synaptic Release in a Subset of Neurons Alters Glomerular Targeting

The expression of tTA under the control of the *OMP* promoter assures that most of the olfactory sensory neurons will express TeTxLC. Under these noncompetitive conditions, there is no apparent perturbation in the sensory map. We also established a situation where only a subpopulation of neurons expressing the P2 receptor are defective in synaptic release in a background in which all other sensory neurons are wild-type. We exploited a mouse strain that we constructed previously that is homozygous for a *P2-IRES-rtTA* allele (Gogos et al., 2000). rtTA is a mutant transcriptional factor that is dependent upon tetracycline for DNA binding and transcriptional activation (Gossen and Bujard, 1992). In these mice, rtTA-mediated expression of reporter genes driven by the *tet<sub>o</sub>* promoter is observed only in the presence of doxycycline. Mice bearing the *P2-IRES-rtTA* allele were crossed with strains bearing the *tet<sub>o</sub>-TeTxLC-IRES-tau-lacZ* transgene, such that only cells that express the P2 receptor would express TeTxLC and tau-lacZ.

The design of this experiment permits us to observe P2 neurons expressing tetanus toxin light chain and tau-lacZ in the neuroepithelium and to follow their projections to the olfactory bulb. In one experimental approach, mice were fed doxycycline during the entire gestation period, inducing the expression of TeTxLC in P2 neurons during development (Figure 3A). Analysis of whole-mount preparations from late embryos and P0 mice revealed that the axons from P2 neurons expressing TeTxLC and tau-lacZ course through the epithelium and traverse the cribriform plate to enter the bulb, where they project to one medial and one lateral glomerulus.

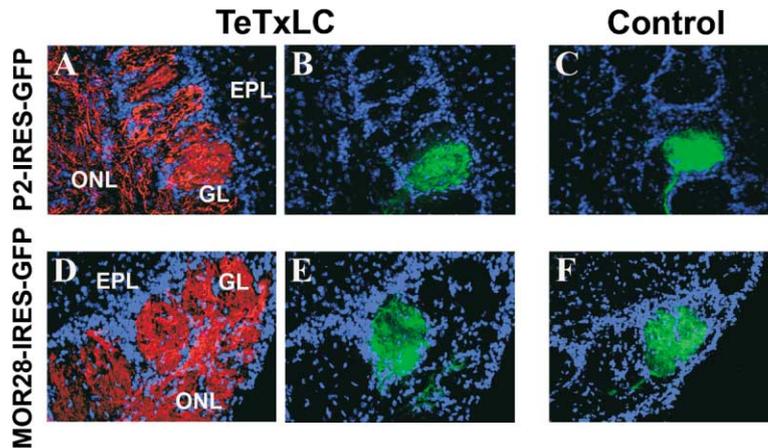


Figure 2. Expression of TeTxLC in the Majority of Sensory Neurons Does Not Disrupt Targeting

(A–C) TeTxLC-expressing (*OMP-IRES-tTA/tet<sub>o</sub>-TeTxLC-IRES-tau-lacZ*) mice (A and B) and control (*OMP-IRES-tTA/tet<sub>o</sub>-IRES-tau-lacZ*) mice (C) containing a *P2-IRES-GFP* allele were stained with antibodies against  $\beta$ -gal (red) and GFP (green), and TOTO-3 nuclear staining (blue) showing the convergence of P2-expressing axons to the medial P2 glomerulus is shown.

(D–F) Olfactory bulb sections from adult TeTxLC-expressing mice containing a *MOR28-IRES-GFP* allele (D and E) and control mice (F) were stained with antibodies against  $\beta$ -gal (red) and GFP (green) and with the nuclear marker TOTO-3 (blue). The medial MOR28 glomerulus is shown. EPL, external plexiform layer; GL, glomerular layer; ONL, outer nerve layer.

However, occasional stray fibers are observed that fail to project to the topographically fixed P2 glomerulus. Between days P3 and P10, an increasing number of axons are observed that fail to converge on the P2 glomerulus, and by day P21, fibers with aberrant trajectories are visible over the entire medial surface of the bulb, many navigating past the P2 glomerulus toward the posterior pins of the bulb. This pattern is in sharp contrast with observations in control mice, in which axons from P2 neurons exhibit strict convergence on one or two medial glomeruli with no stray fibers evident (Figure 3B). Thus, the expression of TeTxLC in a small subpopulation of P2 neurons results in an appropriate convergence on a glomerular target, but this is transient, and the axons begin to stray early in postnatal life and ultimately project to multiple inappropriate targets. A diminution in the number of P2 fibers expressing TeTxLC and tau-lacZ becomes apparent at 3 weeks, and by 6 weeks of age few tau-lacZ<sup>+</sup> fibers persist (Figure 3A).

In a separate experiment, mice carrying the *P2-IRES-rtTA* allele and also the *tet<sub>o</sub>-TeTxLC-IRES-tau-lacZ* transgene were allowed to develop until postnatal day 21 (immediately postweaning) and at this time were fed doxycycline (Figure 3C). The axon trajectories of neurons expressing TeTxLC and tau-lacZ were then examined at various points after doxycycline exposure. This experiment examines the consequence of inhibition of synaptic release after the P2 axons have converged on appropriate glomerular targets. Neurons expressing tetanus toxin light chain begin to stray from the preformed P2 glomerulus and migrate randomly within 2 days after induction. These aberrant migrations presumably result from the dissolution of preexisting synaptic connections and inappropriate axon wandering. After 2 weeks of doxycycline exposure, cells expressing the TeTxLC and tau-lacZ transgenes are significantly reduced in number, and after 4 weeks only a rare lacZ<sup>+</sup> cell is observed in the epithelium. These data indicate that, under competitive conditions, the P2 population of sensory neurons defective in synaptic release project to an appropriate glomerular locus. However, this convergent synaptic structure is transient, and these neurons fail to maintain synaptic connections, migrating inappropriately throughout the olfactory bulb. The in-

ability to maintain stable synapses is followed by a dramatic diminution in cell numbers. This phenotype is not observed under noncompetitive conditions in the majority of the neurons expressing TeTxLC. Under these conditions, axon targeting is normal, the glomerular structure is stable, and there is no obvious diminution in cell numbers.

#### A Conditional Silencing of Olfactory Neurons

We have also examined the consequence of hyperpolarizing the sensory neurons, a process which should inhibit both odor-evoked and spontaneous action potentials. We have generated mice in which the gene encoding the inward rectifying potassium channel, Kir2.1, was placed under the control of a *tTA*-dependent promoter. Previous studies have employed the overexpression of both the delayed rectifier and the inward rectifying potassium channels, which results in a significant hyperpolarization of the neuron and inhibits neuronal firing (Ehrengruber et al., 1997; Johns et al., 1999; Nitabach et al., 2002; Peckol et al., 1999; White et al., 2001). Mice bearing an *OMP-IRES-tTA* allele were crossed with animals containing a *tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* transgene (Figure 4A). Compound heterozygotes express the Kir2.1 and tau-lacZ transgene in 80% of the olfactory sensory neurons (Figure 4B). Doxycycline administration completely suppressed the Kir2.1 and tau-lacZ expression after 2 weeks (Figure 4B).

Two experimental approaches demonstrate that the expression of the inward rectifying potassium channel, Kir2.1, dramatically inhibits the spiking activity of olfactory sensory neurons. First, we have performed extracellular recordings in slices of sensory epithelium that demonstrate a >20-fold diminution in the frequency of spontaneous action potentials in Kir2.1-expressing mice when compared to control animals (Figure 4C). We have also exploited the observation that the level of tyrosine hydroxylase activity by periglomerular cells reflects the level of synaptic activity of the presynaptic sensory neurons (Figure 4D). In mice expressing Kir2.1, the glomeruli are surrounded by only sparse TH-labeled neurons, which display a reduction in TH activity. Although Kir2.1-expressing cells exhibit diminished neural excitability, other markers of cell function appear normal. We have

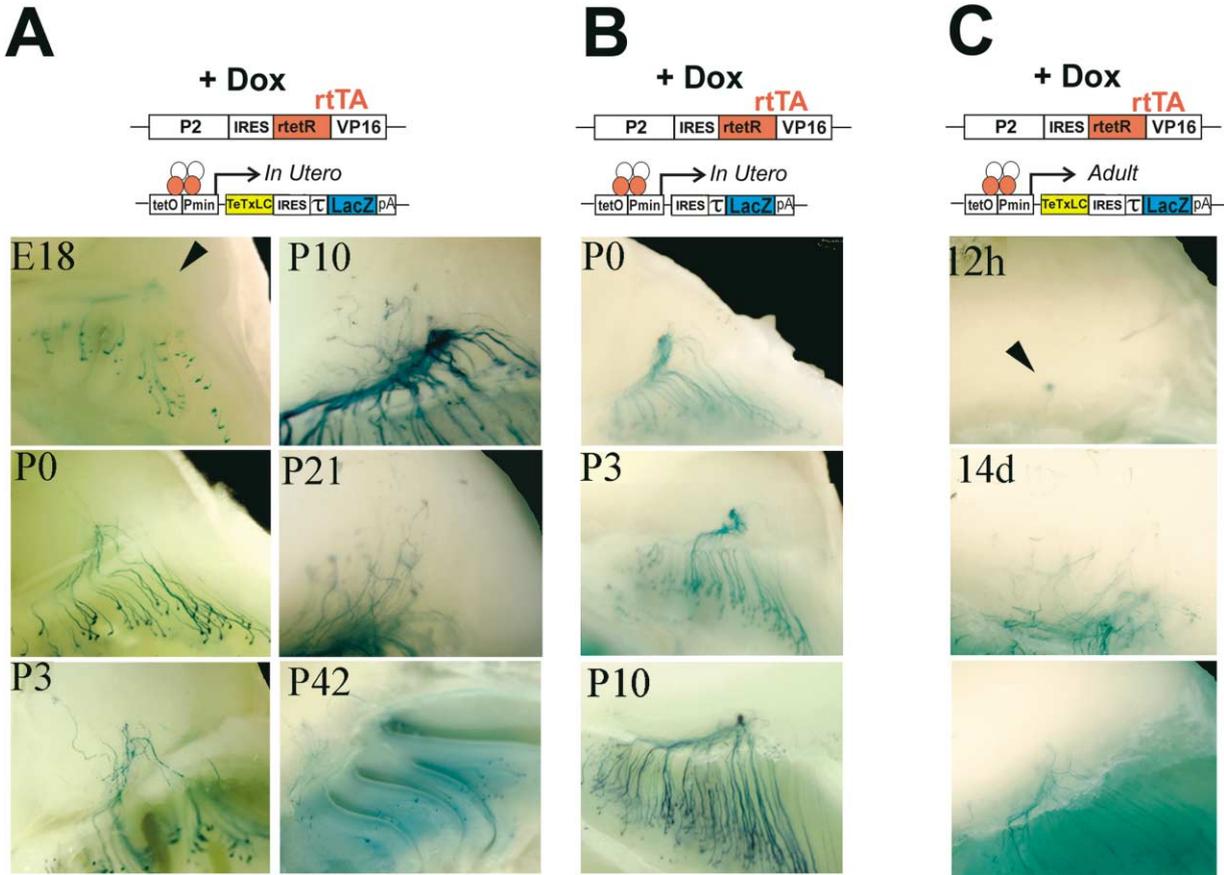


Figure 3. Conditional Expression of TeTxLC in P2 Neurons in the Developing and Adult Olfactory System

(A and B) X-gal-stained whole-mount preparations of olfactory epithelium and bulb of compound heterozygotes containing the *P2-IRES-rtTA* allele and the *tet<sub>o</sub>-TeTxLC-IRES-tau-lacZ* transgene (A) or the control *tet<sub>o</sub>-IRES-tau-lacZ* transgene (B). Expression of TeTxLC and/or tau-lacZ is induced in the presence of doxycycline, administered throughout gestation and the life of the animal. At E18 and P0, “blue” fibers converge to form a protoglomerulus (arrow).

(C) *P2-IRES-rtTA/tet<sub>o</sub>-TeTxLC-IRES-tau-lacZ* compound heterozygote mice were allowed to develop to adulthood in the absence of TeTxLC expression. Expression of TeTxLC and tau-lacZ was then induced by a short duration (12 hr) and long duration (14 days) of doxycycline administration (lower two panels). Signal observed in the bulb after 12 hr (arrow) corresponds to tau-lacZ-positive P2 fibers within the intact P2 glomerulus.

performed electroolfactogram (EOG) recordings from the surface of the sensory epithelium that measure local dendritic field potentials generated by odor stimulation. These recordings indicate that the dendritic response to a variety of odors, a response that is independent of the generation of action potentials, is comparable in Kir2.1-transgenic and wild-type animals (Figure 4E). The synaptic vesicle components also appear unperturbed at sensory termini in these transgenic mice. Immunohistochemistry reveals that the levels of two presynaptic proteins, VAMP2 and synapsin, are comparable in control and transgenic animals (Figures 4F and 4G). Thus, the expression of Kir2.1 in olfactory sensory neurons silences the cell without other signs of neural toxicity.

#### Kir2.1 Overexpression Delays the Development of a Sensory Map

Analysis of the development of the olfactory map in transgenic mice expressing tau-lacZ and Kir2.1 reveals significant delay in the entry of sensory axons into the olfactory bulb (Figure 5). At P0 and P3, we observe a sparse representation of blue axons in the bulb, and

significant innervation is not observed until P11 (Figures 5A and 5B). Even at this stage of development of the sensory map, the posterior dorsal part of the bulb, the site of formation of the necklace glomeruli, remains only sparsely innervated. By P21, the level of innervation of transgenic animals approaches that of wild-type mice. However, we observe a persistent defect in the organization of glomeruli in the dorsal part of the olfactory bulb that is maintained in adult mice. The dorsal bulb remains thinly innervated, the necklace glomeruli are absent, and axons that enter this region of the bulb appear to coalesce and form giant glomerulus-like structures never observed in control animals (Figure 5E).

These developmental defects are also readily apparent upon analysis of sections through the bulb of wild-type and transgenic mice (Figures 5C and 5D). At P0, for example, wild-type animals reveal well-organized glomerular structures surrounded by periglomerular cells. In Kir2.1-expressing animals, the glomeruli remain poorly organized at P0, and sensory axons accumulate in the outer nerve layer, with only rare axons entering the glomerular layer. Innervation of the glomerular layer

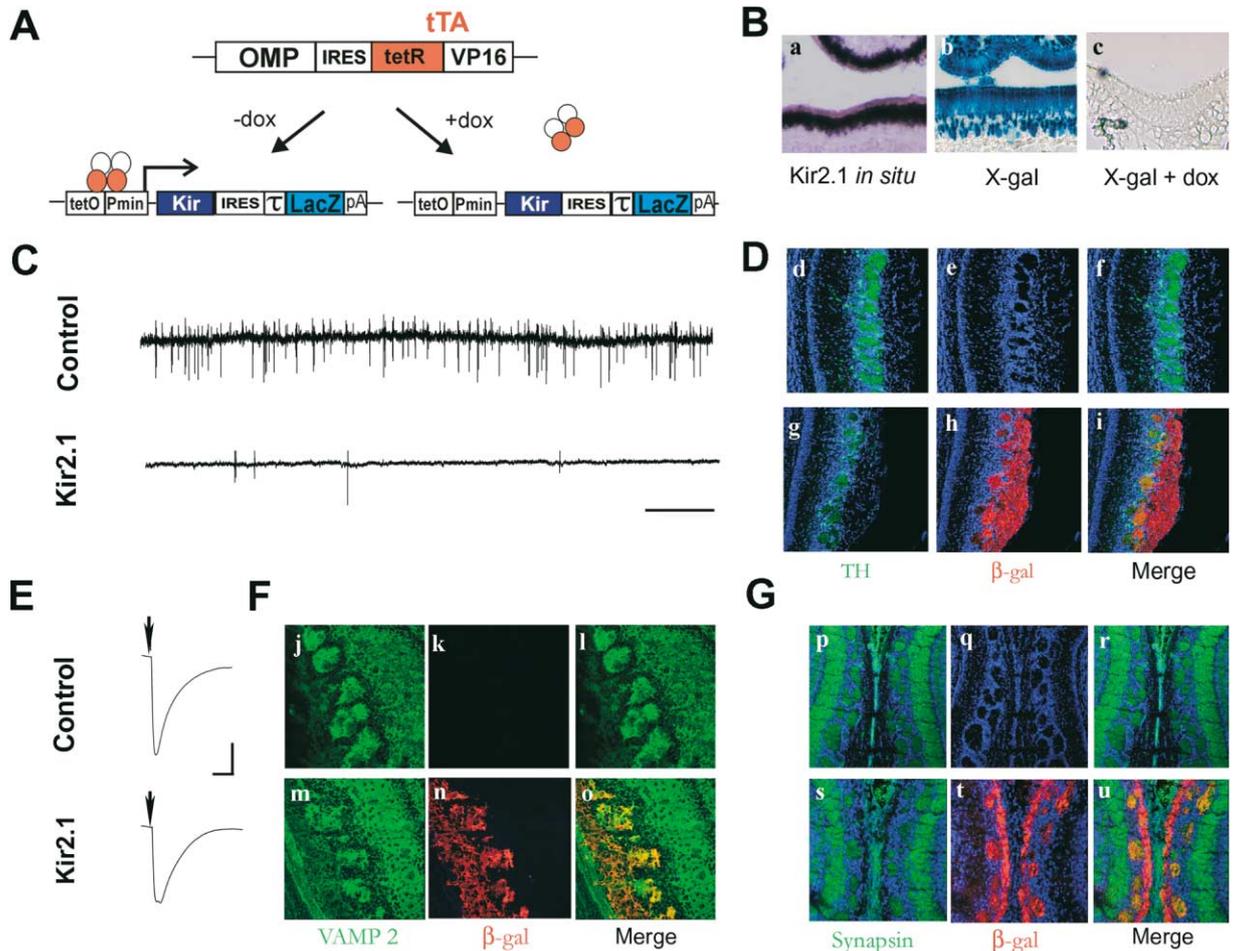


Figure 4. Induced Expression of the Kir2.1 Channel Silences Olfactory Neurons

(A) Schematic illustration of the strategy for inducible expression of Kir2.1 channels in the olfactory neurons. Compound heterozygotes containing *OMP-IRES-tTA* and *tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* alleles express Kir2.1 channels and tau-lacZ marker protein in all olfactory neurons in the absence of doxycycline. Doxycycline suppresses transgene expression.

(B) In situ hybridization with a riboprobe specific for Kir2.1 mRNA (Ba) and X-gal staining (Bb) in sections of the olfactory epithelium of *OMP-IRES-tTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* compound heterozygotes. After 2 weeks of doxycycline diet, the expression of Kir2.1 channels and tau-lacZ is eliminated, as shown with X-gal staining in (Bc).

(C) Extracellular recordings from olfactory neurons show a dramatic reduction in spontaneous neuronal firing in mice expressing the Kir2.1 channel compared to wild-type controls. Vertical deflections in the recordings correspond to individual action potential spikes. Horizontal bar, 2 s.

(D) Immunofluorescent imaging using antibodies against tyrosine hydroxylase (TH) (green) and  $\beta$ -gal (red) in sections of the olfactory bulb from Kir2.1-expressing mice (Dg–Di) and wild-type controls (Dd–Df). The sections are counterstained with the nuclear marker TOTO-3 (blue). (E) EOG recording from the olfactory epithelium of Kir2.1-expressing mice and wild-type controls in response to odor stimulation. x axis scale bar, 1 s; y axis scale bar, 1  $\mu$ A. Arrow indicates time of odor application.

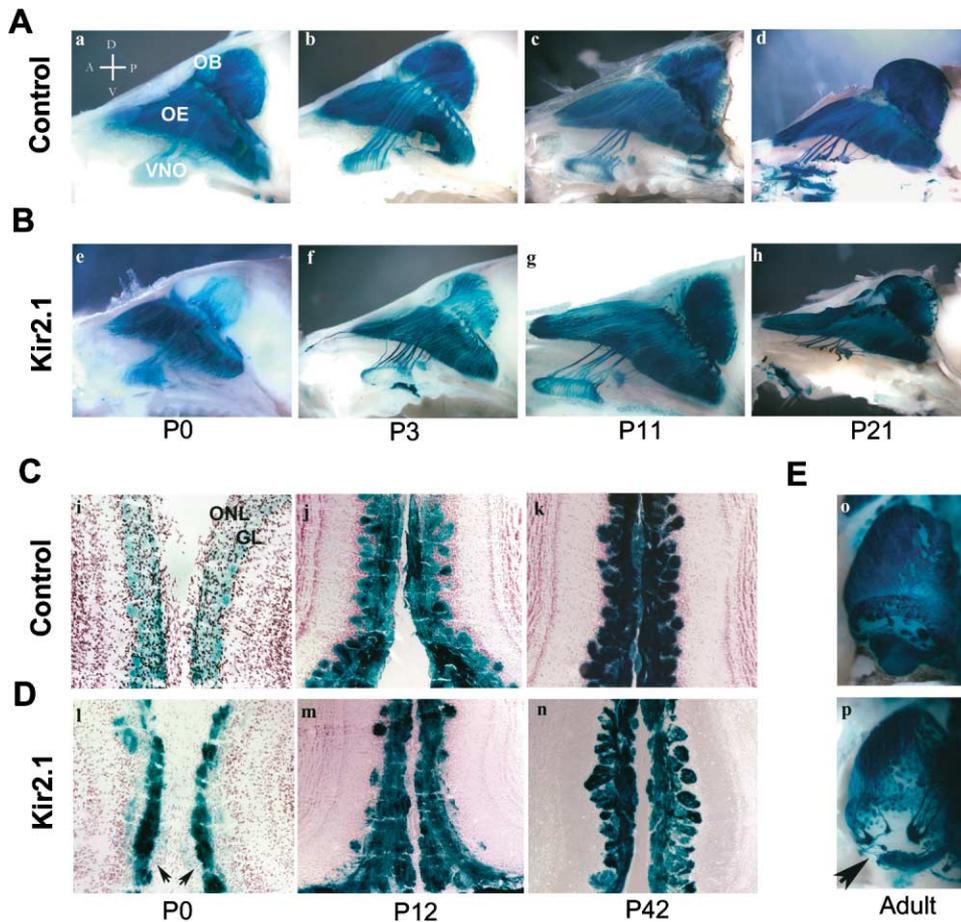
(F) Distribution of presynaptic protein VAMP2 (Fj and Fm) detected by antibody (green) in olfactory bulb sections of Kir2.1-expressing mice (Fm–Fo) and wild-type controls (Fj–Fl). Kir2.1- and tau-lacZ-expressing fibers were labeled with an anti- $\beta$ -gal antibody (red) (Fn).

(G) Distribution of synapsin (Gp and Gs) detected by antibodies to synapsin (green) in olfactory bulb sections of Kir2.1-expressing mice (Gs–Gu) and wild-type controls (Gp–Gr). Kir2.1- and tau-lacZ-expressing fibers were labeled with an antibody against  $\beta$ -gal (red) (Gt), and nuclei were labeled with TOTO-3 (blue) (Gp–Gu).

is apparent by P12, but the number of glomeruli formed is far less than observed in wild-type animals. In adults, the glomeruli in Kir2.1 transgenics appear grossly normal, with perhaps a slight reduction in glomerular number. Thus, the expression of Kir2.1 silences sensory neurons and results in a significant delay in the innervation of the olfactory bulb, with a profound and persistent alteration in glomerular morphology in the dorsal aspect of the bulb.

### Silencing of Neurons Perturbs the Sensory Map

We next examined the precision of targeting of specific olfactory sensory neurons in mice expressing Kir2.1 globally in the olfactory epithelium (Figure 6). Compound heterozygotes containing an *OMP-IRES-tTA* allele and the *tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* transgene were crossed with mice homozygous for the *P2-IRES-GFP* allele. In transgenic animals expressing Kir2.1 at P1, the P2 axons are diffusely distributed, and those that enter the bulb



**Figure 5. Genetically Silencing a Large Number of Olfactory Neurons Causes a Developmental Delay in Axon Growth**

(A and B) Developmental profile of olfactory axons reaching the olfactory bulb after birth (P0–P21), as shown in X-gal-stained whole-mount preparations from control mice expressing tau-lacZ (*OMP-tau-lacZ*) (Aa–Ad), or from Kir2.1 channel- and tau-lacZ-expressing mice (*OMP-IRES-tTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ*) (Be–Bh). Note that in mice expressing Kir2.1 there is sparse innervation within the bulb at P0; the lack of innervation in the dorsal posterior part of the bulb persists throughout development.

(C and D) Sections of olfactory bulb from P0, P12, and P42 *OMP-tau-lacZ* control mice (Ci–Ck) or *OMP-IRES-tTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* compound heterozygotes (Di–Dn). Note that at P0 the accumulation of strong blue staining in the outer nerve layer (arrows in [Di]) reflects the failure of the majority axons to enter the glomerular layer. The glomeruli are not well-formed in mice expressing Kir2.1 until P11 (Di and Dm). (E) Dorsal view of olfactory bulbs from adult *OMP-tau-lacZ* (Eo) and *OMP-IRES-tTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* compound heterozygous (Ep) mice. Note the rare and abnormal innervation patterns and the lack of the necklace glomeruli (arrow) in Kir2.1-expressing mice (Ep). OB, olfactory bulb; OE, olfactory epithelium; VNO, vomeronasal organ; GL, glomerular layer; ONL, outer nerve layer.

sparingly innervate multiple glomeruli in the ventral aspect of the bulb (Figures 6A and 6B). This diffuse pattern of projections becomes even more apparent as postnatal development proceeds, and by P21, P2-expressing neurons innervate an average of 16 medial glomeruli, as opposed to the one medial P2 glomerulus observed in 26 control olfactory bulbs. Projections to multiple glomeruli are also observed for neurons expressing MOR23, which innervate the dorsal aspect of the bulb (Figure 6E).

A similar, but less dramatic phenotype is observed for the subpopulation of neurons expressing the MOR28 receptor. In wild-type animals, neurons expressing the *MOR28-IRES-GFP* allele project to one medial and one lateral glomerulus that reside in the most ventral region of the olfactory bulb. In Kir2.1-transgenic mice, MOR28-

expressing neurons project axons to two to three, rather than to a single lateral glomerulus observed in control animals (Figure 6D). Of 32 lateral MOR28 targets examined, 75% revealed convergence on two or three glomeruli, whereas only 1 of 28 MOR28 targets examined exhibited convergence on two glomeruli in control mice. Interestingly, the medial MOR28 fibers converge on a single glomerulus in both Kir2.1-transgenic and control animals (Figure 6C). Thus, the expression of Kir2.1 broadly in the olfactory sensory neurons results in targeting defects whose severity differs for different subpopulations of neurons.

#### Silencing P2 Neurons in a Competitive Environment

When neural activity is suppressed in the majority of neurons within the sensory epithelium, axons exhibit

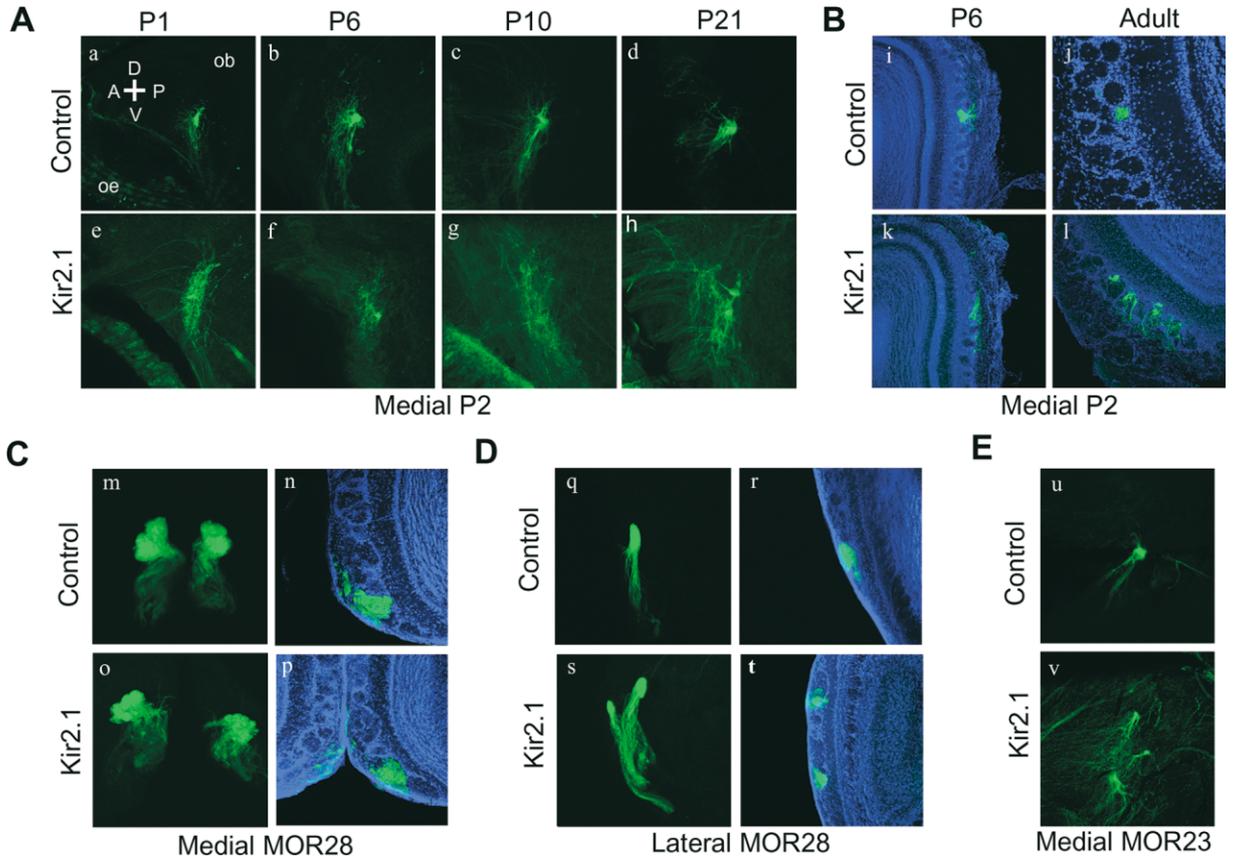


Figure 6. Electrical Silencing of Olfactory Neurons Disrupts the Olfactory Map

(A) Whole-mount fluorescent image of P2 axons projecting to the medial aspect of the olfactory bulb. The upper panels (Aa–Ad) show the developmental profile of *P2-IRES-GFP/OMP-IRES-tTA* control compound heterozygotes from birth (P0) through P21. The lower panels (Ae–Ah) show P2 axons from *P2-IRES-GFP/OMP-IRES-tTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* compound heterozygotes.

(B) Coronal sections of olfactory bulb showing GFP-labeled P2 medial glomeruli in control (Bi and Bj) and Kir2.1-expressing mice (Bk and Bl) during early development (P6) and in adulthood.

(C) Comparison of GFP-labeled MOR28 axon projections in whole mounts (Cm and Co) and olfactory bulb sections (Cn and Cp) between control (*MOR28-IRES-GFP/OMP-IRES-tTA*) (Cm and Cn) and Kir2.1-expressing animals (*MOR28-IRES-GFP/OMP-IRES-tTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ*) (Co and Cp). Ventral whole-mount images in (C) show axons converging onto one of the two bilaterally symmetrical medial glomeruli in the ventral aspect of the two olfactory bulbs. A wild-type medial MOR28 glomerulus is large and lobular (Cm) but still only forms a single convergent structure, as better shown in coronal section (Cn); the structure of MOR28 medial glomeruli in Kir2.1-expressing mice (Co and Cp) is very similar to that seen in wild-type.

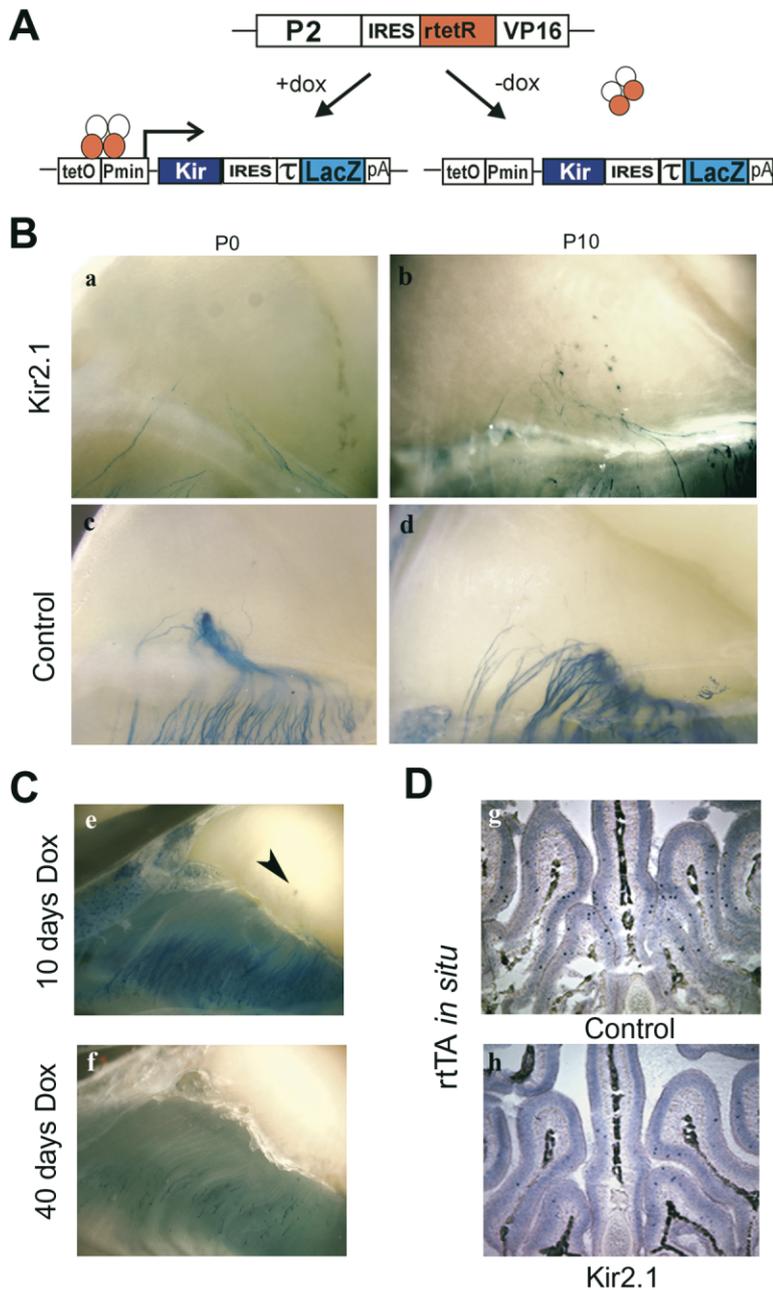
(D) Ventral views of MOR28 axon projections in control (*MOR28-IRES-GFP/OMP-IRES-tTA*) (Dq and Dr) and Kir2.1-expressing animals (*MOR28-IRES-GFP/OMP-IRES-tTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ*) (Ds and Dt) onto the lateral glomeruli.

(E) Whole-mount preparations of GFP-labeled MOR23-expressing neurons in control mice (*MOR23-IRES-GFP/OMP-IRES-tTA*) (Eu) and in mice expressing Kir2.1 (*MOR23-IRES-GFP/OMP-IRES-tTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ*) (Ev). These images correspond to medial MOR23 glomeruli.

targeting defects. We next examined the consequence of silencing the P2 population of neurons in a background in which all other sensory neurons were normal. We crossed transgenic animals bearing a *tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* allele with mice containing the *P2-IRES-rtTA* allele (Figure 7A). In these mice, Kir2.1 expression is dependent upon the administration of doxycycline and should be restricted to P2 neurons. In one experimental design, mice were fed doxycycline during gestation to induce Kir2.1 expression during embryonic development, and the targeting of P2 axons was analyzed in early postnatal life (Figure 7B). Under these conditions, at P0, P2 axons fail to enter the bulb, with only a few fibers crossing the cribriform plate. The rare fibers that enter the bulb do not converge on a single glomerulus

and appear to innervate a broad region of the ventral aspect of the olfactory bulb. This profound defect in axon targeting is also associated with a striking decrease in P2-expressing neurons that is evident even at early postnatal stages. At P21, for example, *in situ* hybridization with a probe for *rtTA* in mice expressing *P2-IRES-rtTA* and Kir2.1 reveals 5-fold fewer cells than in control animals containing only the *P2-IRES-rtTA* allele (Figure 7D).

We have also examined the effects of Kir2.1 expression and neural silencing on the maintenance and stability of the map formed prior to Kir2.1 expression (Figure 7C). Mice bearing a *P2-IRES-rtTA* allele and the *tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* transgene were allowed to develop in the absence of doxycycline. Under these condi-



**Figure 7. Suppression of Activity in a Competitive Environment Severely Disrupts Olfactory Axon Projection**

(A) A schematic illustration of the doxycycline-dependent induction of the *tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* transgene in P2 neurons using reverse tetracycline transactivator (rtTA). Doxycycline induces Kir2.1 channel and tau-lacZ expression specifically in P2 neurons.

(B) Whole-mount X-gal staining of P0 and P10 olfactory epithelia and bulb preparations from Kir2.1 compound heterozygotes (*P2-IRES-rtTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ*) that have been administered doxycycline in utero and throughout postnatal life (Ba and Bb), as well as from control *P2-IRES-tau-lacZ* mice (Bc and Bd). Whole mounts of additional control animals (*OMP-IRES-tTA/tet<sub>o</sub>-IRES-tau-lacZ*) are illustrated in Figure 3B.

(C) Whole-mount X-gal staining of olfactory epithelia and bulb preparations from compound heterozygotes that were allowed to develop in the absence of doxycycline. After weaning (P21), the animals were fed doxycycline to induce Kir2.1 channel and tau-lacZ expression in P2 neurons. The P2 glomerulus (arrowhead) is visible in the medial aspect of the olfactory bulb after a 10 day induction (Ce). After 40 days of transgene induction, the number of neurons expressing the tau-LacZ marker diminished, and the P2 glomerulus disappeared (Cf).

(D) In situ hybridization with probes specific for rtTA in the olfactory epithelium of 3-week-old *P2-IRES-rtTA/tet<sub>o</sub>-Kir2.1-IRES-tau-lacZ* compound heterozygotes continuously fed doxycycline (Dh) and *P2-IRES-rtTA* controls (Dg). The dark purple dots within the olfactory epithelium show cells stained with the in situ probe.

tions, Kir2.1 is not expressed, and the sensory map should develop normally. P21 mice, immediately post-weaning, were fed doxycycline to induce the expression of Kir2.1 and tau-lacZ. After 10 days of exposure to doxycycline, tau-lacZ is observed in P2 neurons, and their axons converge on a single glomerulus in an olfactory bulb (Figure 7C). After 40 days of doxycycline and Kir2.1 expression, convergence on a P2 glomerulus is no longer evident. Only rare stray fibers are observed that stain for tau-lacZ, suggesting that the suppression of neural activity in neurons that converge onto an already formed glomerulus destabilizes the synaptic structure and causes these cells to disappear. Thus, Kir2.1-mediated silencing of neural activity results in a more profound axon phenotype when restricted to the

subpopulation of P2 neurons. Under these competitive conditions, most P2 neurons fail to enter the bulb, and those rare fibers that succeed fail to converge to form a P2 glomerulus. If a glomerulus is allowed to form under control conditions, activation of the Kir2.1 transgene and the persistent suppression of neural activity result in destabilization of the glomerular structure and the disappearance of the P2 neurons.

### Discussion

Neural circuits must exhibit activity-dependent plasticity to allow the organism to respond to a changing environment. The observation that experience shapes the character of specific neural connections immediately poses

the question as to the role of neural activity in the establishment of neural circuits during development. The contribution of activity dependence to the development of neural circuits is complex and may benefit from deconstruction. One important distinction surrounds the question as to how much of the circuitry is governed by developmentally programmed mechanisms, such as the timing of axon growth and the recognition of guidance molecules, and to what extent these deterministic processes are dependent on neural activity. Sensory neurons can exhibit activity independent of sensory input, and we must therefore ask whether activity-dependent events result from spontaneous or experience-evoked firing. Both of these forms of neural activity may either be correlated or noncorrelated, adding yet another parameter to the complexities of activity-dependent processes. Finally, activity may be permissive or instructive. It may merely be required to allow for changes in a circuit or it may direct the establishment and reorganization of neural connections.

We have performed a series of genetic experiments to determine the role of activity in the establishment and maintenance of an olfactory sensory map. Mice lacking the cyclic nucleotide-gated channel, a central component in the olfactory transduction apparatus, fail to exhibit odorant-evoked electrophysiologic responses to a wide range of odor stimuli (Brunet et al., 1996). Nonetheless, the convergence to appropriate target glomeruli by cells expressing either the M50 or P2 odorant receptor is unaffected in CNG channel mutant mice (Lin et al., 2000; Zheng et al., 2000). Twenty percent of neurons expressing the M71 receptor target to inappropriate glomeruli that are close to the predominant M71 glomerulus. These data argue that olfactory experience is not required for the establishment of precise patterns of afferent innervation in the olfactory bulb. Since it is difficult to envisage a mechanism to correlate the activity of randomly distributed neurons expressing the same receptor without odor-evoked stimulation of receptors, these data further imply that correlated neural activity is not required for the generation of the olfactory sensory map. These results are consistent with the observation that mice deficient in the olfactory-specific G protein,  $G_{olf}$ , also exhibit normal patterns of olfactory neural convergence in the olfactory bulb (Belluscio et al., 1998). These experiments were performed under conditions in which odor-evoked activity is inhibited in the entire population of sensory neurons. These data, therefore, do not exclude a role for sensory activity under competitive conditions in the generation and maintenance of the map. Moreover, these experiments do not address the possibility that spontaneous, noncorrelated activity or neurotransmitter release may be required for the establishment or maintenance of a spatial map.

Our current studies employ a genetic strategy to either conditionally suppress synaptic release or inhibit action potentials in both a noncompetitive and competitive environment. Synaptic release is inhibited by the expression of tetanus toxin light chain. Widespread expression of TeTxLC in the vast majority of neurons does not elicit a discernible phenotype in either the sensory epithelium or the olfactory bulb, where the topographic map remains unperturbed. A different result is observed when firing is suppressed by overexpression of the inward

rectifying  $K^+$  channel, Kir2.1. Expression of Kir2.1 in the majority of olfactory neurons causes a delay in axon entry and a gross disorganization of the dorsal collection of glomeruli. Different subpopulations of neurons are disturbed in different ways. P2 and MOR23 neurons do not converge on a single glomerulus, as do wild-type neurons; rather they innervate multiple (12 to 16) glomeruli sparsely in the bulb. MOR28 neurons consistently innervate two or three rather than a single lateral glomerulus observed in control animals. These data suggest a requirement for neural firing for the appropriate timing of axon outgrowth and the precision of axon targeting.

Spike activity in sensory neurons might be required for the appropriate action of extrinsic factors, perhaps by creating an appropriate milieu within sensory neurons to allow the correct developmental program to unfold. Previous work demonstrates that electrical activity dramatically enhances the effects of trophic factors on axon growth in retinal ganglion cells (Goldberg et al., 2002). In these experiments, low levels of electrical stimulation were sufficient to dramatically potentiate axon growth and survival in response to peptide neurotrophic factors. Similarly, electrical activity may promote the responsiveness of olfactory axons to guidance cues elaborated in the olfactory bulb. Whatever the mechanism, the consequence of neural activity is likely to be permissive rather than instructive, since mutations that actively block odor-evoked or correlated neural activity have only minor effects on the targeting of sensory axons.

The expression of TeTxLC or Kir2.1 in small subpopulations of olfactory neurons creates a competitive environment that results in far more severe phenotypes. Inhibition of synaptic release by TeTxLC in subpopulations of neurons expressing a given receptor allows the initial convergence of fibers to an appropriate glomerulus. However, this glomerular aggregate of like axons is not stable; fibers begin to stray, and by 3 weeks these neurons are no longer present in the epithelium. Thus, under competitive conditions, neurons that are deficient in synaptic release fail to maintain stable synapses, and the number of LacZ+ "inactive" cells diminishes dramatically. Expression of Kir2.1 in a subpopulation of P2 neurons results in the failure of olfactory neurons to cross the cribriform plate, and by 3 weeks these neurons are no longer present in the sensory epithelium. If P2 neurons are allowed to develop normally and form a glomerular structure and either Kir2.1 or TeTxLC is then induced in the P2 cells, the synaptic structure is not maintained, the axons stray, and these neurons again appear to die. Under these experimental conditions, we observe that neurons expressing a given receptor are no longer evident within the epithelium by 2–3 weeks. One interpretation of these findings is that inactive neurons fail to survive. An alternative explanation that is consistent with recent data is that these neurons extinguish P2 gene expression and switch to transcribe a different receptor gene, resulting in a loss of LacZ+ cells. Recent genetic experiments suggest a mechanism of OR gene choice in which a cell selects one receptor gene but can switch at low frequency (Lewcock and Reed, 2004; Serizawa et al., 2003; B.M. Shykind et al., personal communication). Expression of a functional receptor elicits a signal that suppresses switching and stabilizes the choice of odorant receptor. Inactive P2

neurons might be preferentially insensitive to this mechanism and extinguish P2 gene expression, resulting in a striking diminution in P2 neurons that results from receptor switching rather than cell death.

Thus, suppression of spike firing and synaptic release under competitive circumstances could result in a striking growth and survival advantage for active neurons when compared with the subpopulation of inactive cells. Alternatively, if switching is the source of the diminution of inactive neurons, then receptor stabilization may be inoperative in inactive neurons in a competitive environment. The simplest interpretation of these data is that both synapse stability and neural survival depend upon limiting factors whose elaboration or action is sensitive to neural activity or synaptic release. When all neurons are equally debilitated, there is no bias and no reward, and the neurons develop normally. Under competitive conditions, neurons that are deficient in synaptic release or spike activity suffer at the hands of the fitter wild-type cell population. In a competitive environment, silenced cells may be defective in a wide range of neuronal functions, including the stability of receptor expression, synaptic stability (Buffelli et al., 2003), axon growth and arborization (Goldberg et al., 2002; Meyer-Franke et al., 1998; Sretavan et al., 1988), and survival (Zhao and Reed, 2001).

Our results are reminiscent of the early observations of Hubel and Wiesel on the generation and maintenance of ocular dominance columns. The projection from the LGN to layer 4 of the primary visual cortex terminates in eye-specific ocular dominance columns (LeVay et al., 1975, 1978). Early physiological experiments, together with recent studies employing improved anatomical and physiological techniques, suggest that the development of ocular dominance columns involves two phases: an initial establishment phase that may utilize innate signals and a later, plastic phase, referred to as “the critical period,” that relies on activity-dependent processes (Crowley and Katz, 1999, 2000; Horton and Hocking, 1996; Katz and Crowley, 2002). This phase requires a competitive environment, since closure of both eyes during this critical period does not perturb column organization, whereas closing one eye vastly expands the cortical area responsive to the open eye (LeVay et al., 1980).

A more complex role for activity has been described in the lateral geniculate, the relay between retina and visual cortex. Early in development, the axons from the two eyes overlap but later segregate into discrete eye-specific layers (Godement et al., 1984). The segregation of retinal axons requires neural activity but occurs before the eyes open, implicating spontaneous firing of retinogeniculate afferents as an essential component in the layering process (Feller et al., 1996; Meister et al., 1991; Penn et al., 1998; Wong et al., 1995). Whereas neural activity is clearly required, it remains unclear whether spontaneous firing plays a permissive or instructive role in axon segregation. An instructive role for neural activity in the absence of visual input requires correlated spontaneous firing. In fact, correlated waves of activity characterize neighboring retinal ganglion cells, but the firing between the two eyes remains uncorrelated. In one study with immunotoxin depletion of amacrine cells, disruption of these correlated waves

without inhibiting the spontaneous firing of retinal ganglion cells did not perturb the patterned segregation of axons into eye-specific layers (Chapman, 2000). A different conclusion emerges from the analysis of a mouse mutant in the  $\beta 2$  subunit of the nicotinic acetylcholine receptor, in which inhibition of waves disrupts lamination (Rossi et al., 2001). These observations suggest that neural activity is essential for the appropriate segregation of axons, but its role as a permissive or instructive determinant remains controversial.

A role for correlated waves of retinal activity has been demonstrated in the refinement of the retinotopic map, both in the geniculate and in the projections to the superior colliculus. A nasotemporal gradient of EphA receptors on retinal axons, combined with overlapping gradients of two ephrins in the colliculus, governs the continuous spatial mapping of retinal ganglion cells (Feldheim et al., 2000; McLaughlin et al., 2003a). Genetic disruption of correlated retinal activity in mice that are deficient in the  $\beta 2$  subunit of the nicotinic acetylcholine receptor results in diffuse terminal arbors that disrupt the fine structure map (Grubb et al., 2003; McLaughlin et al., 2003b).

Our studies suggest that neural activity is likely to play a permissive role in the establishment and maintenance of the precise connectivity inherent in an olfactory sensory map. Whereas activity is necessary for the precision of the topographic map, it cannot instruct the spatial targeting of axons, a process likely to be mediated by a complex repertoire of developmentally programmed guidance molecules. Activity may at one level be necessary to allow a developmental program of axon migration to unfold. Once a sensory map is established, activity-dependent processes may serve to refine the connectivity and correct errors in the innate program, ultimately contributing to the striking precision of the topographic map. Finally, activity-dependent mechanisms might afford a plasticity not inherent in innate developmental programs that may strengthen salient connections to allow the olfactory system to adapt more effectively to a changing environment.

## Experimental Procedures

### Generation of Transgenic Mice and Mice with Targeted Alleles

DNA fragments containing the *tet<sub>o</sub>* promoter followed by an artificial intron and splice site, the tetanus toxin light chain-IRES-tau-lacZ (*TeTxLC-IRES-tau-lacZ*) or *Kir2.1-IRES-tau-lacZ* or *IRES-tau-lacZ* cassette, and an SV40 polyadenylation signal were separated from the vector by digestion at insert-flanking sites, gel purified, and microinjected into the pronuclei of fertilized eggs. These bigenic offspring between responsive founders and tTA/rtTA lines were fed with food pellets containing doxycycline (6 mg/g of food), as stated in the text and analyzed with X-gal. All of the transgenic mice used in this study were maintained in strict accordance with NIH and institutional animal care guidelines. For the generation of mice with targeted alleles, see the Supplemental Experimental Procedures at <http://www.neuron.org/cgi/content/full/42/4/553/DC1>.

### In Situ Hybridization

RNA in situ hybridization using digoxigenin-labeled probes was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993). See Supplemental Experimental Procedures at <http://www.neuron.org/cgi/content/full/42/4/553/DC1> for details.

## Electrophysiology

### Slice Preparation

Mice were killed by CO<sub>2</sub> asphyxiation followed by rapid decapitation. Olfactory epithelia and olfactory bulbs were dissected out in mouse artificial cerebrospinal fluid (mACSF) continuously aerated with 5% CO<sub>2</sub> and 95% O<sub>2</sub> (4°C). The epithelia were embedded in 5% low-melting agarose gel at 37°C, chilled on ice, then mounted to the Vibrotome specimen tray and sectioned into 300 μm slices.

### Extracellular Recording

Extracellular recording was conducted in a loose-patch configuration in the setting for patch clamp. Pipette solution was the same as the internal solution to achieve a shunting of the loose patch to allow for the recording of larger signals.

### Patch Clamp

Olfactory bulb mitral cells were identified through direct visualization under infrared DIC microscope (Olympus BX 50 WI). Input resistance after whole-cell configuration was between 50 and 100 MΩ. Bipolar tungsten electrodes were placed directly in the outer nerve layer of the olfactory bulb, above the mitral cells being patched.

### Local Field Potentials

Local field potentials (EOGs) were recorded from the surface of intact olfactory sensory epithelia. For details, see the Supplemental Experimental Procedures at <http://www.neuron.org/cgi/content/full/42/4/553/DC1>.

### FM1-43 Staining

FM1-43 staining was modified after Pyle et al. (1999). For details, see the Supplemental Experimental Procedures at <http://www.neuron.org/cgi/content/full/42/4/553/DC1>.

### X-gal Staining

The procedure is described in previous publication (Mombaerts et al., 1996). See the Supplemental Experimental Procedures at <http://www.neuron.org/cgi/content/full/42/4/553/DC1> for details.

### Fluorescent Immunohistochemistry

The procedure is described in previous publication (Gogos et al., 2000). For details, see the Supplemental Experimental Procedures at <http://www.neuron.org/cgi/content/full/42/4/553/DC1>.

### Whole-Mount Fluorescent Imaging

Animal heads of mice expressing *P2-IRES-GFP* or *MOR23-IRES-GFP* were bisected to expose the medial aspect of the olfactory bulb and the olfactory epithelium. The heads were chilled on ice and imaged with confocal microscope at 4× or 10× magnifications. Z-series images were collected at 5 μm intervals and collapsed to show the projection of receptor neurons. For *MOR28-IRES-GFP* mice, the whole mouse brain, with the olfactory bulbs, was taken out of the skull and laid on its dorsal side. The ventral aspect of the bulb was imaged with confocal microscope. Z-series were collapsed to show the projection. Following imaging, samples were fixed and stained with X-gal to confirm the expression of transgenes.

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