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Emx2 Stimulates Odorant Receptor Gene Expression

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Abstract

The mechanisms selecting a single odorant receptor (OR) gene for expression in each olfactory sensory neuron (OSN) establish an OR expression pattern critical for odor discrimination. These mechanisms are largely unknown, but putative OR promoters contain homeodomain-like sites, implicating homeobox transcription factors such as Emx2. At embryonic day 18.5, expression of 49–76% of ORs was decreased in mice lacking Emx2, depending on the metric used. The decreases were due to fewer OSNs expressing each OR. Affected ORs showed changes that were disproportionately greater than the 42% reduction in mature neurons and similar decreases in unrelated olfactory neuron-enriched messenger RNAs in Emx2$^{-/-}$ mice. Both Class I and Class II ORs decreased, as did ORs expressed in both the dorsal and ventral regions of the epithelium. Conversely, 7% of Class II ORs tested were expressed more frequently, suggesting that some ORs are independent of Emx2. Emx2 helps stimulate transcription for many OR genes, which we hypothesize is through direct action at OR promoters, but Emx2 appears to have no significant role in regulating other aspects of OR gene expression, including the zonal patterns, OR gene cluster selection mechanisms, and singularity of OR gene choice.

Key words: gene choice, olfaction, olfactory receptor, smell, transcription

Introduction

Odorant receptors (ORs; also known as olfactory receptors) determine the capacity of animals to detect volatile chemical signals. The size of the OR gene family, the largest at more than 1000 functional genes in several mammalian genomes, correlates with the diversity of the many thousands of volatile chemicals that are potential odorants for mammals (Firestein 2001; Rouquier and Giorgi 2007). Although determining which odorants activate each OR is difficult, several studies have now demonstrated that odorants do act as agonists, and even as antagonists, for ORs (Mombaerts 2004; Krautwurst 2008). In addition to detecting odorant compounds, ORs also play a critical part in the further coding of odor signals via their role in the coalescence of olfactory sensory neuron (OSN) axons into the glomeruli of the olfactory bulb (Mombaerts et al. 1996). All axons terminating in a glomerulus originate from OSNs expressing the same OR protein, allowing the glomerular layer to act as a spatial map of odor quality. This mechanism of encoding odor quality depends on restricting OR expression to a single OR gene in each OSN. In addition, because alleles of an OR gene could encode OR proteins with differing pharmacologies, this logic would work best if OR gene expression was monoallelic, which is indeed the case (Chess et al. 1994; Strotmann et al. 2000; Ishii et al. 2001). This logic is also predicated on an ability of small differences in OR sequence to direct OSN axons to different glomeruli. This also proves to be true (Feinstein and Mombaerts 2004). Layered on top of these forces dictating the singularity of OR gene choice by OSNs is the phenomenon OR zonality. Every mammalian OR gene investigated thus far is expressed in a circumscribed region of the olfactory epithelium. For most ORs tested thus far, the expression zone is constrained in the dorsomedial to ventrolateral dimension, forming a band that stretches the rostrocaudal extent of the tissue (Ressler et al. 1994; Vassar et al. 1994; Kubick et al. 1997; Miyamichi et al. 2005). Whether zonality of OR expression depends on signal gradients that endure throughout life or regional specification laid down during development is not known.

Everything we understand about OR function, from tissue- and spatially restricted expression patterns to the singularity of expression in OSNs, argues for the evolution of a tightly regulated mechanism for controlling OR gene expression. This mechanism is perhaps the greatest remaining mystery about ORs. It appears to be hierarchical, acting at the zone, OR gene cluster, single OR gene, and allele levels to select a single OR gene, freeing it from the silencing that must otherwise be experienced by OR genes. To what extent the levels in the hierarchy are interdependent is as yet unknown. We do

know that at levels below the OR expression zone, the mechanisms have random properties. In addition, the selection of a single OR gene for transcription in OSNs appears to involve several pathways that stimulate transcription and at least one suppressive mechanism, whereby the expressed OR protein feeds back negatively upon the expression of other OR genes (Serizawa et al. 2003; Feinstein et al. 2004; Lewcock and Reed 2004; Shykind et al. 2004). That the overall OR gene selection mechanism is complemented by cell-level selection against OSNs that express no OR or multiple ORs may also be possible (Tian and Ma 2008).

Two novel hypothesized mechanisms for activating transcription of single OR alleles now seem unlikely. A unique and conserved 2-kb sequence on mouse chromosome 14 was discovered to be critical for expression of OR genes in the MOR28 gene cluster, which sits 75 kb away (Serizawa et al. 2003). This sequence, called the H-element, was proposed to act as the factor necessary for the singularity of all OR expression in OSNs, requiring it to act in trans upon ORs on other chromosomes (Lomvardas et al. 2006). This mechanism seems implausible, however, because OR expression is normal in mice lacking the H-element, except for reduced expression of the 4 MOR28 cluster genes nearest the H-element (Fuss et al. 2007; Nishizumi et al. 2007). Perhaps instead of selecting individual OR genes, the H-element may be the founding member of a set of enhancer elements that select OR clusters (Rodriguez 2007). Also out of favor is the hypothesis that DNA rearrangement might control OR gene expression. Cloning of mice by transfer of mature OSN nuclei resulted in clones with normal OR expression patterns rather than expression of a single OR in all OSNs (Eggan et al. 2004; Li et al. 2004). Unless nuclear reprogramming during early development was able to reverse DNA rearrangements used to select OR genes for expression, this finding argues that OR expression is largely regulated in a more conventional fashion.

Indeed, investigation of putative promoter regions just upstream of predicted transcriptional start sites of OR genes implicates these regions in the control of OR expression. Transgenes carrying as little as a few hundred base pairs of a putative OR promoter are often able to replicate the native expression pattern of the OR gene (Qasba and Reed 1998; Vassalli et al. 2002; Rothman et al. 2005). Two conserved elements within these putative promoters have been identified (Vassalli et al. 2002; Hoppe et al. 2006; Michaloski et al. 2006). Most OR genes contain Olf-1/Early B-cell factor (O/E)-like sites located upstream of the predicted transcriptional initiation site (Vassalli et al. 2002). O/E-like sites are bound by the Ebf family of transcription factors and are present in the putative promoters of many genes whose expression is largely restricted to the olfactory epithelium (Kudrycki et al. 1993; Wang and Reed 1993; Walters et al. 1996; Dugas and Ngai 2001). The O/E-like site is therefore likely to contribute to the olfactory specificity of OR expression. Immediately upstream of the O/E-like sites typically is a homeodomain-like site that is also implicated in OR gene expression (Vassalli et al. 2002; Rothman et al. 2005). This site can bind several homeobox transcription factors, and one of them, Lhx2, may be necessary for expression of some ORs (Hirota and Mombaerts 2004, 2007; Kolterud et al. 2004). Though it is clear that other sites or mechanisms must also help regulate OR gene expression, these 2 DNA elements and the factors that bind them appear to be important components of the mechanism regulating OR gene expression.

We have investigated a homeobox transcription factor, Emx2, known to bind a putative OR promoter and to be expressed in OSNs (Hirota and Mombaerts 2004; Nedelec et al. 2004). Emx2 has important developmental roles in other tissues, most critically in the patterning of cortical areas of the brain and in formation of the urogenital tract (Miyamoto et al. 1997; Polleux 2004). We have investigated whether Emx2 is necessary for expression of OR genes in OSNs. We found that in Emx2 mutant mice, the olfactory epithelium developed normal pseudostratification, except for a reduction in the number of mature OSNs. OR expression, however, was disproportionately affected. The majority of OR genes showed expression in fewer OSNs, whereas a few OR genes were expressed in more OSNs. These data indicate that Emx2 is necessary for full expression of many OR genes and lend support to the hypothesis that Emx2 does so by acting directly on OR promoters.

Materials and methods

Mice

Mutant mice with targeted disruption of the Emx2 gene were obtained from the RIKEN Center for Developmental Biology, Japan (Yoshida et al. 1997). $Emx2^{-/-}$ mice die soon after birth due to urogenital defects (Pellegrini et al. 1996; Miyamoto et al. 1997). We therefore used mice at embryonic age 18.5 days (E18.5) for our experiments. Embryonic animals were obtained by allowing mating overnight. The morning of vaginal plug detection was considered E0.5. Preliminary experiments revealed no differences between $Emx2^{+/-}$ mice and $Emx2^{+/+}$ mice, so these genotypes were considered phenotypically equivalent in the analyses performed. Olfactory marker protein (OMP)–green fluorescent protein (GFP) mice were obtained from Dr Peter Mombaerts (Max Planck Institute of Biophysics, Frankfurt, Germany). All mouse procedures were performed in accordance with an approved institutional animal care and use committee protocol.

In situ hybridization

In situ hybridizations (ISHs) were performed as described previously (Shetty et al. 2005; Yu et al. 2005). A detailed protocol is available from the authors. In brief, mouse heads were fixed overnight in paraformaldehyde, cryoprotected, mounted in OCT (Sakura Finetek USA, Inc., Torrance, CA) and stored at –80 °C. Coronal sections of 10-μm thickness were cut on a cryostat and mounted on Superfrost Plus

slides (Fisher Scientific, Pittsburg, PA). Digoxygenin-labeled riboprobes were prepared from cDNA fragments that ranged from 500 to 1000 bp in length. In cases where preparing probes that react with more than 1 OR was unavoidable, the results are described as detection of multiple ORs. Riboprobes were hybridized in 50% formamide in 10 mM Tris–HCl (pH 8.0), 10% dextran sulfate, 1× Denhardt's solution, 600 mM NaCl, 0.25% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, and 200 μg/ml yeast tRNA at 65 °C (1 ng/μl per riboprobe). Washes were done in phosphate-buffered saline (PBS). Detection was done using an alkaline phosphatase-conjugated antibody to digoxygenin and hydrolysis of nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine. Sense-strand probes were used as controls and were invariably negative. All comparisons between genotypes were done using slides processed together on the same date and under identical conditions. Digital wide-field images were obtained using a Spot 2e camera on a Nikon Diaphot 300 inverted microscope. Images were processed in Adobe Photoshop by adjusting size, brightness, and contrast. Images were then combined and labeled using Deneba Canvas.

Cell counts

All cell counts are reported as means with their standard deviations. Counts of OSNs expressing an OR gene were done from ISH experiments using 3 $Emx2^{-/-}$ and 3 $Emx2^{+/-}$ mice. For each OR tested, 8 coronal sections were matched for anterior–posterior position. All labeled OSNs, irrespective of location in the olfactory epithelium, were counted and summed across the 8 sections. The length of epithelium in each section used was measured to allow calculation of the labeled OSNs per unit distance for each OR tested. To count $Gap43^{+}$ immature OSNs, labeled cells in images of ISH for Gap43 messenger RNA (mRNA) were counted in 200-μm long sections of septal epithelia from $Emx2^{-/-}$($n = 2$) and $Emx2^{+/-}$ ($n = 3$) mice. To count total cells per linear dimension of the olfactory epithelium, fluorescent images of nuclei stained with Hoechst 33258 were prepared, the location of the basement membrane marked, and nuclei apical to this membrane were counted in 200-μm long sections of the epithelium.

To facilitate the counting of mature OSNs, we bred $Emx2^{+/-}$ mice onto an OMP–GFP homozygous background (Potter et al. 2001) to obtain $Emx2^{-/-}$:OMP–$GFP^{-/-}$, $Emx2^{+/-}$:OMP–$GFP^{-/-}$, and $Emx2^{+/+}$:OMP–$GFP^{-/-}$, littermates. These genotypes were used only for accurate counting of GFP fluorescent mature OSNs. Mouse heads were fixed and sectioned as described for ISH. Slides were washed with PBS for 15 min and stained with Hoechst 33258 for 5 min followed by a 5-min PBS wash. Digital dual fluorescent (GFP and Hoechst 33258) images were obtained from the coronal sections matched across genotypes for anterior–posterior position. Cells were counted in 200-μm regions of the dorsal and ventral septum.

mRNA abundance

GeneChip® assessment of mRNA abundance was done using procedures previously established (Shetty et al. 2005; Sammeta et al. 2007). Olfactory epithelium was isolated from mice at age E18.5 using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). Pooled samples consisting of 2.7 μg of olfactory epithelium RNA from each of 3 $Emx2^{+/+}$ and 3 $Emx2^{-/-}$ mice ($n = 3$ pools) were prepared. Labeling, hybridization, and scanning were performed according to standard Affymetrix protocols by the University of Kentucky Microarray Core Facility using Affymetrix GeneChip® Mouse Exon 1.0 Sense Target Arrays. Affymetrix Expression Console software was used for analysis and generation of gene-level robust multichip analysis (RMA) values from exon probe sets. Gene-level data derived from clusters of exons that belong to a single gene are termed transcript clusters. These were analyzed at the Core annotation level (the most conservative level), limiting analysis to exon-level probe sets that map to BLAST alignments of mRNAs with annotated full-length open reading frames. Gene-level data were then manipulated in Excel (Microsoft, Redmond, WA). The microarray data have been deposited at Gene Expression Omnibus (Accession No. GSE12135). Due to the similarity of some OR genes, a few transcript clusters may detect mRNAs from multiple ORs, a fact that prevents exact identification of every OR affected and, therefore, calculating the exact number of ORs affected.

To eliminate background, we deleted any mRNAs that failed to give a signal of at least 9% of the overall mean gene-level signal on at least one GeneChip®. This eliminated 1793 transcript clusters. We verified that this eliminated background by assessing the correlation between variance and average signal intensity. The size of the variance should become independent of signal intensity at low signals where differences in the biological samples are not the primary source of variation. Testing for differences for each gene was done using Student's *t*-test at an α level of 0.05, followed by correction for multiple testing using a false discovery rate of 10%. That these criteria were rigorous was indicated by ORs whose *P* values exceeded 0.05 yet were documented by ISH to differ between $Emx2^{-/-}$ and $Emx2^{+/+}$ mice.

Genes

To avoid ambiguity, the official gene symbols provided by the National Center for Biotechnology Information (NCBI) are used for all genes described herein. Table 1 lists all genes mentioned in this paper, along with their NCBI Gene IDs and any synonyms with functional significance.

As a comparison for the behavior of OR mRNAs in the microarray data, we used genes identified by Sammeta et al. (2007) as being expressed primarily in OSNs. This population consists of more than 4700 genes that are expressed in both immature and mature OSNs. These mRNAs are sufficiently enriched in purified mature OSNs to indicate

Table 1 Gene reference table

Gene Symbol	Gene Name	Mouse Gene ID	Chr.	Synonyms
Adcy3	*adenylate cyclase 3*	104111	12	*AC3*
Ano2	*anoctamin 2*	243634	12	*Tmem16b, N64J*
Bbs2	*Bardet-Biedl syndrome 2*	67378	8	
Bbs4	*Bardet-Biedl syndrome 4*	102774	9	
Cnga2	*cyclic nucleotide gated channel alpha 2*	12789	X	*Cnca, Cncg4, OCNC1*
Cyp2g1	*Cytochrome P450, family 2, subfamily g, polypeptide 1*	13108	7	
Dnali1	*dynein, axonemal, light intermediate polypeptide 1*	75563	4	
Ebf1	*early B-cell factor 1*	13591	11	*O/E-1, Olf-1*
Ebf2	*early B-cell factor 2*	13592	14	*Mmot1, O/E-3*
Ebf3	*early B-cell factor 3*	13593	7	*O/E-2*
Ebf4	*early B-cell factor 4*	228598	2	*Ebf3, O/E-4, Olf-1*
Emx2	*empty spiracles homolog 2*	13797	19	*Pdo*
Gap43	*growth associated protein 43*	14432	16	*B-50, Basp2, GAP-43*
Hydin	*hydrocephalus inducing*	244653	8	*hy-3, hy3*
Ift172	*Intraflagellar transport 172 homolog*	67661	5	*Slb, wim*
Ift74	*intraflagellar transport 74 homolog*	67694	4	*Ccdc2, Cmg1*
Lhx2	*LIM homeobox protein 2*	16870	2	*LH2A, Lh-2, Lim2, ap, apterous*
Ncam1	*neural cell adhesion molecule 1*	17967	9	*CD56, E-NCAM, Ncam*
Neurog1	*neurogenin 1*	18014	13	*Ngn1, Math4C, Neurod3*
Nphp1	*nephronophthisis 1*	53885	2	
Olfr121	*olfactory receptor 121*	258622	17	*MOR263-4*
Olfr129	*olfactory receptor 129*	258324	17	*MOR263-9*
Olfr1440	*olfactory receptor 1440*	258679	19	*MOR215-1*
Olfr15	*olfactory receptor 15*	18312	16	*MOR256-17; OR3*
Olfr1507	*olfactory receptor 1507*	57269	14	*MOR244-1, Mor28*
Olfr1508	*olfactory receptor 1508*	57270	14	*MOR244-2*
Olfr151	*olfactory receptor 151*	406176	9	*MOR171-2; M71*
Olfr156	*olfactory receptor 156*	29846	4	*MOR262-6; OR37B*
Olfr160	*olfactory receptor 160*	80706	9	*MOR171-3; M72; Olfr7b*
Olfr17	*olfactory receptor 17*	18314	7	*MOR263-15; P2*
Olfr2	*olfactory receptor 2*	18317	7	*MOR103-15; I7; I54*
Olfr270	*olfactory receptor 270*	258600	4	*MOR262-9*
Olfr272	*olfactory receptor 272*	258836	4	*MOR262-7*
Olfr273	*olfactory receptor 273*	258821	4	*MOR222-8*
Olfr308	*olfactory receptor 308*	258614	7	*MOR104-1*
Olfr544	*olfactory receptor 544*	257926	7	*MOR42-3*
Olfr545	*olfactory receptor 545*	258837	7	*MOR42-1*
Olfr6	*olfactory receptor 6*	233670	7	*MOR103-16; M50*
Olfr615	*olfactory receptor 615*	259084	7	*MOR19-2*

Table 1 Continued

Gene Symbol	Gene Name	Mouse Gene ID	Chr.	Synonyms
Olfr642	*olfactory receptor 642*	258326	7	*MOR13-6*
Olfr90	*olfactory receptor 90*	258469	17	*MOR256-21*
Omp	*olfactory marker protein*	18378	7	*OMP*
Spa17	*sperm autoantigenic protein 17*	20686	9	*Sp17*
Spag6	*sperm associated antigen 6*	50525	16	*axoneme protein*
Tekt1	*tektin 1*	21689	11	*MT14*
Umodl1	*uromodulin-like 1*	52020	17	*olfactorin, N8*

Chr., mouse chromosome.

that they are more abundant in mature OSNs than in immature OSNs, but, like ORs, they are usually present at lower amounts in immature OSNs (Iwema and Schwob 2003; Sammeta et al. 2007). We randomly selected 600 of these genes to obtain 340 that had signal above background on the exon microarray.

Results

Olfactory epithelia of Emx2$^{-/-}$ mice were morphologically normal but had fewer mature OSNs

The nasal cavities of age E18.5 Emx2$^{-/-}$ mice contained easily identifiable landmarks and were nearly normal in appearance (Figure 1A,B). The most noticeable difference from wild-type littermates was in the shortening of the septum, presumably due to the slightly decreased size of the entire frontal–nasal region of the head. Most importantly, for this study, the extent of the olfactory epithelium across the surface of the cavity was normal, and the epithelium contained mature neurons expressing the OMP gene (Figure 1). The pseudostratification of the olfactory epithelium was also normal (Figure 2A–J). Specific markers for several cell types identified mature neurons (Figure 2A,B), immature neurons (Figure 2C,D), both immature and mature neurons (Figure 2E,F), sustentacular cells (Figure 2G,H), and a subtype of globose basal cells (Figure 2I,J) in their appropriate positions. However, the thickness of the epithelium was reduced by an average of 15% compared with heterozygous and wild-type littermates (Table 2), a statistically significant decrease ($P < 0.00001$; Student's $t = 10.266$). A decrease in thickness of the olfactory epithelium indicates that fewer cells are present in the epithelium, often due to a decrease in OSN number. A reduction in mature OSNs was apparent from ISH for OMP in Emx2$^{-/-}$ mice compared with wild-type littermates (Figures 1A,B and 2A,B). To more easily quantify this decrease, we bred Emx2$^{-/-}$ mutant mice with OMP–GFP mice (Potter et al. 2001). Compared with Emx2$^{+/+}$:OMP–GFP$^{-/-}$ littermates, Emx2$^{-/-}$:OMP–GFP$^{-/-}$ mice had 42% fewer OMP^{+} mature OSNs (Table 2 and Figure 1C,D), a significant difference ($P < 0.01$; Student's $t = 5.086$). The number of OMP^{+} OSNs in heterozygous Emx2$^{+/-}$:OMP–GFP$^{-/-}$ mice did not differ from wild-type littermates. The decrease in the number of mature OSNs was shared equally by the dorsomedial and ventrolateral regions of the epithelium. For example, the average cell counts of OMP^{+} mature OSNs

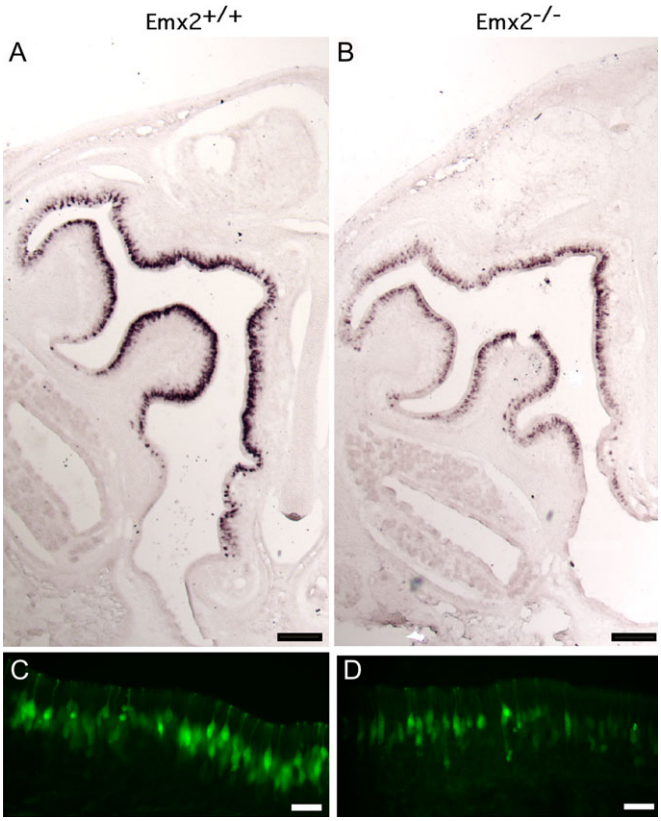

Figure 1 Emx2$^{-/-}$ mice at age E18.5 had olfactory epithelia containing mature OSNs over the same extent of the nasal cavity as wild-type littermates. **(A, B)** ISH for OMP mRNA to identify mature OSNs. GFP expression from the OMP locus was used to identify and count mature OSNs **(C, D)**. (C) Emx2$^{+/+}$:OMP–GFP$^{-/-}$ genotype. (D) Emx2$^{-/-}$:OMP–GFP$^{-/-}$ genotype. Scale bars, (A, B) 200 μm; (C, D) 20 μm.

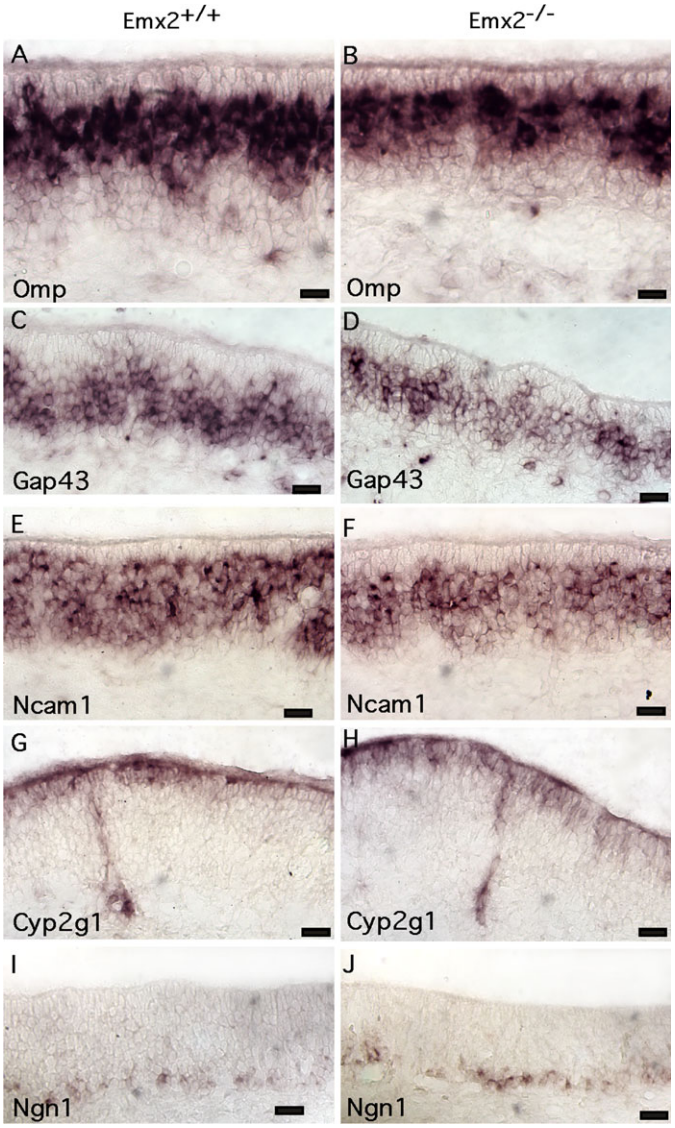

Figure 2 Mice lacking Emx2 had normal pseudostratification of the cell body layers in the olfactory epithelium. **(A, B)** ISH for OMP mRNA to label mature OSNs. **(C, D)** ISH for Gap43 to label immature OSNs. **(E, F)** ISH for Ncam1 to label both developmental stages of OSNs. **(G, H)** ISH for Cyp2g1 to label sustentacular cells and Bowman's glands (the labeled structure stretching from the lamina propria across the entire depth of the olfactory epithelium. (**I, J**) ISH for Ngn1 (Neurog1) to label a subpopulation of globose basal cells. Scale bars, 20 μm.

in dorsal and ventral zones of the septa of $Emx2^{-/-}$:OMP–$GFP^{-/-}$ mice were 77.5 and 77.0 per mm, respectively.

The loss of mature OSNs appeared to account for nearly all of the decrease in thickness of the epithelium. Total cell counts within the olfactory epithelium were reduced by 17% in $Emx2^{-/-}$ mice compared with wild-type and heterozygous littermates (Table 2), similar to the 15% decrease in thickness. ISH for markers of immature OSNs, sustentacular cells, and globose basal cells labeled cell body layers that were similar in extent to the labeling in littermate controls (Figure 2C–J). Counts of immature OSNs by ISH labeling

Table 2 Olfactory epithelium thickness and number of mature OSNs (OMP^+) were reduced in $Emx2^{-/-}$ mice

Genotype	Number of mice	Mean olfactory epithelium thickness (μm)	OMP^+ cell count	Total cell count
+/+	2	98 ± 3	125.5 ± 20.0	1358 ± 61
+/–	5	98 ± 2	137.5 ± 25.5	1360 ± 65
–/–	6	83 ± 3	77.0 ± 16.5	1132 ± 36

Cell counts are means and standard deviations per mm of epithelium.

for Gap43 mRNA found no difference between $Emx2^{+/-}$ and $Emx2^{-/-}$ mice, with 390 ± 30 cells and 355 ± 120 cells per mm of epithelium, respectively,

Many ORs were expressed by fewer OSNs in $Emx2^{-/-}$ mice

Small upstream regions of OR genes containing the homeodomain-like site that presumably binds Emx2 are often sufficient to support normal expression patterns of OR genes in transgenic mice (Qasba and Reed 1998; Vassalli et al. 2002; Hirota and Mombaerts 2004; Rothman et al. 2005). This finding suggests that Emx2 might globally promote OR gene transcription. If so, the absence of Emx2 should reduce OR expression. OR mRNAs are readily detected by ISH because they are among the most abundant mRNAs in an OSN, so we used ISH to test whether ORs were expressed in fewer OSNs. We observed little evidence of any decrease in OR mRNA abundance within individual OSNs (insets in Figures 3A,B and 4A,B), a change that we detect in 2 ways: as increases in the time necessary for reaction products to become visible and as decreases in signal intensity. Instead, 13 of the 17 ORs we tested were detected in many fewer OSNs in $Emx2^{-/-}$ mice compared with $Emx2^{+/+}$ and $Emx2^{+/-}$ littermates (Table 3 and Figure 3). Conversely, the other 4 ORs were observed in an increased number of OSNs in $Emx2^{-/-}$ mice (Table 3 and Figure 4), suggesting that not all ORs need Emx2 to help activate their transcription.

ORs from all expression zones and both OR classes were affected

The mammalian OR gene family contains 2 phylogenetic classes (Glusman et al. 2001; Zhang and Firestein 2002). Class I ORs appear to be more ancient, having homology to fish ORs, and nearly all of them are expressed only in the dorsomedial zone of the mammalian olfactory epithelium. Class II receptors evolved more recently are more numerous, and their expression spans all regions of the olfactory epithelium. We observed a decrease in the frequency of expression for 3 Class I and 10 Class II ORs, whereas all 4 ORs that increased were from Class II (Table 3).

The overall pattern of OR expression in $Emx2^{-/-}$ mice appeared normal. Sections from multiple levels of the nasal cavity provided no evidence that the ORs detected in fewer OSNs had merely shifted their expression to different regions

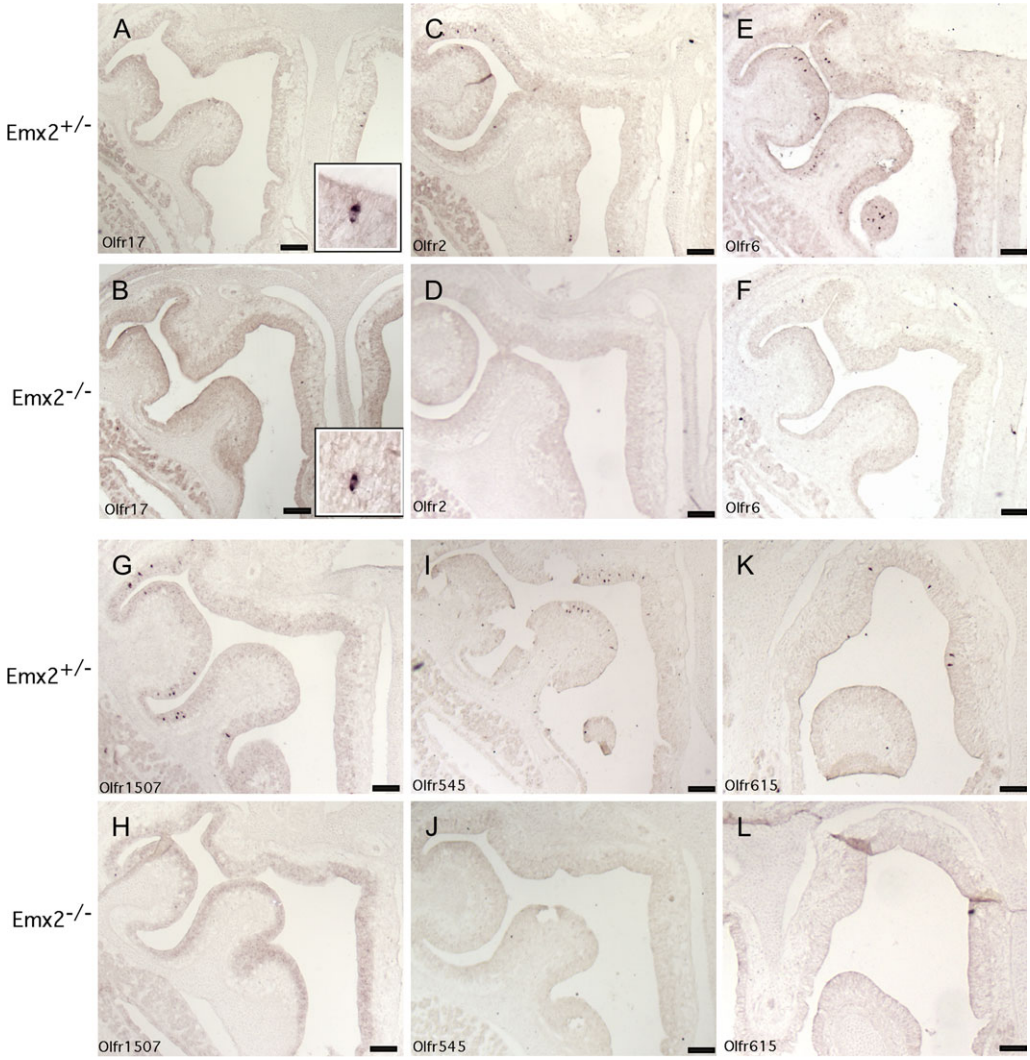

Figure 3 Frequency of expression of many ORs decreased in Emx2$^{-/-}$ mice. **(A, B)** Olfr17, a Class II OR expressed in the ventrolateral region. Insets, the intensity of signal for an Olfr17 mRNA within each neuron was not altered by the absence of Emx2. **(C, D)** Olfr2, a Class II OR expressed in the ventrolateral region. **(E, F)** Olfr6, a Class II OR expressed in the ventrolateral region. **(G, H)** Olfr1507, a Class II OR expressed in the ventrolateral region. **(I, J)** Olfr545, a Class I OR expressed in the dorsomedial region. **(K, L)** Olfr615, a Class I OR expressed in the dorsomedial region. Half the bilaterally symmetric nasal region is shown in each image, with septum at the right. Scale bars, 200 μm.

or zones in the olfactory epithelium. For the ORs detected with increased frequency, the expression zones were similarly stable, though small expansions may have occurred. For example, the expression of Olfr15 in the ventrolateral region in wild-type mice spreads into the dorsomedial region in Emx2$^{-/-}$ mice (Figure 4A,B).

Expression of many ORs decreased in Emx2$^{-/-}$ mice

To gain a more comprehensive view of whether OR expression depends on Emx2, we used Affymetrix GeneChip® Mouse Exon 1.0 ST Arrays to compare the olfactory epithelia of Emx2$^{-/-}$ and Emx2$^{+/+}$ mice (n = 3). Unlike other GeneChip microarrays we have tested, which detect OR mRNAs poorly, this exon microarray detected many OR mRNAs (Shetty et al. 2005; Sammeta et al. 2007). The gene-level analysis of these data identified 677 OR transcript clusters, representing 734 OR genes, with mRNA signals above background (Supplementary Table 1). Of these, 336 transcript clusters (representing 365 OR genes) were significantly reduced in the Emx2$^{-/-}$ samples. Only 22 transcript clusters were significantly increased. Of the 13 ORs that were decreased in our ISH data, 9 were significantly decreased and 1, Olfr17, was not represented on the microarray (Table 3). The remaining 3 that showed decreases by ISH did not reach significance in the microarray data, an indication that the statistical analysis of the microarray data was conservative. All 4 ORs that increased in our ISH data were significantly increased in the microarray data.

The absence of Emx2 disproportionately impacted OR mRNAs compared with other mRNAs in the olfactory

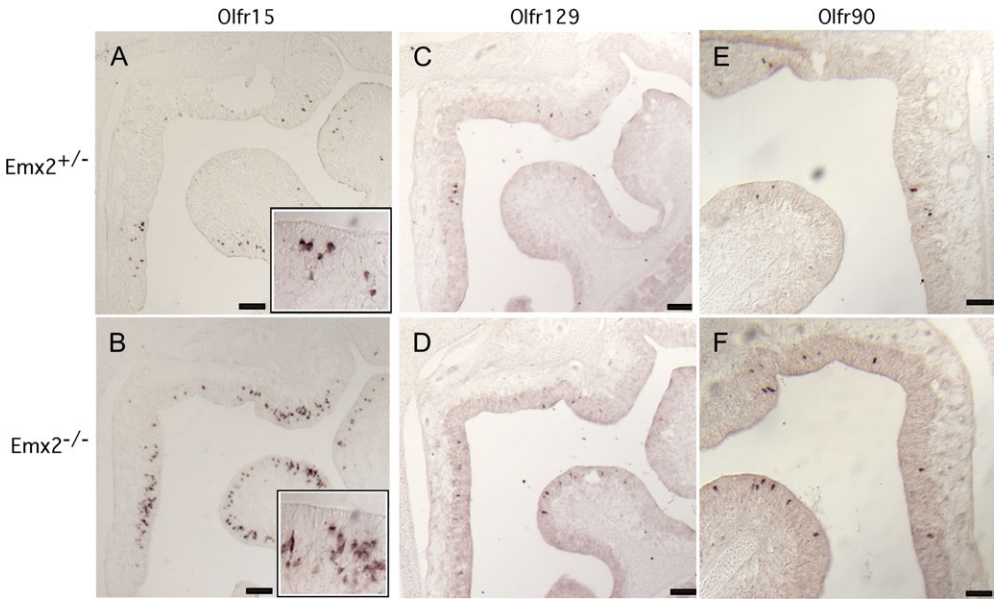

Figure 4 Frequency of expression of a few ORs increased in Emx2$^{-/-}$ mice. **(A, B)** Olfr15, a Class II OR expressed in the ventrolateral region. The region of expression of Olfr15 appeared to expand in Emx2$^{-/-}$ mice. Insets, the intensity of signal for Olfr15 mRNA within each neuron was not altered by the absence of Emx2. **(C, D)** Olfr129, a Class II OR expressed in the ventrolateral region. **(E, F)** Olfr90, a Class II OR expressed in the ventrolateral region. Scale bars, (A–D) 200 μm; (E–F) 80 μm.

epithelium. The 336 OR transcript clusters that were significantly less abundant in the Emx2$^{-/-}$ samples represented 28% of the transcript clusters that had significant decreases. OR mRNAs represent about 10% of the mRNA species expressed in mouse OSNs (Sammeta et al. 2007). OR mRNAs were also the most strongly affected mRNAs. Of the 250 transcript clusters with the greatest fold decreases in this data set, 217 were ORs. Even more compelling was a comparison of fold changes for all ORs detected on the array against the fold changes detected in an equivalent population of mRNAs—340 randomly selected OSN-enriched mRNAs (Sammeta et al. 2007). Compared with OR mRNAs, the abundance of these OSN-enriched mRNAs was only slightly decreased by the 42% reduction in mature OSNs (Figure 5). To illustrate this fact at the level of individual genes, our cell count data predicted that mRNAs expressed solely in mature OSNs should have decreased by approximately 42%. Indeed, this prediction was borne out as OMP mRNA was reduced by 44%, Adcy3 by 28%, Cnga2 by 38%, Ano2 by 56% (Yu et al. 2005), and Umodl1 by 52% (Yu et al. 2005). We conclude that the decrease in mature OSN number could have accounted for only a small fraction of the ORs with decreased expression in Emx2$^{-/-}$ mice.

Emx2 regulates OR genes independently of OR gene cluster organization

Most OR genes occur in clusters on the chromosomes. We analyzed 4 of these clusters: 17-1, 7-3, 11, and 14-1. The absence of Emx2 did not have the same effect on all OR genes within any of these clusters. OR genes whose mRNAs decreased coexisted with OR genes whose mRNAs increased in Emx2$^{-/-}$ mice in all 4 clusters. For example, of the 50 ORs in cluster 17-1, the microarray detected 3 increases, 16 decreases, 19 that had no significant change, 10 that were not represented on the microarray, and 2 that were not above background. Supplementary Table 2 contains a complete listing of the ORs in these clusters.

Discussion

By comparing expression of Emx2$^{-/-}$ mice with wild-type and heterozygous littermates, we detected reduced expression of many ORs and increased expression of a few ORs. Unlike markers of OSN maturity, the reduction in OR expression was disproportionately greater than a 42% reduction in mature OSNs, indicating that the absence of Emx2 is not altering OR expression through some general defect in OSN phenotype. Emx2 therefore appears to contribute to transcriptional activation of many, perhaps most, mouse ORs. We hypothesize that the action of Emx2 on OR expression is direct, consistent with previous evidence that Emx2 can bind an OR promoter and that most of the OR promoter regions predicted thus far have homeodomain-like elements that would be necessary for direct action of Emx2 on OR gene transcription (Vassalli et al. 2002; Hirota and Mombaerts 2004; Hoppe et al. 2006; Michaloski et al. 2006). A few ORs increased in abundance in Emx2$^{-/-}$ mice, arguing that some ORs may be transcribed independently of Emx2. These OR genes appeared to be chosen for expression more often in the absence

Table 3 OR mRNAs tested by ISH

Gene Symbol	Class	OSNs/mm (wild-type)	ISH ratio	GeneChip ratio	Region
Olfr2	Class II	2.6	0.03	0.4*	Ventral
Olfr6	Class II	0.8	0.02	0.2*	Ventral
Olfr15	Class II	3.5	5.70	3.1*	Ventral
Olfr17	Class II	0.9	0.10	NP	Ventral
Olfr90	Class II	1.0	2.10	1.5*	Ventral
Olfr129; Olfr121	Class II	2.0	2.10	2.9*	Ventral
Olfr156	Class II	3.1	0.02	0.4	OR37 region
Olfr160; Olfr151	Class II	1.6	0.40	0.3	Dorsal
Olfr270	Class II	0.7	0.07	0.3*	OR37 region
Olfr272	Class II	0.1	0.00	0.5*	OR37 region
Olfr273	Class II	0.5	0.00	0.2*	OR37 region
Olfr308	Class II	0.6	0.00	0.6*	Ventral
Olfr545; Olfr544	Class I	2.6	0.03	0.4*	Dorsal
Olfr615	Class I	1.0	0.00	0.2*	Dorsal
Olfr642	Class I	0.5	0.00	1.0	Dorsal
Olfr1440	Class II	1.2	1.80	1.7*	Ventral
Olfr1508; Olfr1507	Class II	1.9	0.05	0.5*	Ventral

OSNs/mm, the number of OSNs expressing the OR per mm of olfactory epithelium in $Emx2^{+/+}$ mice. Ratios are $Emx2^{-/-}$ divided by $Emx2^{+/+}$. *, Significant difference between $Emx2^{-/-}$ and $Emx2^{+/+}$ mice; NP, not present on the microarray; region, the zone of expression within the olfactory epithelium.

of Emx2, perhaps compensating for a reduction in the frequency of choice of most other OR genes.

OSN maturity is unaffected in the absence of Emx2

Four lines of evidence argue that a decrement in OSN maturity was not the cause of reduced OR expression. First, the ISH data indicated that both reductions and increases were due to changes in the number of OSNs expressing an OR rather than in the amounts of OR mRNA per OSN. In other words, the absence of Emx2 altered the frequency with which an OR gene was chosen for expression. Second, the mRNAs of genes expressed specifically in mature OSNs showed reductions in abundance that corresponded closely with the 42% reduction in the number of mature OSNs. In contrast, more than 250 OR mRNAs had reductions of more than 100%, a highly disproportionate effect. Third, the elaboration of cilia is one of the final events in the maturation of OSNs (Cuschieri and Bannister 1975; Schwarzenbacher et al. 2005) and therefore should be one of the events most susceptible to defective maturation of OSNs, but we observed no evidence of this at the level of expression of cilia-related genes in $Emx2^{-/-}$ mice. For example, Dnali1, Tekt1, Hydin, Ift172, Spag6, Spa17, Ift74, Bbs4, Bbs2, and Nphp1, which are all documented cilia-related mRNAs expressed by OSNs, were present at normal amounts in the olfactory epithelia of $Emx2^{-/-}$ mice (Kulaga et al. 2004; Nishimura et al. 2004; McClintock et al. 2008). Fourth, some ORs showed expression in significantly more OSNs, as would be expected if OR gene choice mechanisms were acting normally and free to favor those ORs least dependent on Emx2. If a general defect in OSN development was affecting OR gene expression, then all ORs should show reduced expression.

Transcription of many OR genes depends on Emx2

Measuring the number of OSNs expressing an OR by ISH showed decreases for 76% of the ORs tested. The broader experiment using microarray analysis to rapidly test larger numbers of ORs, albeit less sensitive for any given OR mRNA, gave similar results, finding significant decreases in 49% of the OR transcript clusters detected. We believe that the microarray data underestimated the number of affected ORs. First, both of the ORs that failed to reach significance in the microarray data but were also tested by ISH were detected in many fewer OSNs in $Emx2^{-/-}$ mice. Second, ORs were disproportionately affected in $Emx2^{-/-}$ mice compared with other genes expressed primarily by OSNs. Third, homeodomain-like sites are found in the predicted promoter regions of nearly all OR genes analyzed thus far, so if Emx2 is acting directly on OR promoters, the vast majority of OR promoters have potential binding sites for Emx2 (Vassalli et al. 2002; Hoppe et al. 2006;

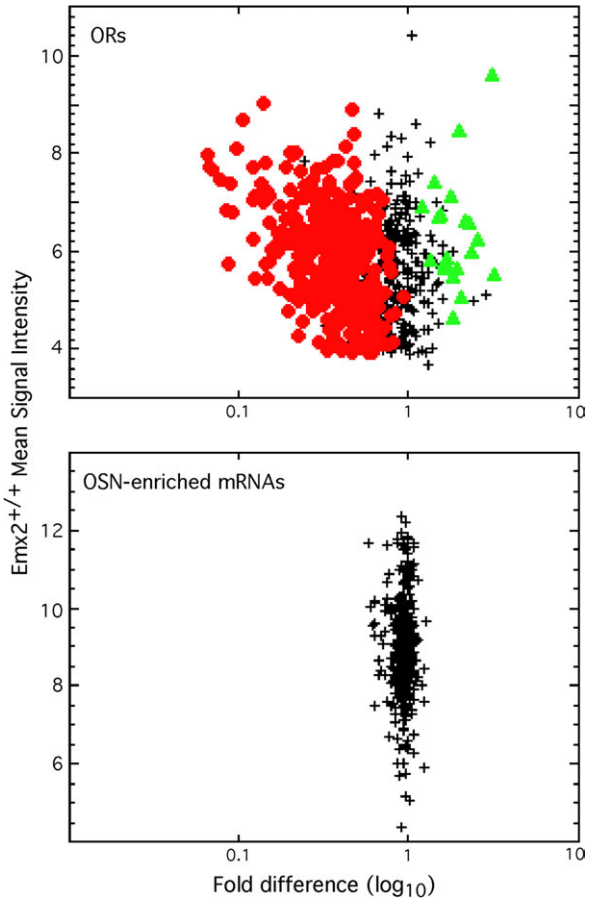

Figure 5 Abundances of OR mRNAs were disproportionately altered compared with other OSN-enriched mRNAs in mice lacking Emx2. The mean signals from GeneChip mouse exon arrays for $Emx2^{+/+}$ mice (log_2) are plotted against the log_{10} of the fold difference between $Emx2^{-/-}$ and $Emx2^{+/+}$ mice.

Michaloski et al. 2006). These facts argue that Emx2 helps stimulate transcription of at least a majority of OR genes.

Identifying all OR genes affected by the absence of Emx2 was not possible from the data obtained. First, our methods assessed many, but not all, OR genes. Second, some OR transcript clusters on the exon array detect multiple OR mRNAs due to sequence similarity between certain ORs. For the ORs in this category, therefore, we cannot be certain which of the OR mRNAs represented in a transcript cluster were decreased, forcing us to calculate conservatively. If we limit the calculation to ORs that decreased at least 2-fold in order to avoid counting any ORs that might have decreased due solely to the 42% reduction in mature neurons, the number of ORs for which we had evidence of a decrease was 280. Similarly, our data identified at least 19 ORs whose frequency of expression increased.

The dependence of chemosensory receptor genes on Emx2 may not be limited to OR genes. The microarray data detected significant decreases in abundance in $Emx2^{-/-}$ mice for 5 trace amine-associated receptor (Taar) transcript clusters, representing 7 of the 15 intact mouse Taar genes (Supplementary Table 1). Taar genes are expressed in subsets of OSNs, and at least some of them encode proteins that detect amine odors in urine (Liberles and Buck 2006).

Emx2 appears to be the predominant homeobox protein for OR genes

If Emx2 was not more important for stimulating OR gene transcription than other homeobox proteins, we should not have observed decrements in the expression of most ORs tested. However, the dependence of OR genes on Emx2 was only rarely absolute. Only 5 of the OR mRNAs tested by ISH failed to be observed in at least one OSN in $Emx2^{-/-}$ mice. Consistent with this observation, some of the OR mRNAs that decreased in the microarray analysis were detected at levels above background in $Emx2^{-/-}$ mice. Therefore, we expect that other homeobox proteins contribute to OR gene expression. A few dozen other homeobox transcription factor mRNAs are present in OSNs (Sammeta et al. 2007). The most promising candidate is Lhx2, a Lin11, Isl-1, and Mec-3 (LIM)-homeobox transcription factor reported to contribute to OR gene expression (Hirota et al. 2007). Like Emx2, Lhx2 binds to an OR promoter that contains a homeodomain-like site (Hirota and Mombaerts 2004). In $Lhx2^{-/-}$ mice, which die in utero at about age E15.5, differentiation of OSNs appears to be halted at a stage where OR expression has just been initiated and very few mature OSNs form (Kolterud et al. 2004). Only in the dorsal zone of the epithelium do mature OSNs form and only at 10% of their normal numbers. OR expression can be detected in immature OSNs (Iwema and Schwob 2003), but if differentiation halts within the immature OSN stage, this is a potential explanation for why expression of few ORs can be detected in $Lhx2^{-/-}$ mice and correlates exactly with the finding that 2 Class I ORs normally expressed ventrally cannot be detected in $Lhx2^{-/-}$ mice while at least some dorsal zone Class I ORs can be detected, albeit at reduced levels (Hirota et al. 2007). In $Lhx2^{-/-}$ mice, therefore, whether decreased expression of ORs could result from the significant reduction in the number of sufficiently differentiated OSNs, from loss of direct positive action at OR promoters, or both is difficult to assess.

For Emx2, the situation is more easily interpreted. Effects on OSN development were limited to a reduction in the number of mature OSNs in $Emx2^{-/-}$ mice, so the amount of OR expression measured, which included increased, decreased, and unaffected OR genes, was most likely due to transcriptional events rather than OSN differentiation or survival. Overall, the data are most consistent with the interpretation that the ORs with reduced expression in $Emx2^{-/-}$ mice depend on Emx2 to stimulate their transcription. Whether this dependence is direct, as Emx2 binding to the Olfr15 (M71) promoter would suggest (Hirota and Mombaerts 2004), or indirect cannot yet be concluded. However, the effects of Emx2 deletion on OR expression were not due to loss of Lhx2. Lhx2 expression, which is primarily in immature OSNs, was normal in $Emx2^{-/-}$ mice (Supplementary Figure 1). If we presume that

Emx2 does act directly on OR promoter elements, then the idea that these other homeobox transcription factors might stimulate the same OR genes as Emx2 at varying efficacies seems reasonable. However, whether these hypothetical mechanisms are normally active or are instead merely compensating mechanisms that are irrelevant in a wild-type mouse is impossible to predict at this time. We also note that the homeodomain-like site of putative OR promoters may not be the only avenue for compensation in Emx2$^{-/-}$ mice. At present, we interpret our findings to indicate that Emx2 is the most important homeobox protein for OR genes in general and that other homeobox proteins can only partially substitute for Emx2 to drive expression of most OR genes.

For OR genes that appeared to be independent of Emx2, their promoters may be more sensitive to other homeobox proteins, such as Lhx2, or alternatively do not depend on homeobox proteins at all (Michaloski et al. 2006). However, we cannot completely rule out the possibility that these ORs do normally depend on Emx2 and are merely better compensated than other OR genes in the absence of Emx2. This would mean that all ORs normally depend on Emx2 for activation. To clarify these questions, future experiments will need to investigate the ability of Emx2 to act directly on putative promoters of ORs that were sensitive, versus those that were insensitive, to the absence of Emx2.

Implications for OR gene choice

Two of our findings seem relevant to the problem of how an OSN selects an OR gene for expression. First, some ORs showed expression in increased numbers of OSNs in Emx2$^{-/-}$ mice. This is consistent with the hypothesis that differentiating OSNs may serially express several ORs before locking in the expression of 1 OR gene (Shykind et al. 2004). This idea depends on the demonstrated ability of expressed ORs to suppress expression of other OR genes, such that in Emx2$^{-/-}$ mice this ratcheting mechanism would have reduced probability of locking on the ORs most dependent on Emx2 (Serizawa et al. 2003; Feinstein et al. 2004; Lewcock and Reed 2004; Shykind et al. 2004). Alternatives exist, however, such as explanations in which the absence of Emx2 leads to disinhibition or relaxing the competition for some limiting factor, thereby increasing the selection of OR genes for which Emx2 is not the dominant positive factor.

Emx2 has several critical roles in OSNs

Our evidence that Emx2 is important for OR gene expression adds to previous evidence that Emx2 is critical for OSN development and function. In addition to altering OR expression, the absence of Emx2 causes OSN axons to terminate at the surface of the olfactory bulb where they form a fibrous cellular mass (Yoshida et al. 1997). OR expression in OSNs that lack contact with their targets is consistent with previous evidence of recovery of OR expression in bulbectomized rodents and with evidence that OR expression precedes contact of OSN axons with the bulb (Strotmann et al. 1995; Sullivan et al. 1995; Konzelmann et al. 1998). The lack of axonal contact with the olfactory bulb was therefore unlikely to have caused the changes of OR expression we observed in Emx2$^{-/-}$ mice.

Our evidence is similarly inconsistent with the interpretation that the axonal targeting defect in Emx2$^{-/-}$ mice was caused by the reduced expression of OR genes, largely because we did not find evidence that OSNs lack OR expression or have reduced transcription of the OR gene expressed, but rather the absence of Emx2 changed the frequency with which many OR genes were selected for expression. However, Emx2 has another putative function in OSNs that may be more relevant. Emx2 is reported to interact with eIF4E and may therefore regulate translation of proteins in OSNs (Nedelec et al. 2004). This interaction was detected in OSN axons, which also contain OR mRNAs (Vassar et al. 1994; Ressler et al. 1994), so it is possible to envision a scenario whereby changes in OR protein translation in OSN axons result in altered OSN axon behavior. ORs are important for the coalescence of OSN axons expressing the same OR, and they might also be involved in the generation of cyclic adenosine 3′,5′-monophosphate that is important for OSN axon extension during development (Imai et al. 2006). If translation of OR mRNAs in OSN axons is reduced in the absence of Emx2, then OSN axon behavior could be compromised, leading to defects in both axon extension and fasciculation. However, alternative causes, such as changes in the reception or processing of external guidance signals in Emx2$^{-/-}$ mice, are perhaps even more plausible.

The place of Emx2 in the hierarchy of OR gene regulation

Emx2 was not necessary for the zonality of OR gene expression. Neither did it appear to be necessary for the choice of a single OR gene by each OSN, as we would then have expected to observe widespread increases in the frequency of OR expression. Our data revealed no evidence implicating Emx2 in regulating clusters of OR genes, in the silencing of OR genes, or in the random inactivation of one parental allele of each OR gene. Instead, we conclude that Emx2 is a transcriptional activator for OR genes. Though it is necessary for producing normal frequencies of expression of many OR genes, it is perhaps best viewed as a permissive factor whose stimulatory action is gated by the contributions of other factors that control the singularity, zonality, and monoallelism of OR gene expression.

Supplementary Material

Supplementary material can be found at: http://www.chemse.oxfordjournals.org/.

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