Cell, Vol. 117, 801–815, June 11, 2004, Copyright ©2004 by Cell Press

Gene Switching and the Stability of Odorant Receptor Gene Choice

Benjamin M. Shykind, S. Christy Rohani, Sean O'Donnell, Adriana Nemes, Monica Mendelsohn, Yonghua Sun, Richard Axel,* and Gilad Barnea
Department of Biochemistry
and Molecular Biophysics and
Howard Hughes Medical Institute
College of Physicians and Surgeons
Columbia University
New York, New York 10032

Summary

Individual olfactory sensory neurons express only a single odorant receptor from a large family of genes, and this singularity is an essential feature in models of olfactory perception. We have devised a genetic strategy to examine the stability of receptor choice. We observe that immature olfactory sensory neurons that express a given odorant receptor can switch receptor expression, albeit at low frequency. Neurons that express a mutant receptor gene switch receptor transcription with significantly greater probability, suggesting that the expression of a functional odorant receptor elicits a feedback signal that terminates switching (Lewcock and Reed, 2004; Serizawa et al., 2003). This process of receptor gene switching assures that a neuron will ultimately express a functional receptor and that the choice of this receptor will remain stable for the life of the cell.

Introduction

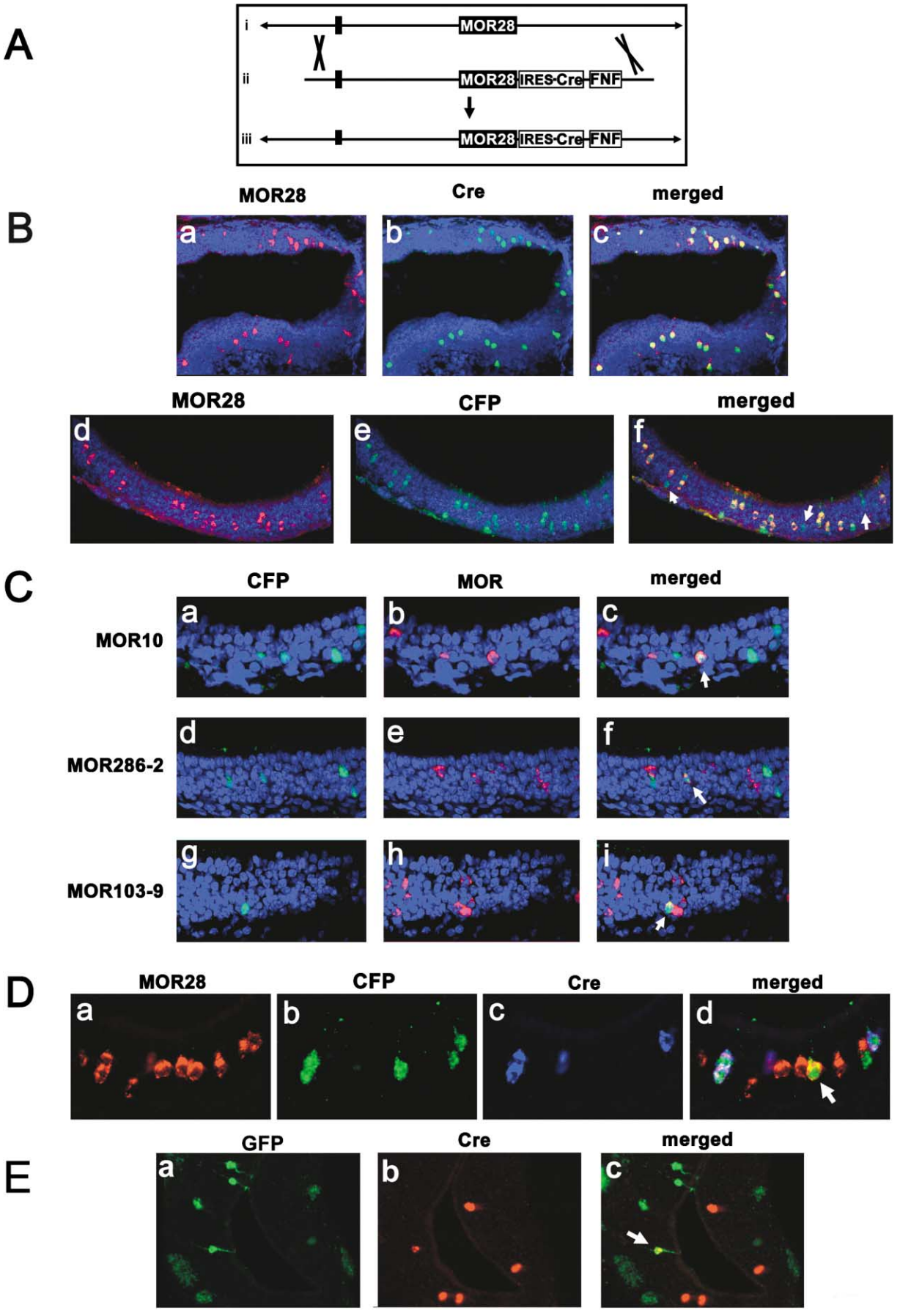
Olfactory perception requires both the recognition of a diverse repertoire of odorous molecules in the periphery and more central mechanisms that allow the discrimination of odors. In mice, the recognition of odors in the environment is accommodated by a large family of odorant receptor molecules that are encoded by an estimated 1500 genes (Young and Trask, 2002; Zhang and Firestein, 2002). The discrimination of olfactory information, however, requires neural mechanisms that are capable of distinguishing which of the numerous receptors have been activated by a given odorant. In mice, each of the two million olfactory sensory neurons expresses only one of the approximately 1500 odorant receptor genes, from only one allele (Chess et al., 1994; Malnic et al., 1999). Although randomly distributed in the epithelium within one of four broad but circumscribed zones, neurons expressing a given receptor project their axons with precision to two discrete synaptic structures, or glomeruli, within the olfactory bulb (Mombaerts et al., 1996a; Ressler et al., 1994; Vassar et al., 1994). The pattern of projections is spatially invariant, providing a two-dimensional representation of receptor activation in the brain. Imaging studies demonstrate that different odors elicit defined patterns of glomerular activation, revealing a functional representation of the anatomic map (Belluscio et al., 2002; Malnic et al., 1999; Rubin and Katz, 1999; Uchida et al., 2000; Wachowiak and Cohen, 2001). The quality of an odorant may therefore be reflected by different spatial patterns of activity, first in the olfactory bulb and ultimately in higher centers in the brain.

The observation that individual sensory neurons express only a single receptor gene is an essential feature of this model of olfactory perception. This immediately poses the question as to which mechanism has evolved to assure the expression of only one out of approximately 1500 genes in a sensory neuron. One model for the control of olfactory receptor (OR) expression invokes the existence of more than 1000 different cell types, each expressing a unique combination of regulatory factors that governs the choice of a different OR gene. This deterministic model predicts that all OR genes will contain different *cis*-regulatory sequences that are recognized by distinct sets of transcription factors. An alternative, stochastic model of receptor gene selection suggests that all odorant receptor genes within a zone contain the same *cis*-regulatory information and are controlled by the same set of transcription factors. In this model, a special mechanism must exist in order to assure that only one receptor gene is expressed from only one of the two alleles in a given neuron. However, once a specific receptor is chosen for expression, this transcriptional choice must be stable for the life of the cell, because receptor switching after stable synapse formation would seriously perturb odor discrimination.

Recent experiments that examine the expression of wild-type and mutant OR transgenes (Lewcock and Reed, 2004; Serizawa et al., 2003) suggest that the stable transcription of an OR gene is dependent on the expression of a functional receptor. Sensory neurons that express a transgene bearing a nonfunctional OR also transcribe other OR genes, whereas cells that express a wild-type receptor do not transcribe additional OR genes. Expression of a functional receptor is therefore likely to elicit a feedback signal that stabilizes OR gene choice. These data have led to the suggestion that the choice of a nonfunctional receptor gene leads to the coordinate expression of one mutant and one wild-type receptor in a single neuron, abrogating the "one receptor-one neuron" rule.

We have devised a different genetic strategy to examine the stability of wild-type and pseudogene OR choice. We have employed a lineage tracer to map the fate of sensory neurons that express either an intact or deleted MOR28 gene. We observe that switching is an inherent property of wild-type receptor gene choice. Immature neurons that express an intact MOR28 receptor but have not yet formed stable synapses in the brain can switch receptor choice, albeit at low frequency. Neurons that initially choose to express a mutant MOR28 receptor subsequently extinguish its expression and switch at high frequencies to express alternate receptors, such that a given neuron transcribes only a single receptor

*Correspondence: ra27@columbia.edu


A
i
MOR28
ii
MOR28
IRES-Cre
FNF
iii
MOR28
IRES-Cre
FNF
B
MOR28
Cre
merged
a
b
c
MOR28
CFP
merged
d
e
f
C
CFP
MOR
merged
MOR10
a
b
c
MOR286-2
d
e
f
MOR103-9
g
h
i
D
MOR28
CFP
Cre
merged
a
b
c
d
E
GFP
Cre
merged
a
b
c

gene. These observations suggest that the expression of a functional odorant receptor is likely to signal the termination of switching. This mechanism assures that all neurons will ultimately express a functional receptor and that the choice of this receptor will remain stable for the life of the cell. This provides a solution to the "pseudogene problem" and has implications for the mechanisms assuring the singularity of receptor gene choice and monoallelic OR gene expression.

Results

The Stability of Receptor Gene Choice

We have developed a gene targeting strategy that allows us to examine the stability of receptor gene choice over the life of an olfactory sensory neuron. A construct containing an internal ribosome entry site (IRES) driving the translation of Cre recombinase was introduced into the 3′ untranslated region of the MOR28 receptor gene by gene targeting in mouse ES cells to generate recombinant animals (Figure 1A). These mice were then crossed with strains bearing the reporter Rosa-loxP-stop-loxP-CFP (Srinivas et al., 2001). In double mutant strains, cells that express MOR28 will also express Cre, which will direct loxP-mediated recombination and CFP expression. Since Cre-mediated recombination is irreversible, CFP expression will persist even if MOR28 expression is extinguished, and, therefore, CFP serves as a lineage tracer for all cells that have chosen this OR at any time in their life.

MOR28 mRNA is one of the most abundant odorant receptor RNAs detected in the epithelium (J. Young, personal communication) and is expressed in about 10% of the neurons in zone I, the most ventral zone of the epithelium (Tsuboi et al., 1999). MOR28 expression in the sensory epithelium of mice homozygous for the MOR28-IRES-Cre allele was detected using an antibody directed against an intracellular epitope of the MOR28 receptor (Barnea et al., 2004). Double staining with antibody to MOR28 and Cre reveals that all cells that express MOR28 receptor also express Cre, and all Cre^+ cells are also $MOR28^+$ ($n > 5000$) (Figures 1Ba–1Bc). Cre-mediated recombination is highly efficient in neurons expressing the modified MOR28 allele, since all $MOR28^+$ cells also express CFP ($n > 2500$) (Figures 1Bd–1Bf). Approximately 90% of the CFP^+ neurons are also $MOR28^+$, indicating that, once the MOR28 allele is chosen, its expression is stable for the life of the majority of neurons. However, roughly 10% of the CFP^+ neurons no longer express MOR28 receptor (Figures 1Bd–1Bf), and we have examined the fate of this population of cells.

Several independent experiments indicate that these cells have extinguished MOR28 expression and have switched to express different receptor genes. First, we used antibodies directed against CFP together with in situ hybridization using RNA probes for ten receptor genes expressed in zone I of the epithelium. CFP^+ cells express one of the ten receptor genes at frequencies ranging from 0.05% to 0.2% (Figure 1C). Hybridization with probes to receptors expressed outside of zone I is never observed in CFP^+ cells (data not shown). If 10% of the $MOR28^+$ CFP^+ cells extinguish MOR28 expression, and these cells are capable of switching to any of the estimated 100 receptors expressed in zone I, we would expect a given receptor probe to hybridize to 0.1% of the CFP^+ cells. This value is in accord with our observations suggesting that extinction of MOR28 results in switching to the repertoire of receptors permitted in zone I.

In a second experiment, we asked whether cells that have extinguished expression of the MOR28-IRES-Cre allele can switch to the unmodified MOR28 allele in a heterozygote animal (Figure 1D). Immunofluorescent staining with antibodies directed against CFP was coupled with double in situ hybridization using RNA probes against MOR28 and Cre in mice bearing the MOR28-IRES-Cre and Rosa-loxP-stop-loxP alleles. Approximately 90% of the CFP^+ neurons also express Cre. The CFP^+ Cre^- neurons presumably reflect cells that have extinguished expression of the MOR28-IRES-Cre-allele. The detection of CFP^+ Cre^- cells that also express MOR28 would therefore identify neurons that have switched expression from the modified to the unmodified MOR28 allele. Roughly 10% of the CFP^+ Cre^- cells express the MOR28 receptor (Figures 1Da–1Dd), indicating that switching to the second MOR28 allele occurs with a frequency that approximates the frequency of MOR28 expression in zone I of wild-type animals.

Figure 1. Targeted Generation of MOR28-IRES-Cre Allele and Characterization of Expression Stability

(A) The targeted generation of MOR28-IRES-Cre allele. (Aa) Genomic locus of MOR28. (Ab) Targeting construct that generates bicistronic MOR28-Cre mRNA. FNF selection cassette contains neomycin resistance gene flanked by frt sites. (Ac) Genomic locus after homologous recombination.

(B) Stability of MOR28 choice examined in coronal sections through olfactory epithelium of homozygous MOR28-IRES-Cre mouse exposed to (Ba) anti-MOR28 anti-serum (red fluorescence) and (Bb) anti-Cre antibody (green fluorescence), and (Bc) merged signal from (Ba) and (Bb). Coronal sections (20 μm) through olfactory epithelium of homozygous MOR28-IRES-Cre mouse crossed to Rosa-loxP-stop-loxP-CFP line stained with (Bd) anti-MOR28 (red fluorescence) and (Be) anti-CFP (green fluorescence), and (Bf) merged signal of (Bd) and (Be), CFP^+MOR28^- cells shown with arrows. Nuclei were counterstained (blue fluorescence) with Toto-3.

(C) Receptor switching demonstrated by fluorescent RNA in situ hybridization carried out with olfactory receptor riboprobes followed by immunohistochemistry with anti-CFP antibody performed on 15 μm coronal sections of MOR28-IRES-Cre, Rosa-loxP-stop-loxP-CFP mouse. (Ca, Cd, and Cg) CFP signal (green fluorescence). (Cb, Ce, and Ch) Fluorescent RNA in situ signal (red), and (Cc, Cf, and Ci) merged signals, double positives shown with arrows. Nuclei were counterstained (blue fluorescence) with Toto-3.

(D) Interallele switching shown by double RNA fluorescent in situ hybridization followed by immunohistochemistry with anti-CFP antibody on 15 μm coronal sections of MOR28-IRES-Cre, Rosa-loxP-stop-loxP-CFP mouse. (Da) MOR28 riboprobe (red), (Db) anti-CFP (green), (Dc) Cre riboprobe (blue), and (Dd) merged signal of (Da)–(Dc), $CFP^+Cre^-MOR28^+$ cell shown with arrow.

(E) Evidence of interallele switching in progress shown by RNA fluorescent in situ hybridization carried out with Cre-riboprobe, followed by immunohistochemistry with anti-GFP antibody on 15 μm coronal sections of MOR28-IRES-Cre/MOR28-IRES-GFP mouse. (Ea) anti-GFP (green), (Eb) Cre riboprobe (red), (Ec) merged signal of (Ea) and (Eb). GFP^+Cre^+ cell shown with arrow.

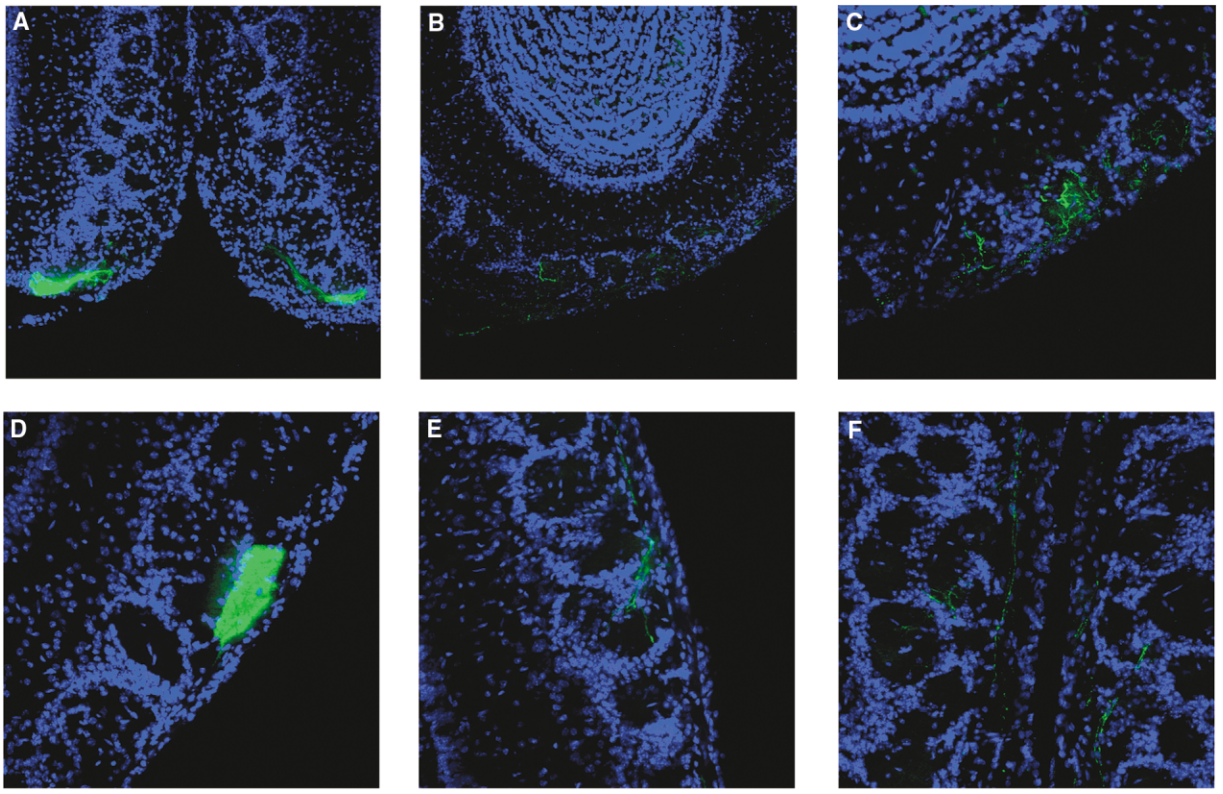


Figure 2. Innervation of Glomeruli by GFP-Marked Axons

Coronal sections through the olfactory bulb in MOR28-IRES-Cre mice crossed into the Z/EG lineage-marking line. Sections (20 μm) exposed to anti-GFP antibody (green fluorescence) and counterstained for nuclei with Toto-3 (blue fluorescence) show that fibers predominantly target symmetric ventral glomeruli (A) and aberrant innervation is shown (B and C). Coronal sections through the olfactory bulb in MOR28-IRES-GFP. Sections (20 μm) exposed to anti-GFP antibody (green fluorescence) and counterstained for nuclei with Toto-3 (blue fluorescence) show that fibers predominantly target ventral glomerulus (D), aberrant innervation is shown (E and F).

In a third experiment, we exploited the observation that neurons expressing a given receptor converge on one medial and one lateral glomerulus within the olfactory bulb (Mombaerts et al., 1996b; Ressler et al., 1994; Vassar et al., 1994). Mice bearing the MOR28-IRES-Cre allele were crossed to the reporter line Z/EG, which expresses GFP at high levels upon Cre-mediated activation (Novak et al., 2000). Most GFP^+ fibers converge on two glomeruli within the bulb (Figure 2A); however, a small but significant number of GFP^+ fibers are also observed in approximately 20 ventral glomeruli (Figures 2B and 2C). We presume that GFP^+ axons that project diffusely reflect neurons that have extinguished MOR28 and have switched to express one of several alternate receptors that direct axonal projections to their cognate glomeruli. Adult mice bearing a MOR28-IRES-GFP allele project to one medial and one lateral glomerulus with no stray fibers observed (data not shown). These data suggest that 90% of the neurons that have chosen MOR28 continue to stably transcribe this gene for the life of the cell. However, 10% of these neurons extinguish MOR28 expression, switch to a second receptor, and innervate the glomeruli appropriate for this receptor choice.

An alternative interpretation of these data posits that the CFP^+ neurons that do not express MOR28 arose through low-level aberrant expression of Cre rather than from the high-level expression that arises from the normal expression of the MOR28-IRES-Cre allele. We have performed two experiments that render this interpretation unlikely. First, we asked whether we could identify neurons in the process of switching. Cells that are in the act of switching from the MOR28-IRES-GFP allele to a second OR gene should contain high levels of GFP along with nascent mRNA encoding the second receptor. We therefore examined olfactory sensory neurons from mice bearing both an MOR28-IRES-Cre and an MOR28-IRES-GFP allele in the absence of a Cre-responsive reporter gene. Neurons that expressed the MOR28-IRES-GFP allele at a high level were identified with antibodies to GFP. This population of GFP^+ cells was analyzed for the expression of a second receptor by RNA in situ hybridization using probes either against Cre or against four receptors expressed in zone I.

In three-week-old mice bearing both an MOR28-IRES-Cre and an MOR28-IRES-GFP allele, we observe 11 cells that express high levels of both GFP and Cre RNA (Figure 1E, $n \approx 10{,}000$ cells). We observe a similar number of GFP^+ cells that are positive in in situ hybridization experiments with a pool of antisense RNAs against four receptors expressed in zone I (data not shown, $n = 12{,}294$ cells). These double-positive cells presumably reflect differentiating neurons in the process of receptor switching. No double-positive neurons are observed in

adult mice in which the fraction of differentiating neurons is far smaller (data not shown, n = 11,892 cells)

The frequency of MOR28-IRES-GFP-expressing cells in the act of switching is low but not surprising. The process of switching is likely to be restricted to a narrow window in developmental time (see below). Thus, the frequency of GFP^+ cells expressing a second receptor is predicted to be far lower than the frequency of switching observed using the lineage tracer, which reports all cells that have switched during their lifetime. If 10% of the MOR28-IRES-GFP cells switch during their lifetime, then each of the estimated 100 zone I receptors should be expressed in 0.1% of the GFP^+ cells. Only a small subset of these cells will be observed as double-positive neurons actively engaged in the switching process. Of the ~15,000 $MOR28^+$ neurons in the three-week-old sensory epithelium, we observe about ten cells positive for both GFP and a second receptor, a value in accord with our estimates of switching frequency. A similar frequency was reported for the expression of the endogenous MOR28 gene in neurons also expressing an MOR28 transgene (Serizawa et al., 2000).

Independent experiments that examine aberrant axonal targeting provide further evidence for a switch in receptor choice in neurons expressing functional receptors. The vast majority of GFP^+ fibers in mice with an MOR28-IRES-GFP allele target to a single medial and lateral glomerulus in the olfactory bulb (data not shown and Figure 2D). However, several stray GFP^+ fibers are also observed entering multiple glomeruli on the ventral and ventromedial aspects of the bulb in young animals. (Figures 2E and 2F). Stray fibers reflect cells that express high levels of the MOR28-IRES-GFP allele, since low levels of GFP would not permit visualization of single axons. Aberrant targeting is likely to result from switching to a second receptor that now dictates the projection to its cognate glomerular locus. Taken together, these data provide evidence for switching at the MOR28 locus. Thus, lineage-tracing experiments indicate that 90% of the neurons that have chosen MOR28 continue to transcribe this gene for the life of the cell. Analysis of the 10% of the neurons that extinguish MOR28 expression suggest that these cells have switched to a second OR and innervate the glomeruli appropriate for this receptor choice.

The Stability of Receptor Choice Depends upon Expression of a Functional Receptor

Receptor gene switching affords a mechanism to assure that sensory neurons each express a functional odorant receptor. We have therefore examined the fate of cells that have chosen a mutated MOR28 allele that does not encode functional receptor. Mouse strains were generated by gene targeting that harbor an MOR28Δ-Cre allele, which contains a substitution of the coding region of MOR28 with the Cre recombinase gene (Figure 3A). This strain was crossed with mice bearing a Rosa-loxP-stop-loxP-CFP allele. Double staining with antibodies to the MOR28 receptor and CFP reveals that the frequency of CFP^+ cells in the sensory epithelium of these mice is about equal to the frequency of cells expressing the wild-type MOR28 allele (Figures 3Bb–3Bd and 3Bf–3Bh). Thus, cells choose the wild-type and mutant MOR28 alleles with roughly equal frequency.

We next asked whether this population of cells continues to express the MOR28Δ-Cre allele or switches to express a second receptor. The expression of the MOR28Δ-Cre allele was examined by staining with antibody directed against the Cre-recombinase. At 2 weeks, about 10% of the cells in zone I are Cre^+ (Figure 3Ba). This frequency drops to about 0.3% 12 weeks after birth (Figure 3Be). In contrast, the frequency of CFP^+ cells does not change over this time period (compare Figures 3Bb and 3Bc with 3Bf and 3Bg), indicating that the population of cells initially choosing the nonfunctional MOR28Δ-Cre allele persists in the epithelium. Control experiments reveal that the relative frequency of cells expressing the wild-type MOR28 allele remains unchanged between 2 and 12 weeks (Figures 3Bc and 3Bg). Thus, cells that have initially chosen to express the MOR28Δ-Cre allele subsequently extinguish its expression.

Additional experiments indicate that the CFP^+ cells express alternate OR genes. First, we used antibodies against MOR28 and CFP to determine the frequency with which cells from mice heterozygous for the MOR28Δ-Cre allele switch to express the wild-type MOR28 allele. The frequency of CFP^+ cells that are also $MOR28^+$ is approximately 10% (Figure 4A), a value roughly equal to the frequency of $MOR28^+$ cells in zone I in the wild-type animal. Thus, the frequency with which cells switch to the unmodified MOR28 allele is equivalent to the frequency with which neurons initially choose this gene.

We next stained with antibody against CFP together with in situ hybridization using RNA probes to ten receptor genes to examine the fate of cells that have expressed the MOR28Δ-Cre allele. CFP^+ cells express one of the ten receptor gene probes at frequencies ranging from 0.2% to 10%, five of which are shown (Figure 4B). CFP^+ cells that express the unmodified MOR28 allele, for example, are observed at a frequency of 10% (Figures 4Ba–4Bc), a value in accord with the frequency of switching detected by immunohistochemistry. CFP^+ cells that express MOR10, a gene in the same genomic cluster as MOR28, are present at a frequency of 2% (Figures 4Bd–4Bf), a figure that approximates the representation of MOR10 in the epithelium. Thus, the frequency of receptor gene choice upon switching approximates the frequency with which any given receptor is chosen in zone I in the epithelium. CFP^+ cells express several other receptor genes including MOR83, also in the MOR28 cluster (Tsuboi et al., 1999), in addition to receptor genes located outside of this cluster (Figures 4Bg–4Br). In situ hybridization experiments with probes for receptor genes expressed in other zones reveal no expression in CFP^+ cells (data not shown). These experiments indicate that CFP^+ cells that have expressed the MOR28Δ-Cre allele extinguish expression of this gene and switch transcription to the repertoire of OR genes permitted in zone I of the epithelium.

If cells that once expressed the MOR28Δ-Cre allele switch to express one of many other receptor genes, we would expect that the projections from lineage-marked neurons would innervate multiple glomeruli. We observe that these cells project axons diffusely to numerous glomeruli that are largely restricted to the ventral region of the olfactory bulb (Figure 5A). Significant variation in the density of innervation of individual glomeruli is

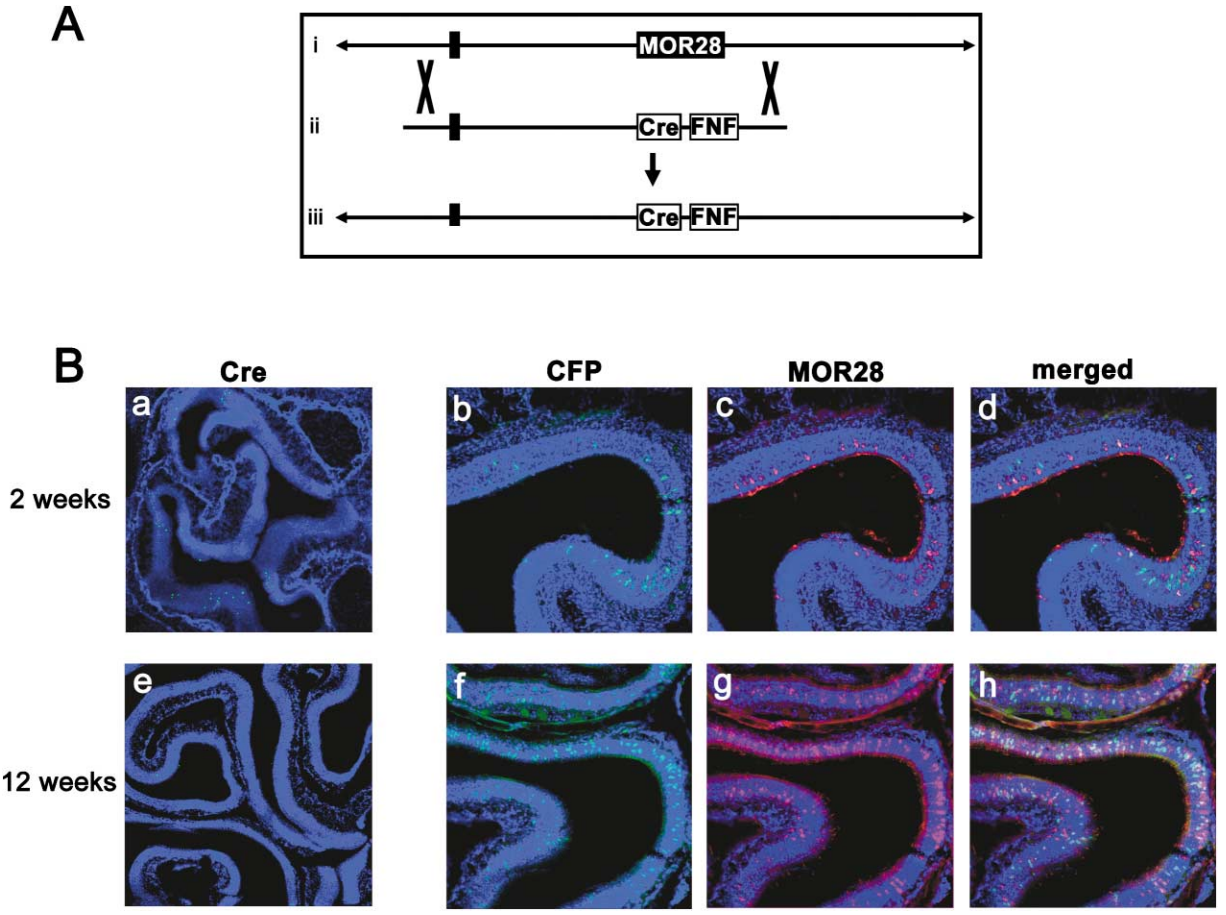


Figure 3. Generation and Fate of MOR28Δ-Cre Allele

(A) The targeted generation of MOR28Δ-IRES-Cre allele. (i) Genomic locus of MOR28. (ii) Targeting construct: deletes the MOR28 coding region and replaces it with Cre recombinase. FNF selection cassette contains neomycin resistance gene flanked by frt sites. (iii) Genomic locus after homologous recombination between targeter and genomic locus.

(B) Immunohistochemical analysis of olfactory epithelium of MOR28Δ-IRES-Cre mouse crossed into Rosa-loxP-stop-loxP-CFP reporter line at 2 weeks (Ba–Bd) and 12 weeks (Be–Bh). Coronal sections, 20 μm, exposed to anti-Cre antibody (Ba and Be) and anti-CFP antibody (Bb and Bf) in green fluorescence and stained with anti-MOR28 antiserum (Bc and Bg) in red fluorescence, and merged signals (Bd and Bh). Nuclei are counterstained with Toto-3 (blue fluorescence).

observed (Figures 5Aa–5Ac). Some glomeruli contain relatively few GFP$^+$ fibers, whereas others are more richly innervated, suggesting that, upon switching, different receptors are chosen with different frequencies. Further, in animals heterozygous for the MOR28Δ-Cre allele, we observe that CFP$^+$ fibers innervated the wild-type MOR28 glomerulus as well as a number of neighboring glomeruli (Figures 5Ad–5Af). The high density of innervation of the wild-type MOR28 glomerulus is consistent with the observation that the MOR28Δ allele switches frequently to the other MOR28 allele. These experiments indicate that an MOR28Δ-Cre allele, encoding a nonfunctional receptor, is chosen for expression at a frequency equivalent to that of the wild-type allele; however, its expression is extinguished and receptor switching is observed in the vast majority of these neurons. The observation that switching occurs at high frequency in cells expressing the mutant MOR28 allele suggests that a functional receptor is required for stable receptor gene expression.

Switching at the P2 Locus

We have previously generated mice that express a modified P2 allele, P2Δ-IRES-tau-lacZ, which contains a deletion of the entire P2 receptor coding region (Wang et al., 1998). At birth, the frequency of cells transcribing this mutant P2 allele is roughly equal to the frequency of cells expressing a functional P2-IRES-tau-lacZ allele. However, the axons from neurons expressing this mutant P2 allele fail to converge on discrete glomerular targets and project diffusely within the olfactory bulb. Moreover, the number of lacZ$^+$ cells diminishes dramatically over time such that, by ten weeks, less than 1% of the lacZ$^+$ neurons present at birth remain in the epithelium. One interpretation of these findings is that neurons that fail to express a functional receptor ultimately die. An alternative explanation consistent with the current data is that neurons expressing the mutant P2 allele switch to transcribe a different receptor from the repertoire of olfactory genes expressed in its zone. If receptor choice upon switching is random, then lacZ$^+$ neurons

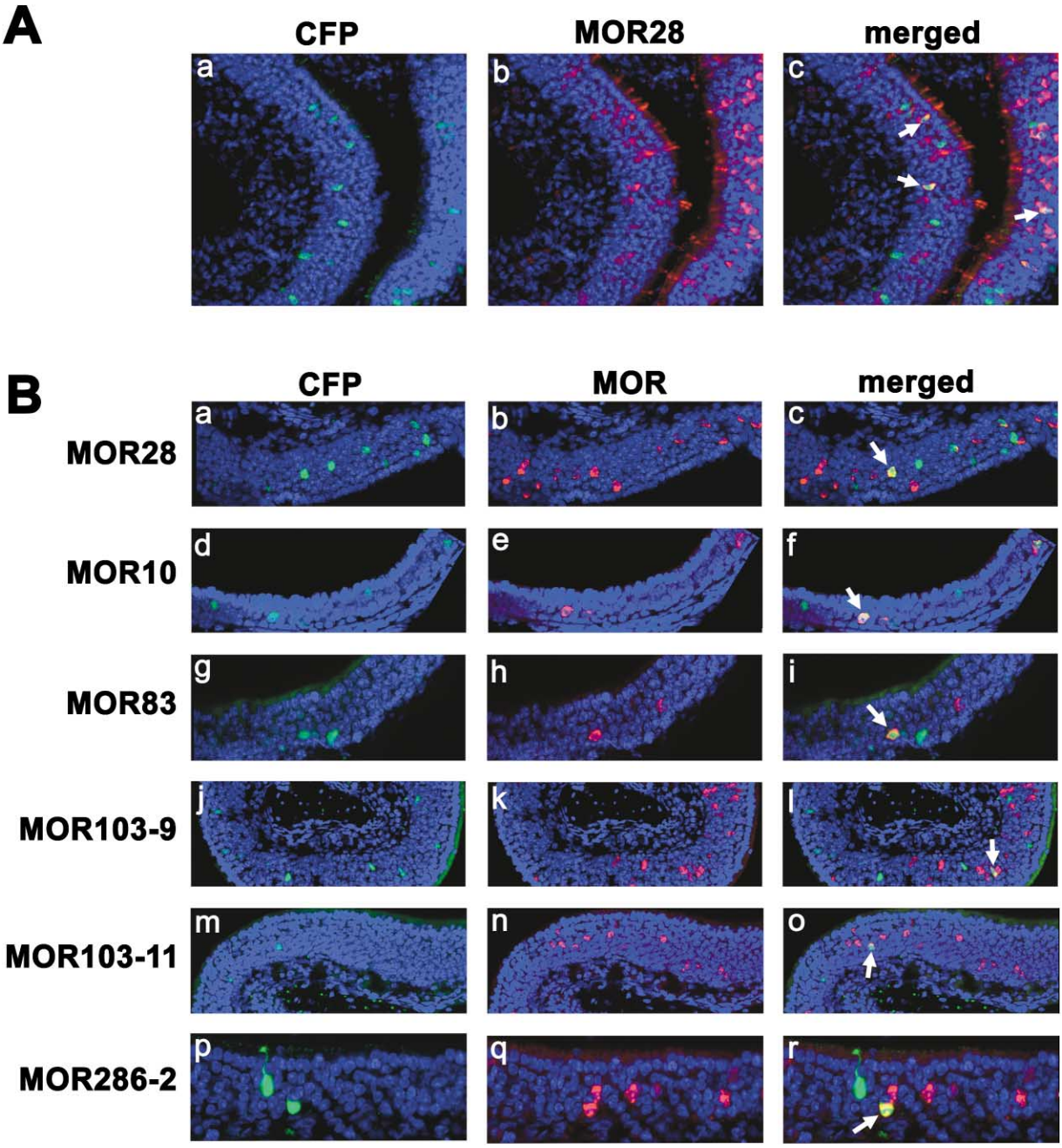


Figure 4. Neurons that Choose MOR28Δ-Cre Allele Switch to Express Other Receptors

(A) Immunohistochemical analysis of olfactory epithelium of MOR28Δ-IRES-Cre mouse crossed into Rosa-loxP-stop-loxP-CFP reporter line shows inter-allele switching. Coronal sections (20 μm) exposed to (Aa) anti-CFP antibody (green fluorescence) and (Ab) anti-MOR28 antiserum (red fluorescence) and (Ac) merged signals from (Aa) and (Ab), $MOR28^{+}CFP^{+}$ cells shown with arrows. Nuclei are counterstained with Toto-3 (blue fluorescence).

(B) RNA in situ hybridization followed by immunohistochemistry carried out on olfactory epithelium of MOR28Δ-IRES-Cre mouse crossed into Rosa-loxP-stop-loxP-CFP reporter line. Coronal sections of 15 μm (panels Ba, Bd, Bg, Bj, Bm, and Bp) exposed to anti-CFP antibody (green fluorescence) and probed with assorted odorant receptor riboprobes shown by red fluorescence (panels Bb, Be, Bh, Bk, Bn, and Bq). Signals from in situ and antibody staining merged in last column of panels (Bc, Bf, Bi, Bl, Bo, and Br), double positives shown with arrows.

might choose all receptors permitted within zone III and project to multiple glomeruli.

We have therefore analyzed the neurons expressing the P2Δ-IRES-tau-lacZ allele to discern whether receptor switching or cell death is responsible for the observed phenotype. We first asked whether neurons expressing the P2Δ-IRES-tau-lacZ allele undergo apoptotic cell death by performing TUNEL assays on the sensory epithelium at 3 weeks (Figure 6). As a positive control for our TUNEL assays, a small segment of the

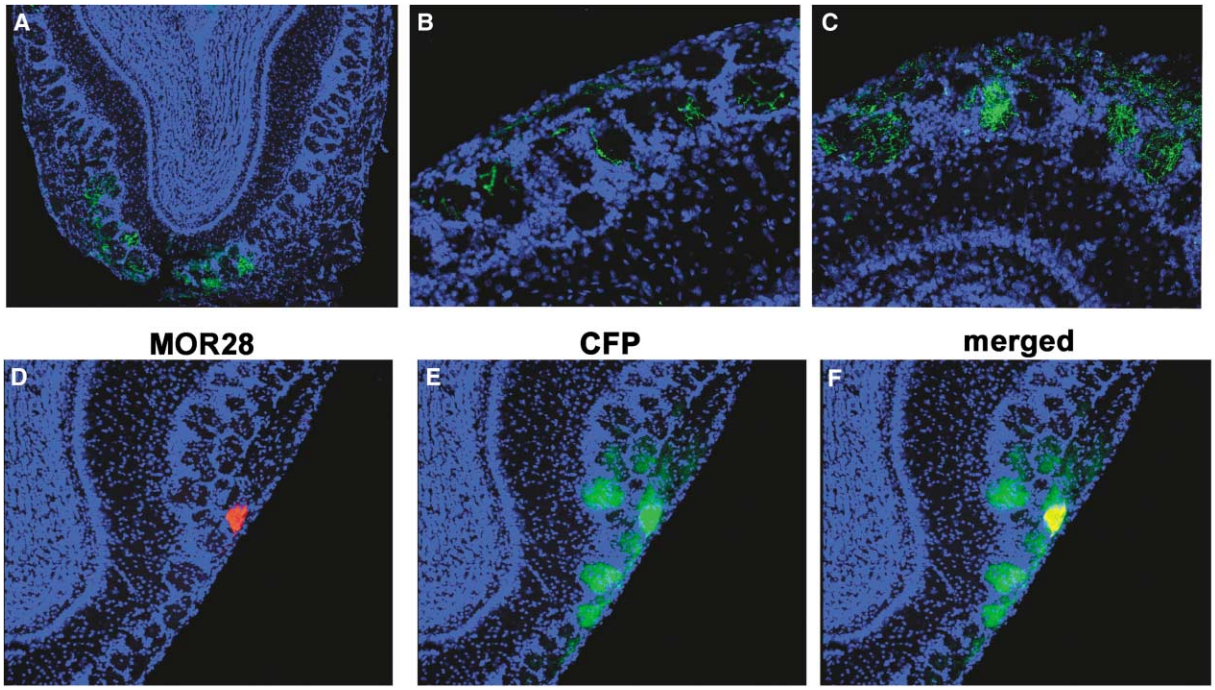


Figure 5. Innervation of Olfactory Bulb by Lineage-Marked Axons in MOR28Δ-Cre Mice

Coronal sections of the olfactory bulb in MOR28Δ-Cre mice crossed into the Z/EG lineage-marking lines. Sections (20 μm) exposed to anti-GFP antibody (green fluorescence) and nuclei were counterstained (blue fluorescence) with Toto-3 (A–C). Analysis of coronal sections of the olfactory bulb in MOR28Δ-Cre mice crossed to the Rosa-loxP-stop-loxP-CFP reporter line. (D) Stained with the anti-MOR28 antibody (red fluorescence) and (E) exposed to anti-CFP antibody (green fluorescence) and (F) merged signals from (D) and (E).

olfactory nerve from a P2Δ-IRES-tau-lacZ mouse was unilaterally severed, a procedure that elicits sensory neuron death (Holcomb et al., 1995). Under these conditions, apoptotic cells are observed 24 hr after surgery only on the axotomized side of the epithelium (Figure 6A). Immunohistochemistry for lacZ, combined with TUNEL on the contralateral side, does not reveal evidence of apoptosis in the population of cells expressing the P2Δ-IRES-tau-lacZ allele (Figure 6B). Thus, cells expressing the P2Δ-IRES-tau-lacZ allele do not appear to undergo apoptosis at frequencies greater than neurons expressing wild-type receptor genes.

We next asked whether neurons transcribing the mutant P2 allele switch receptor expression. We first crossed mice bearing P2Δ-IRES-tau-lacZ with mice containing a P2-IRES-GFP allele (Gogos et al., 2000) to generate compound heterozygotes. We observe GFP expression in lacZ$^+$ cells at a frequency of 0.2% (Figures 7A and 7B). These observations are consistent with a mechanism in which lacZ$^+$ cells have extinguished transcription of P2Δ-IRES-taulacZ allele and switch to express the P2-IRES-GFP allele at frequencies roughly equal to the frequency with which this gene is chosen in the epithelium. This interpretation is in accord with the demonstration that 5% of the cells expressing lacZ protein in P2Δ-IRES-tau-lacZ mice no longer express lacZ mRNA as revealed by coupled in situ hybridization-immunostaining (Figure 7H). This suggests that lacZ protein persists after the shutoff of transcription of the deletion allele and is consistent with the long half-life of the protein (C. Yu, personal communication). As a control, in compound heterozygotes bearing the P2-IRES-GFP and P2-IRES-tau-lacZ alleles, we have not observed double-positive cells. The low frequency of cells expressing P2, compared to the number expressing MOR28, is likely to preclude the detection of P2 cells in the process of switching to the second P2 allele. This not withstanding, these data suggest that switching is a far more frequent event in cells that choose a nonfunctional receptor (Figures 7D and 7E).

We have also performed in situ hybridization with RNA probes for five receptors expressed in zone III together with antibody staining for lacZ in mice bearing the P2Δ-IRES-taulacZ allele. LacZ$^+$ cells express zone III receptors at frequencies of about 0.2% (Figure 7G). We also examined the pattern of axonal projections from neurons expressing the P2Δ-IRES-tau-lacZ allele and observed that, at 3 weeks, lacZ$^+$ fibers enter multiple glomeruli, including the wild-type P2 glomerulus (Figure 7C), consistent with the interpretation that these neurons extinguish P2Δ expression and switch to a large repertoire of receptors. This pattern of innervation is in contrast to that of compound heterozygous mice expressing both P2-IRES-tau-lacZ and P2-IRES-GFP alleles in which tight convergence of lacZ$^+$ and GFP$^+$ fibers is seen and rare fibers innervating aberrant glomeruli are observed (Figure 7F; data not shown; Royal and Key, 1999; Wang et al., 1998).

We have asked when the P2Δ-IRES-tau-lacZ allele is extinguished during the differentiation of the sensory neuron. Neurons are generated in the basal layer of the epithelium throughout the life of the animal and migrate

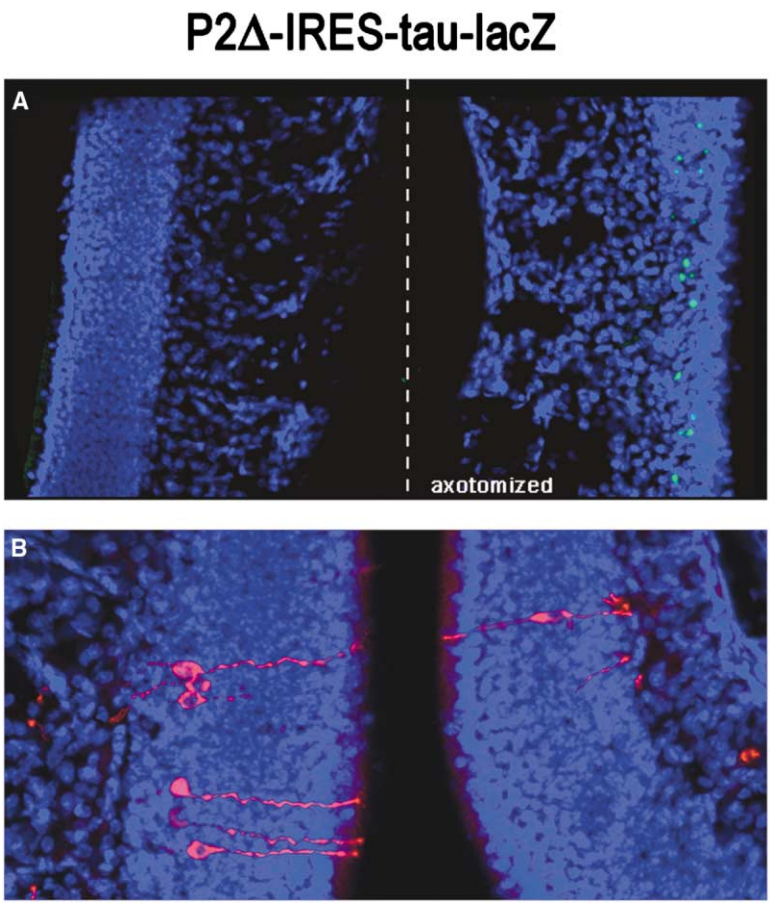


Figure 6. TUNEL Analysis of P2Δ-IRES-tau-lacZ$^+$ Sensory Neurons

Unilateral axotomy (denoted by dotted line in [A]) was performed on mice bearing the P2Δ-IRES-taulacZ allele. (A and B) TUNEL assay (green fluorescence) coupled with anti-lacZ immunohistochemisty (red fluorescence) was carried out 24 hr post surgery on 20 μm coronal sections of olfactory epithelia. Nuclei were counterstained (blue fluorescence) with Toto-3.

apically as they mature. The olfactory marker protein (OMP) is expressed in mature olfactory neurons that reside in the apical layers of the olfactory epithelium (Farbman and Margolis, 1980) and is expressed after sensory axons have reached their glomerular targets (Iwema and Schwob, 2003; Schwob et al., 1999). We therefore performed in situ hybridization followed by immunohistochemistry to GFP, in mice heterozygous for P2Δ-IRES-tau-lacZ and bearing a modified allele of OMP, which coexpresses GFP (OMP-IRES-GFP, see Experimental Procedures), with RNA probes to lacZ and P2 coding region. At 18 days of age, approximately 72% of neurons expressing the unmodified P2 allele were found in the OMP$^+$, apical layer of the epithelium (Figure 7I). In contrast, only 20% of cells expressing the P2Δ-IRES-tau-lacZ allele were located in the in the OMP$^+$ layer (Figure 7J). Thus, the extinction of the nonfunctional P2Δ-IRES-tau-lacZ allele largely occurs early, before OMP expression in the epithelium. These data are consistent with those obtained for the MOR28Δ-Cre allele, which showed a similar pattern of shutoff (data not shown).

The Instability of Pseudogene Expression

In the mouse, 340 of the estimated 1500 odorant receptor gene sequences are pseudogenes, and several of these are transcribed (Young et al., 2002, 2003). If the expression of a large number of pseudogenes were to persist, a significant subpopulation of sensory neurons would be nonfunctional. The observations that neurons expressing nonfunctional MOR28 and P2 alleles extinguish mutant gene expression and switch to express a functional receptor suggest that a similar process will accompany the choice of a naturally occurring pseudogene. We have examined this prediction by performing RNA in situ hybridization with an expressed pseudogene, MOR135-15. At 3 weeks, the expression of the pseudogene is zonally restricted and is observed in about 0.2% of the neurons (Figure 8A). By 12 weeks of age, however, pseudogene expression drops in frequency to about 10% of that observed in the young epithelium (Figure 8B). In contrast, the frequency of neurons expressing a functional receptor (M50) is maintained (Figures 8C and 8D). Thus, neurons that express a pseudogene shut off its expression, and the epithelium is depleted of cells that express it within the first few months of life.

In previous experiments, we observed switching in neurons marked by the expression of genetically modified OR alleles. It is possible that modification at the OR loci inadvertently results in the misregulation of the OR genes. The observations with pseudogenes provide a natural scenario that recapitulates the extinction of nonfunctional OR genes. Thus, neurons that choose to express a pseudogene will, early in their life, extinguish expression and presumably switch until a functional odorant receptor gene is selected. This mechanism would assure that neurons expressing nonfunctional re-

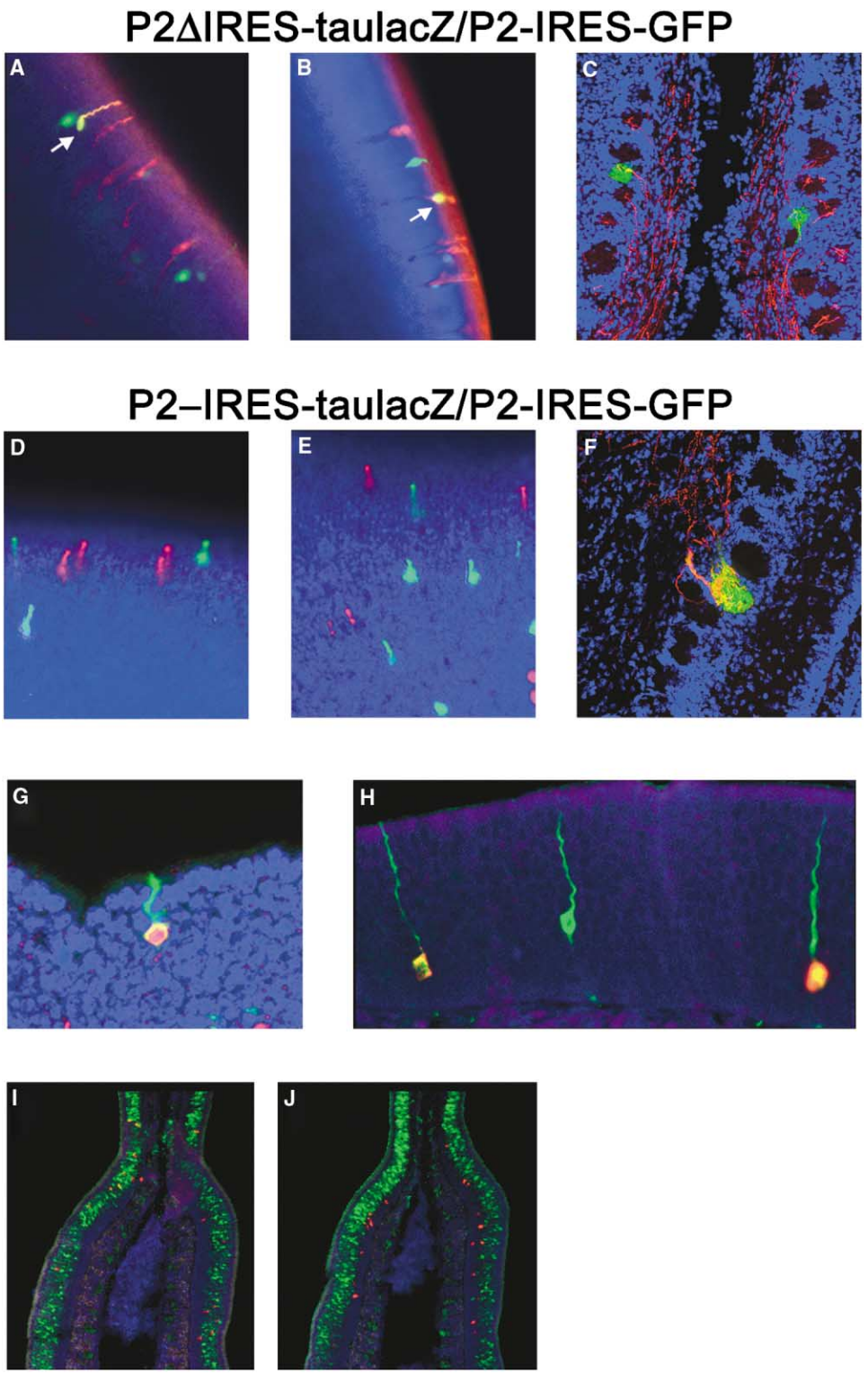


Figure 7. Characterization of Switching from P2Δ-IRES-taulacZ Allele

Whole mount immunohistochemistry on compound heterozygous epithelia. (A and B) Epithelium of P2Δ-IRES-taulacZ/P2-IRES-GFP mouse double stained with anti-lacZ (red fluorescence) and anti-GFP antibodies (green fluorescence) and counterstained with Toto-3 (blue fluorescence) to visualize nuclei. $LacZ^{+}GFP^{+}$ cells are indicated with arrows. (D and E) Epithelium of P2-IREStaulacZ/P2-IRES-GFP mouse double stained with anti-lacZ (red fluorescence) and anti-GFP antibodies (green fluorescence) and counterstained with Toto-3 (blue fluorescence) to visualize nuclei. (C) Coronal section of olfactory bulb showing axons of P2Δ-IRES-taulacZ and P2-IRES-GFP expressing cells double stained with anti-lacZ (red fluorescence) and anti-GFP antibodies (green fluorescence) and counterstained with Toto-3 (blue fluorescence) to visualize nuclei. (F) Coronal section, 20 μm, of olfactory bulb showing axons of P2-IRES-taulacZ and P2-IRES-GFP expressing cells double stained with anti-lacZ (red fluorescence) and anti-GFP antibodies (green fluorescence) and counterstained with Toto-3 (blue fluorescence) to visualize

ceptor, incapable of recognizing odorant, would not persist in the sensory epithelium.

Discussion

Switching and the Stability of Receptor Gene Choice

An olfactory neuron in the mouse expresses only one of about 1500 odorant receptor genes. We have devised a genetic strategy that allows us to examine the stability of receptor expression. We observe that the majority of neurons that have chosen a specific receptor continue to express this receptor for the life of the cell. However, a subpopulation of cells switches receptor gene choice. Cells that initially transcribe a mutant receptor switch with far greater probability and ultimately stably express a functional receptor gene. These observations suggest a mechanism of OR gene choice in which a cell selects only one receptor allele but can switch at low frequency. Expression of a functional receptor likely elicits a signal that suppresses switching and stabilizes odorant receptor expression. Neurons that initially express a mutant receptor fail to receive this signal and switch genes until a functional receptor is chosen. This model of serial monogamy assures that neurons will express a single receptor throughout their life.

The coordinate expression of MOR28 receptor and Cre recombinase in our genetically modified mice results in a DNA recombination event that provides a lineage marker, allowing us to examine the stability of receptor choice and the fate of neurons initially choosing the modified MOR28 allele. Immature neurons expressing the wild-type MOR28 allele switch receptors at low frequency. In contrast, the vast majority of neurons expressing the MOR28 deletion allele extinguish expression of the mutant gene and ultimately choose a functional receptor. Recent studies have shown that cells expressing mutant OR transgenes also transcribe other receptors; however, these studies could not distinguish between coordinate expression of two receptor genes and switching (Lewcock and Reed, 2004; Serizawa et al., 2003). The introduction of lineage tracers allows us to map the fate of MOR28Δ-expressing cells and demonstrates the extinction of MOR28Δ expression. Moreover, this experimental approach also reveals that switching is inherent in the mechanism of receptor choice of wild-type OR genes. A similar lineage-tracing scheme has recently been employed to mark olfactory sensory neurons used in the generation of clonal mice through nuclear transfer (Li et al., 2004). These authors examined the stability of expression of the modified M71 OR allele, used in these studies, and failed to observe switching to the other M71 allele. However, due to the small number of cells examined and the low frequency at which M71 is chosen in the epithelium, it is unlikely that switching could have been detected.

This feedback model, in which expression of a functional odorant receptor suppresses switching to other OR genes, is reminiscent of one mechanism of allelic exclusion in T0 and B-lymphocytes. In pre-B cells, for example, immunoglobulin expression initiates with the rearrangement and transcription of the heavy chain gene. A functional rearrangement results in the surface expression of μ chain, in association with surrogate light chains. The formation of this pre-B cell receptor complex downregulates the expression of Rag-1 and Rag-2 recombinases via a cascade of tyrosine phosphorylation events and prevents subsequent rearrangement of the second allele to assure the expression of a single heavy chain per cell. The generation of a nonfunctional μ chain fails to form the pre-B cell receptor complex and allows continued recombination, enhancing the probability that a pre-B cell will ultimately express functional antigen receptor (Nemazee, 2000). Our data suggest that a conceptually similar circuit is operative in olfactory neurons, suppressing gene switching rather than DNA recombination.

A Time and a Place for Switching

Our data demonstrate that switching is inherent in the process of receptor gene choice. Switching must be restricted in time to a window early in the development of sensory neurons, prior to migration of axons to specific glomeruli in the olfactory bulb. Switching after a stable synapse is formed would perturb odor perception. Odorant receptor expression is observed in maturing postmitotic sensory neurons before their axons make contact with the brain (Sullivan et al., 1995). Receptor expression is followed by the transcription of OMP, a marker of more mature sensory neurons (Iwema and Schwob, 2003; Schwob et al., 1999). Expression of the mutant MOR28 and P2 genes is primarily observed in immature neurons that have not yet expressed OMP. These data imply that there is a critical window early in sensory neuron development during which switching can occur. This time window will assure that cells will have switched receptor expression prior to extensive axon outgrowth. Such a mechanism would prevent neurons from switching receptors after they form stable synapses, since this would confound odor discrimination by altering the identity of the odorant activating a specific glomerulus.

These observations are in accord with recent data that suggest that receptor choice is stabilized by a feedback signal elicited by functional receptor (Lewcock and Reed, 2004; Serizawa et al., 2003). We have no insight into the nature of the signal, but previous experiments

nuclei. (G) RNA in situ hybridization of coronal section of P2Δ-IRES-taulacZ epithelium followed by immunohistochemistry: mixed riboprobes (red fluorescence) and anti-lacZ antibody (green fluorescence) counterstained with Toto-3 (blue fluorescence). (H) Persistence of lacZ protein after shutdown of P2Δ-IRES-taulacZ allele. RNA in situ hybridization of coronal section through P2Δ-IRES-taulacZ heterozygote epithelium followed by immunohistochemistry: LacZ riboprobe (red fluorescence) and anti-lacZ antibody (green fluorescence) counterstained with Toto-3 (blue fluorescence). (I and J) Timing of P2Δ-IRES-taulacZ shutdown. RNA in situ hybridization of coronal sections through P2Δ-IRES-taulacZ heterozygote, OMP-IRES-GFP heterozygote epithelium followed by immunohistochemistry. (I) P2 coding region riboprobe (red) and anti-GFP antibody (green), counterstained with Toto-3 (blue fluorescence). (J) LacZ riboprobe (red) and anti-GFP antibody (green), counterstained with Toto-3 (blue fluorescence).

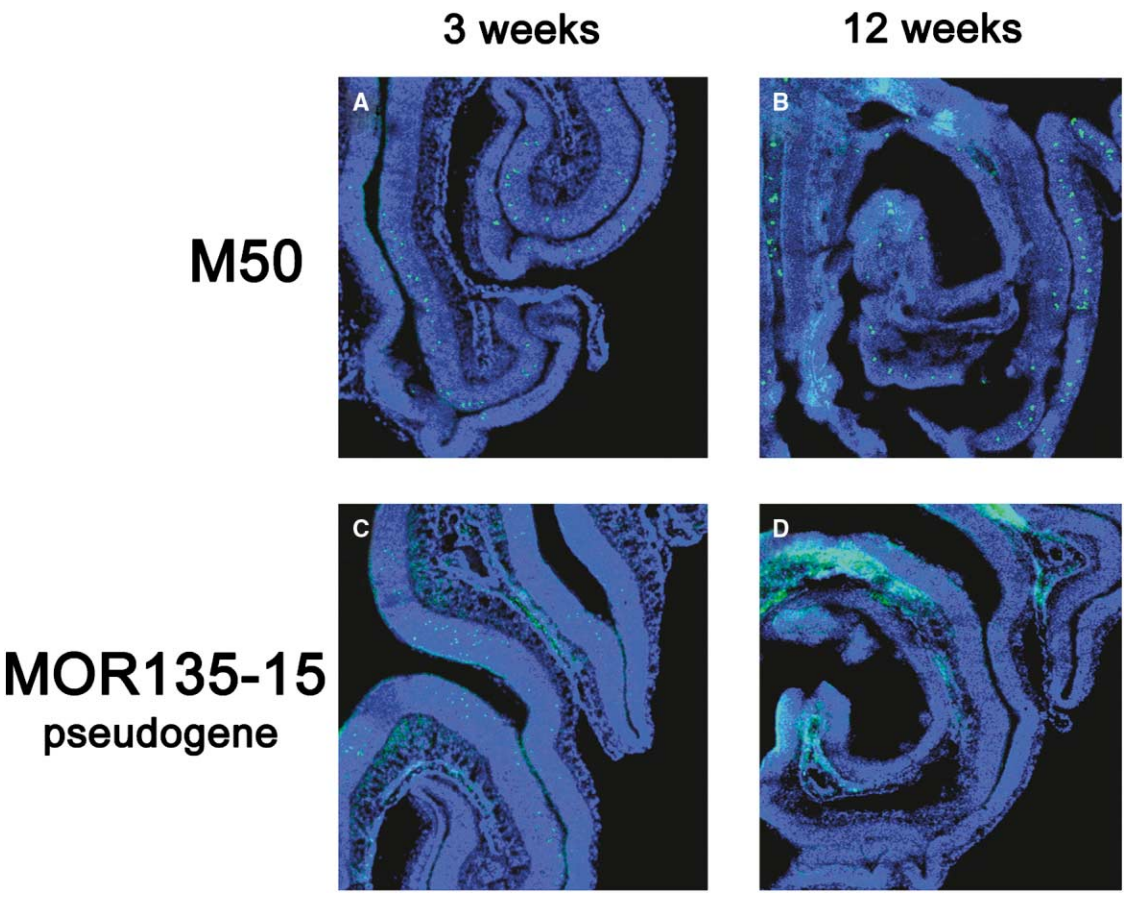


Figure 8. Extinction of Naturally Occurring Olfactory Receptor Pseudogene Expression

Fluorescent RNA in situ hybridizations (green fluorescence) carried out on 20 μm coronal sections of wild-type olfactory epithelia and counterstained with Toto-3 to visualize nuclei. Indicated riboprobes were used on 3-week-old ([A] and [C]) and 12-week-old ([B] and [D]) epithelia.

suggest that it may be mediated by receptor localized to axons rather than dendrites. Antibody staining reveals the presence of odorant receptor on dendrites, where they recognize odors, as well as on axons, where they may serve as guidance molecules (Barnea et al., 2004). Genetic disruption of either G_{olf} or the cyclic nucleotide-gated (CNG) ion channel, essential components that transduce receptor occupancy on dendrites, does not alter the stability of receptor expression (Belluscio et al., 1998; Brunet et al., 1996). In these mutants, expression of a P2-IRES-tau-lacZ allele is maintained, and $lacZ^{+}$ fibers innervate two glomeruli. Thus, it is likely that stabilization of receptor choice does not employ the transduction pathway activated by odorants on dendrites. However, it remains possible that receptor choice is stabilized by odor binding at the dendrite that activates an alternate signal transduction pathway. In this manner, olfactory experience could sculpt the repertoire of ORs. Alternatively, the feedback signal mediating stabilization of receptor choice could result from the activation of ORs on axon termini (Barnea et al., 2004)

Receptor Switching and the Pseudogene Problem

The mouse genome contains an estimated 1500 OR genes, of which 340 are pseudogenes, whereas the human genome contains 550 pseudogenes out of 900 identified ORs (Young and Trask, 2002; Zhang and Firestein, 2002). The rapid rate of evolutionary change in OR genes is reflected in the persistent transcription of a significant number of pseudogenes. If expression of pseudogenes is maintained, this would result in the generation of sensory neurons incapable of odor recognition. A mechanism that allows switching provides a solution to this "pseudogene problem." Switching in immature sensory neurons assures that another transcriptional opportunity is provided if pseudogenes are chosen, so that each neuron may ultimately express a functional receptor.

There is a second consequence of the switching process: the maintenance of a large repertoire of pseudogenes. The absence of a switching mechanism would result in a large frequency of nonfunctional olfactory neurons, a feature likely to be selected against over evolutionary time by pseudogene loss. Pseudogenes, however, provide a potential repository for the generation of new receptors that may be important in allowing a species to rapidly adapt to changes in the environment. For many organisms, smell is the primary sensory modality used to identify food, predators, and mates. The ability to recognize novel odors could be enhanced by a rapidly evolving repertoire of olfactory receptors, a process facilitated by a large collection of pseudogenes. The maintenance of such a reservoir of pseudogenes,

capable of gene conversion or reversion to generate functional receptor, may facilitate evolutionary adaptation. This OR gene repertoire provides an example of "evolvability" or the capacity to select for properties that afford rapid evolutionary change (Poole et al., 2003). Thus, the switching mechanism prevents the persistence of nonfunctional neurons and may also obviate the evolutionary drive to eliminate valuable pseudogenes.

The Problem of Monoallelism

An olfactory sensory neuron expresses a single OR gene from one of the two alleles (Chess et al., 1994; Malnic et al., 1999). Within an individual cell, monoallelic expression of a given OR may result from the inactivation of one of the two alleles. By analogy to X inactivation, the alleles of an OR gene may be differentially marked such that only one of the two may ever be chosen in an individual cell. In this scenario, OR allele choice would be random in a population of cells but nonrandom in an individual neuron. Our data suggest an alternative model in which both the maternal and paternal alleles of OR genes have the potential to be transcribed, but only one can be expressed at a given moment in time. We demonstrate that, in an individual neuron, switching can occur between the two alleles of an OR gene, indicating that both are transcriptionally competent.

Switching and the Mechanisms of Receptor Choice

The demonstration of receptor switching also provides insight into the mechanism by which cells express only a single OR gene. Two extreme models can be invoked to explain the singularity of receptor gene choice. One model argues that receptor expression is deterministic, that each OR gene contains distinct *cis*-regulatory sequences that are recognized by one of 1500 different combinations of transcription factors. A second model argues that receptor choice is stochastic, such that receptor genes within a zone share common *cis*-regulatory elements that are controlled by a single transcriptional apparatus that activates only one OR gene in each neuron.

Cells expressing a mutant OR switch and express the repertoire of OR genes permitted in a given zone. We observe that the frequency with which cells expressing the MOR28Δ allele switch to alternate OR genes is roughly equal to the frequency with which those genes are chosen in the epithelium. Thus, a cell expressing MOR28Δ retains the potential to express all other receptor genes allowed in its zone. This finding is most consistent with a stochastic model in which a common set of regulatory factors recognize *cis*-control features shared by all OR genes. This common regulatory machinery could be displaced from one receptor promoter and activate a second OR gene to accomplish switching. The stochastic choice of a single OR gene will result in the expression of the full repertoire of receptors by a limited set of regulatory factors shared by all olfactory sensory neurons.

One mechanism to assure the expression of only one receptor in a cell could involve DNA rearrangement events at a single active expression site for odorant receptor genes in the chromosome. We recently generated cloned mice by transfer of nuclei of postmitotic olfactory sensory neurons expressing the P2 receptor into oocytes (Eggan et al., 2004). These mice express the full repertoire of odorant receptors in a pattern indistinguishable from wild-type animals, demonstrating that irreversible genetic alterations do not accompany odorant receptor gene choice. Rather, we favor a model in which OR gene choice is accomplished by a rate-limiting transcriptional process and the stability of this choice is assured by feedback mechanisms mediated by the expression of a functional odorant receptor.

Experimental Procedures

Generation of Targeted Mutations

MOR28-IRES-Cre and MOR28-IRES-GFP

A genomic clone containing the MOR28 receptor was modified by recombinant PCR (Stratagene) to introduce an AscI site 50 base pairs downstream of the STOP codon. Into the AscI site, a cassette containing an IRES followed by nuclear Cre-Flip Recombinase Target (FRT)-Neo^R-FRT (IRES-nCre-FNF) was inserted (Dymecki, 1996). For MOR28-IRES-GFP, a cassette containing IRES-GFP followed by Angiotensin-Converting Enzyme-Cre-LoxP-Neo^R-LoxP (ACN) (Bunting et al., 1999) was introduced into the AscI site. Constructs were linearized using XhoI and electroporated into E14 129/SvEv ES cells (Hooper et al., 1987). Homologous recombinant clones were determined by using a 3′ external probe on Southern blots of EcoRV-digested ES cell genomic DNA, as described previously (Wang et al., 1998). Targeted clones were identified and injected into C57BL/6 blastocysts to produce chimeras that transmitted the mutant MOR28 allele through the germline. Mice are in a mixed (129 X C57BL/6) background.

MOR28Δ-Cre

A genomic clone containing the MOR28 receptor was modified by recombinant PCR (Stratagene), to remove the entire coding region and introduce an AscI site immediately after the START codon, 50 base pairs upstream of the STOP codon. Into the AscI site, a cassette containing nuclear Cre-Flip Recombinase Target (FRT)-Neo^R-FRT (IRES-nCre-FNF) (Mombaerts et al., 1996a) was inserted in frame with the MOR28 ATG (Dymecki, 1996). The construct was linearized using XhoI and electroporated into E14 129/SvEv ES cells (Hooper et al., 1987). Homologous recombinant clones were determined using a 3′ external probe on Southern blots of EcoRV-digested ES cell genomic DNA, as described previously (Wang et al., 1998). One targeted clone was identified and injected into C57BL/6 blastocysts to produce chimeras that transmitted the mutant MOR28 alleles through the germline. Mice are in a mixed (129 X C57BL/6) background.

OMP-IRES-GFP

A genomic clone containing the OMP receptor (Mombaerts et al., 1996a) was modified by reintroducing the NcoI flanked OMP coding region, and by introducing an AscI site directly downstream of the STOP codon by recombinant PCR (Stratagene). A cassette containing IRES-GFP followed by Angiotensin-Converting Enzyme–Cre–LoxP–Neo^R–LoxP (ACN) (Bunting et al., 1999) was introduced into the AscI site. The construct was linearized using NotI and electroporated into 30×10^6 129/SvEv ES cells (Hooper et al., 1987). Homologous recombinant clones were determined by using a 5′ external probe on Southern blots of HindIII-digested ES cell genomic DNA, as described previously (Wang et al., 1998). Targeted clones were identified, one of which was injected into C57BL/6 blastocysts to produce chimeras that transmitted the mutant OMP allele through the germline. Mice are in a mixed (129 X C57BL/6) background.

In Situ Hybridization

RNA probes used for in situ hybridization were labeled via in vitro transcription with T7 RNA polymerase (Roche) in the presence of digoxigenin or fluorescein-labeled dUTP. Olfactory turbinates were dissected, fixed overnight in 4% paraformaldehyde, and decalcified overnight in 0.5 M EDTA in 1X PBS. Turbinates were then frozen in OCT (Tissue Tek), and 15 μm coronal sections were collected on Superfrost slides (Fisher). Hybridizations were carried out as de-

scribed previously (Vassar et al., 1993), and fluorescent detection was performed using the TSA system (Perkin Elmer) according to the manufacturer's protocol. Images were acquired using a Bio-Rad MRC 1024ES confocal microscope.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Gogos et al., 2000). Anti-GFP/CFP was used at a dilution of 1:1000 (rabbit, Molecular Probes), anti-Cre 1:1000 (rabbit, Novagen), anti-lacZ 1:1000 (goat, BioGenesis), anti-MOR28 used at 1:4000 (guinea pig) (Barnea et al., 2004). Primary antibodies were visualized using Alexa 488 or Alexa 546 conjugated donkey anti-rabbit and donkey anti-goat (Molecular Probes) except for donkey anti-guinea pig Cy3 (Jackson Laboratories) as secondary antibodies. For immunohistochemistry following in situ hybridization, sections were first blocked in PBS with 0.1% Triton X-100 and 5% horse serum for 30 min followed by an incubation with a polyclonal rabbit α-GFP antibody (Molecular Probes) in the presence of NaN_3, 0.05%, overnight at 4°C. Subsequently, slides were incubated with an HRP (horse radish peroxidase)-conjugated secondary antibody (Chemicon) for 1 hr, and detection was performed using the TSA system (Perkin Elmer) according to the manufacturer's protocol. Stained sections were visualized using a Bio-Rad MRC 1024ES confocal microscope. TUNEL (Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End-labeling) assay was performed with in situ cell death detection kit (Roche Molecular Biochemicals), according to the manufacturer's protocol. Immunohistochemistry following TUNEL was performed as described above.

Surgery

Unilateral bulb axotomy was performed on 3-week-old C57BL/6 mice (Holcomb et al., 1995) with the following modifications: mice were anesthetized via intraperitoneal injection of 0.016 mg/g body weight of Avertin (1.77 mM 2,2,2-tribromoethanol and 2.5% tert-amyl alcohol dissolved in water). A sterile microprobe was used to sever a minimal number of axons transversing the cribiform plate. Mice were euthanized and dissected 24 hr postsurgery.

Acknowledgments

The authors would like to thank P.J. Kisloff for assistance in preparation of the manuscript. We thank T. Jessell, A. Fleischmann, and J. Montez for helpful discussions and critical reading of the manuscript. We also thank X. Sun for technical assistance; and C.R. Yu for helpful discussions. We are grateful to J. Young for providing OR probes. This work was supported by the Howard Hughes Medical Institute and a grant from the NIH, number 2P01CA23767 (R.A.).

Received: January 30, 2004
Revised: April 8, 2004
Accepted: May 20, 2004
Published: June 10, 2004

References

Barnea, G., O'Donnell, S., Mancia, F., Sun, X., Nemes, A., Mendelsohn, M., and Axel, R. (2004). Odorant receptors on axon termini in the brain. Science, in press.

Belluscio, L., Gold, G.H., Nemes, A., and Axel, R. (1998). Mice deficient in G(olf) are anosmic. Neuron *20*, 69–81.

Belluscio, L., Lodovichi, C., Feinstein, P., Mombaerts, P., and Katz, L.C. (2002). Odorant receptors instruct functional circuitry in the mouse olfactory bulb. Nature *419*, 296–300.

Brunet, L.J., Gold, G.H., and Ngai, J. (1996). General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. Neuron *17*, 681–693.

Bunting, M., Bernstein, K.E., Greer, J.M., Capecchi, M.R., and Thomas, K.R. (1999). Targeting genes for self-excision in the germ line. Genes Dev. *13*, 1524–1528.

Chess, A., Simon, I., Cedar, H., and Axel, R. (1994). Allelic inactivation regulates olfactory receptor gene expression. Cell *78*, 823–834.

Dymecki, S.M. (1996). Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. Proc. Natl. Acad. Sci. USA *93*, 6191–6196.

Eggan, K., Baldwin, K., Tackett, M., Osborne, J., Gogos, J., Chess, A., Axel, R., and Jaenisch, R. (2004). Mice cloned from olfactory sensory neurons. Nature *428*, 44–49.

Farbman, A.I., and Margolis, F.L. (1980). Olfactory marker protein during ontogeny: immunohistochemical localization. Dev. Biol. *74*, 205–215.

Gogos, J.A., Osborne, J., Nemes, A., Mendelsohn, M., and Axel, R. (2000). Genetic ablation and restoration of the olfactory topographic map. Cell *103*, 609–620.

Holcomb, J.D., Mumm, J.S., and Calof, A.L. (1995). Apoptosis in the neuronal lineage of the mouse olfactory epithelium: regulation in vivo and in vitro. Dev. Biol. *172*, 307–323.

Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987). HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature *326*, 292–295.

Iwema, C.L., and Schwob, J.E. (2003). Odorant receptor expression as a function of neuronal maturity in the adult rodent olfactory system. J. Comp. Neurol. *459*, 209–222.

Lewcock, J.W., and Reed, R.R. (2004). A feedback mechanism regulates monoallelic odorant receptor expression. Proc. Natl. Acad. Sci. USA *101*, 1069–1074.

Li, J., Ishii, T., Feinstein, P., and Mombaerts, P. (2004). Odorant receptor gene choice is reset by nuclear transfer from mouse olfactory sensory neurons. Nature *428*, 393–399.

Malnic, B., Hirono, J., Sato, T., and Buck, L.B. (1999). Combinatorial receptor codes for odors. Cell *96*, 713–723.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996a). Visualizing an olfactory sensory map. Cell *87*, 675–686.

Mombaerts, P., Wang, F., Dulac, C., Vassar, R., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996b). The molecular biology of olfactory perception. Cold Spring Harb. Symp. Quant. Biol. *61*, 135–145.

Nemazee, D. (2000). Receptor selection in B and T lymphocytes. Annu. Rev. Immunol. *18*, 19–51.

Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C.G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis *28*, 147–155.

Poole, A.M., Phillips, M.J., and Penny, D. (2003). Prokaryote and eukaryote evolvability. Biosystems *69*, 163–185.

Ressler, K.J., Sullivan, S.L., and Buck, L.B. (1994). Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. Cell *79*, 1245–1255.

Royal, S.J., and Key, B. (1999). Development of P2 olfactory glomeruli in P2-internal ribosome entry site-tau-LacZ transgenic mice. J. Neurosci. *19*, 9856–9864.

Rubin, B.D., and Katz, L.C. (1999). Optical imaging of odorant representations in the mammalian olfactory bulb. Neuron *23*, 499–511.

Schwob, J.E., Youngentob, S.L., Ring, G., Iwema, C.L., and Mezza, R.C. (1999). Reinnervation of the rat olfactory bulb after methyl bromide-induced lesion: timing and extent of reinnervation. J. Comp. Neurol. *412*, 439–457.

Serizawa, S., Ishii, T., Nakatani, H., Tsuboi, A., Nagawa, F., Asano, M., Sudo, K., Sakagami, J., Sakano, H., Ijiri, T., et al. (2000). Mutually exclusive expression of odorant receptor transgenes. Nat. Neurosci. *3*, 687–693.

Serizawa, S., Miyamichi, K., Nakatani, H., Suzuki, M., Saito, M., Yoshihara, Y., and Sakano, H. (2003). Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. Science *302*, 2088–2094.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

Sullivan, S.L., Bohm, S., Ressler, K.J., Horowitz, L.F., and Buck,

L.B. (1995). Target-independent pattern specification in the olfactory epithelium. Neuron *15*, 779–789.

Tsuboi, A., Yoshihara, S., Yamazaki, N., Kasai, H., Asai-Tsuboi, H., Komatsu, M., Serizawa, S., Ishii, T., Matsuda, Y., Nagawa, F., and Sakano, H. (1999). Olfactory neurons expressing closely linked and homologous odorant receptor genes tend to project their axons to neighboring glomeruli on the olfactory bulb. J. Neurosci. *19*, 8409–8418.

Uchida, N., Takahashi, Y.K., Tanifuji, M., and Mori, K. (2000). Odor maps in the mammalian olfactory bulb: domain organization and odorant structural features. Nat. Neurosci. *3*, 1035–1043.

Vassar, R., Ngai, J., and Axel, R. (1993). Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. Cell *74*, 309–318.

Vassar, R., Chao, S.K., Sitcheran, R., Nunez, J.M., Vosshall, L.B., and Axel, R. (1994). Topographic organization of sensory projections to the olfactory bulb. Cell *79*, 981–991.

Wachowiak, M., and Cohen, L.B. (2001). Representation of odorants by receptor neuron input to the mouse olfactory bulb. Neuron *32*, 723–735.

Wang, F., Nemes, A., Mendelsohn, M., and Axel, R. (1998). Odorant receptors govern the formation of a precise topographic map. Cell *93*, 47–60.

Young, J.M., and Trask, B.J. (2002). The sense of smell: genomics of vertebrate odorant receptors. Hum. Mol. Genet. *11*, 1153–1160.

Young, J.M., Friedman, C., Williams, E.M., Ross, J.A., Tonnes-Priddy, L., and Trask, B.J. (2002). Different evolutionary processes shaped the mouse and human olfactory receptor gene families. Hum. Mol. Genet. *11*, 535–546.

Young, J.M., Shykind, B.M., Lane, R.P., Tonnes-Priddy, L., Ross, J.A., Walker, M., Williams, E.M., and Trask, B.J. (2003). Odorant receptor expressed sequence tags demonstrate olfactory expression of over 400 genes, extensive alternate splicing and unequal expression levels. Genome Biol. *4*, R71.

Zhang, X., and Firestein, S. (2002). The olfactory receptor gene superfamily of the mouse. Nat. Neurosci. *5*, 124–133.