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Single cell transcriptomics reveals receptor transformations during olfactory neurogenesis

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Abstract

The sense of smell allows chemicals to be perceived as diverse scents. We used single neuron RNA-Sequencing (RNA-Seq) to explore developmental mechanisms that shape this ability as nasal olfactory neurons mature in mice. Most mature neurons expressed only one of the roughly 1000 odorant receptor genes (*Olfrs*) available, and that at high levels. However, many immature neurons expressed low levels of multiple Olfrs. Coexpressed *Olfrs* localized to overlapping zones of the nasal epithelium, suggesting regional biases, but not to single genomic loci. A single immature neuron could express *Olfrs* from up to seven different chromosomes. The mature state in which expression of *Olfr* genes is restricted to one per neuron emerges over a developmental progression that appears independent of neuronal activity requiring sensory transduction molecules.

Odor detection in mammals is mediated by odorant receptors on olfactory sensory neurons (OSNs) in the nasal olfactory epithelium (1, 2). In mice, approximately 1000 odorant receptor genes (*Olfrs*) and 350 pseudogenes reside at dozens of distinct loci on 17/21 chromosomes (3–5). Each *Olfr* is expressed by a small subset of OSNs scattered in one epithelial spatial zone (6–8). Previous studies suggest that each mature OSN expresses one intact *Olfr* allele, but some coexpress an *Olfr* pseudogene (9–11). In a prevailing model of "OR (*Olfr*) gene choice", the developing OSN selects a single *Olfr* allele for expression and

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Author Contributions.

N.K.H., C.T., and L.B.B. designed research. N.K.H. and C.T. performed research, N.K.H., C.T., K.K., Z.L., D.K., X.Y., X.Q., and L.B.B. analyzed data, L.P. provided guidance, and N.K.H, C.T., and L.B.B. wrote the paper.

Supplementary Materials:
Materials and Methods
Figure S1–S5
Tables S1–S7
References (28–35)

the encoded receptor provides feedback that prevents expression of other *Olfrs* (12–17). OSNs are generated from a developmental progression from progenitors to precursors to immature OSNs to mature OSNs (18, 19). Here we investigated when and how the developing OSN selects one *Olfr* for expression.

We used single cell RNA sequencing (RNA-Seq) (20) to analyze the transcriptomes of single epithelial neurons during development. We first prepared cDNA libraries from single isolated cells (10) and analyzed the libraries for markers of the four stages of OSN development using PCR. We then conducted Illumina sequencing (21) of libraries from multiple cells of each stage, as well as duplicate libraries from some cells. We used TopHat (22) and Cufflinks (23) to identify genes expressed in each cell and estimate their relative mRNA abundances (see Fig. S1 for technical quality metrics).

We compared 85 cell transcriptomes using Monocle, an unsupervised algorithm that determines each cell's stage of differentiation in "pseudotime", which represents progress through gene expression changes during development (24). Monocle showed a linear, nonbranching trajectory of development (Fig. 1A). Based on cell stage markers in individual transcriptomes, the trajectory reflects the developmental progression from progenitors to precursors to immature OSNs to mature OSNs. The markers used were: Progenitor, *Ascl1* (achaete-scute complex homolog 1); Precursor, *Neurog1 (*neurogenin 1) and/or *Neurod1 (*neurogenic differentiation 1); Immature OSN, *Gap43 (*growth associated protein 43) and/or *Gng8 (*guanine nucleotide binding protein gamma 8); Mature OSN, *Omp* (olfactory marker protein) and four olfactory sensory transduction molecules downstream of odorant receptors: *Gnal* (guanine nucleotide binding protein, alpha stimulating, olfactory type), *Adcy3* (adenylate cyclase 3), *Cnga2* (cyclic nucleotide gated channel alpha 2), and *Cnga4* (cyclic nucleotide gated channel alpha 4) (18, 19).

Immature OSNs were further divided into two subsets based on their expression of olfactory sensory transduction molecules. Early immature OSNs lacked one or more olfactory transduction molecules while late immature OSNs expressed all four (Fig. 2D).

A total of 3830 genes were differentially expressed over development. Clusters of genes changed in expression during specific developmental periods, suggesting sequential large and coordinated changes in gene expression during OSN development (Fig. 1B and table S1). By gene ontology, most clusters contained genes associated with transcriptional regulation and/or chromatin modification, suggesting potential regulators of development (table S1). In kinetic diagrams, markers of early and late developmental stages show peak expression early and late in the developmental progression, respectively (Fig. 1C and S2).

Olfr expression first appeared at the late precursor to early immature OSN stage (Fig. 2). *Olfr* transcripts were found in 1/9 precursors, 38/40 immature OSNs, and 25/25 mature OSNs (Fig. 2). None were seen in two non-neuronal epithelial supporting cells or 3 cells of undetermined type. Overall, the number of *Olfr* transcripts per cell increased over OSN development (Fig. 2A). In early immature, late immature, and mature OSNs, they were detected at an average of 735, 1329, and 6376 FPKM (fragments per kilobase of transcript per million mapped reads), respectively, with median values of 87, 517, and 2635 FPKM.

These studies indicate that the developing OSN can initially express multiple Olfrs. Roughly half (52%, 13/25) of early immature OSNs expressed >1 *Olfr*. Coexpression of different *Olfrs* in single neurons declined as development progressed, with 38.5% (5/13) of late immature and 24% (6/25) mature OSNs expressing >1 *Olfr*. Moreover, single early immature OSNs expressed up to 12 different Olfrs, whereas mature OSNs with >1 *Olfr* expressed two or at most three (Fig. 2B).

Early immature and mature OSNs with >1 *Olfr* also differed in the relative abundance of different *Olfr* transcripts. Most (11/13) of the early immature OSNs had similar low levels of different *Olfrs*. The most abundant *Olfrs* were detected at 55–396 FPKM and the next highest at, on average, 63.1% this level (median: 67.3%). However, in 3/6 mature OSNs with >1 *Olfr*, the most abundant was detected at 14,557–18,056 FPKM, with the next highest, on average, only 3.3% as abundant (median: 0.5%).

In mature OSNs, *Olfr* and *Omp* transcripts averaged 6376 and 10,167 FPKM per cell, respectively. However, 6/12 early immature OSNs that expressed >1 *Olfr* did not express *Omp* (Fig. 2D), arguing against the possibility that the *Olfr* transcripts detected were due to contamination from mature OSNs.

Data from 8 duplicate cell samples (technical replicates) were analyzed (Fig. S3–S4). The duplicates confirmed the expression of >1 *Olfr* in specific OSNs (table S2). The data were consistent with reported stochastic losses of low copy number transcripts in single cell RNA-Seq data. *Olfrs* present in both replicates tended to be expressed at higher levels and those present in only one replicate at lower levels.

The above results indicated that early immature OSNs can express low levels of multiple *Olfrs*, but, during subsequent development, two changes typically occur. Expression favors one *Olfr* by up to 100-fold or more, and the expression of additional *Olfrs* declines or disappears.

To validate these findings, we used dual fluorescence RNA in situ hybridization (RNA-FISH) with nasal tissue sections. At postnatal day 3 (P3), a peak time of OSN neurogenesis (19, 25), 0.22 ± 0.05 to 0.22 ± 0.12% of neurons labeled for a single *Olfr* were colabeled with a mix of probes for other *Olfrs* expressed in the same nasal zone (Fig. 3A and table S3). No colabeled cells were seen in adults, where neurogenesis has decreased. Using a highly sensitive RNA-FISH method with branched DNA signal amplification (26), 0.41 ± 0.10 to 0.60 ± 0.13% of cells labeled for one *Olfr* were colabeled for another *Olfr* at P3 and 0.11 ± 0.02 to 0.18 ± 0.05% at P21 (Fig. 3B and table S4). Among neurons labeled for one *Olfr*, the percentage colabeled for the immature OSN markers *Gap43* and *Gng8* also changed, respectively, from 80.0 ± 3.1% and 62.8 ± 0.9 % at P3 to 19.5 ± 0.5% and 14.3 ± 1.1% at P21 (table S5). These results confirm that single OSNs can express more than one *Olfr* and suggest that *Olfr* coexpression occurs predominantly, if not exclusively, in immature OSNs.

To examine whether odorant receptor-induced neuronal activity might be involved in the observed developmental shift in *Olfr* expression, we analyzed transcriptome data for the expression of olfactory sensory transduction molecules: *Gnal* (or *Gnas*, which may substitute for *Gnal*), *Adcy3*, *Cnga2*, and *Cnga4*. All four molecules were expressed in 5/18 immature

OSNs and 6/6 mature OSNs with >1 *Olfr* (Fig. 2D). Furthermore, one or more were absent in data from 12/20 immature and 3/19 mature OSNs with only one *Olfr*. These results suggest that odorant-receptor induced neuronal activity is neither necessary nor sufficient for the decline in coexpressed *Olfrs* during development.

We next asked if the developing OSN is restricted to activating *Olfrs* expressed in a particular nasal zone. Using dual RNA-FISH, we compared the nasal expression patterns of 12 pairs of *Olfrs* coexpressed in 7 different OSNs. In every case, the paired *Olfrs* were expressed in either the same spatial zone or partially overlapping zones (Fig. 3C and table S6). These results indicate that the developing neuron is restricted to the expression of a particular *Olfr* zonal gene set, which can include *Olfrs* with only partially overlapping expression patterns in the adult.

To investigate whether early coexpression of multiple *Olfrs* could result from chromatin changes at a single genomic locus containing those *Olfrs*, we determined the chromosome locations of *Olfrs* coexpressed in individual OSNs (Fig. 4 and table S7). For OSNs expressing 4–12 *Olfrs*, coexpressed *Olfrs* mapped to 3–7 different chromosomes and 4–9 distinguishable *Olfr* gene loci (Fig. 4 and table S7). Thus, the immature OSN is not restricted to expressing *Olfrs* from a single chromosomal region.

Odor detection in the mouse nose is mediated by 1000 different odorant receptors, each expressed by a different subset of sensory neurons. We have asked when and how the neuron comes to express a single *Olfr*. We find that the developing neuron can express low levels of multiple *Olfrs*. As development proceeds, this ability declines. The mature neuron typically expresses high levels of a single *Olfr*. Coexpressed *Olfrs* tend to be expressed by other neurons in the same region of the olfactory epithelium, suggesting regional biases in *Olfr* gene choice, but they can reside at multiple chromosomal locations.

How does the developing OSN transition from expressing low levels of multiple *Olfrs* to expressing a high level of a single *Olfr*? One possibility is a “winner-takes-all” mechanism. In this model, multiple *Olfrs* are initially expressed, but one becomes dominant, for example by the capture of limiting factors required for high level *Olfr* expression (Fig. S5). In a second model, selection of a single *Olfr* for high level expression would occur independently of those initially expressed. In either model, early low level expression of other *Olfrs* could subside owing to the closing of a developmental time window or to feedback signals generated by the highly expressed *Olfr*. OSNs expressing multiple *Olfrs* are probably not pruned by apoptosis, as suggested for OSNs in the nasal septal organ (27), given genetic evidence that some OSNs expressing one *Olfr* previously expressed another (13). This *Olfr* “switching” may reflect the early expression of more than one *Olfr* per immature OSN, as observed in the present studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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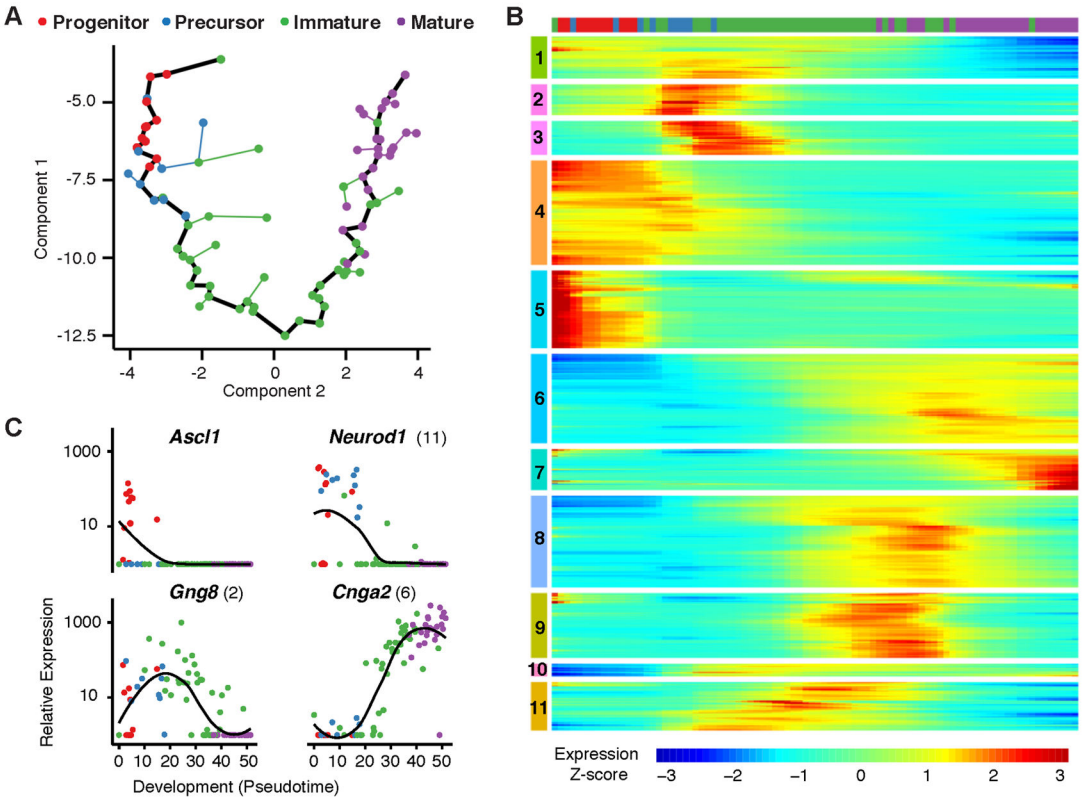

Figure 1. Olfactory neurons exhibit large-scale shifts in gene expression during development

A. Unsupervised analysis of single cell gene expression profiles with Monocle revealed a linear trajectory (black line) along which cells develop in a dimension referred to as “pseudotime”. Coloring of cells based on the expression of developmental markers shows that the trajectory corresponds to a stepwise development from olfactory progenitors to precursors to immature OSNs to mature OSNs.

B. Global analysis of gene expression kinetics along the trajectory identified 3,830 genes that vary significantly over pseudotime development (FDR < 5% by a Tobit-valued generalized linear model likelihood ratio test; see Methods). Hierarchical clustering of these genes via Ward’s method recovered 11 non-redundant groups that covary over the trajectory. Cluster analysis indicates that multiple large shifts in gene expression occur as neurons progress through development. The bar on top shows the locations of individual cells, colored by stage of development, along this developmental trajectory.

C. Kinetic diagrams show the expression of known markers of different developmental stages over the developmental progression. Parentheses indicate the groups in which genes are found in part B. Dots indicate individual cells colored according to developmental stage. Black lines indicate loess smoothing (span = 0.75, degree = 2) of log-transformed FPKM values over developmental pseudotime.

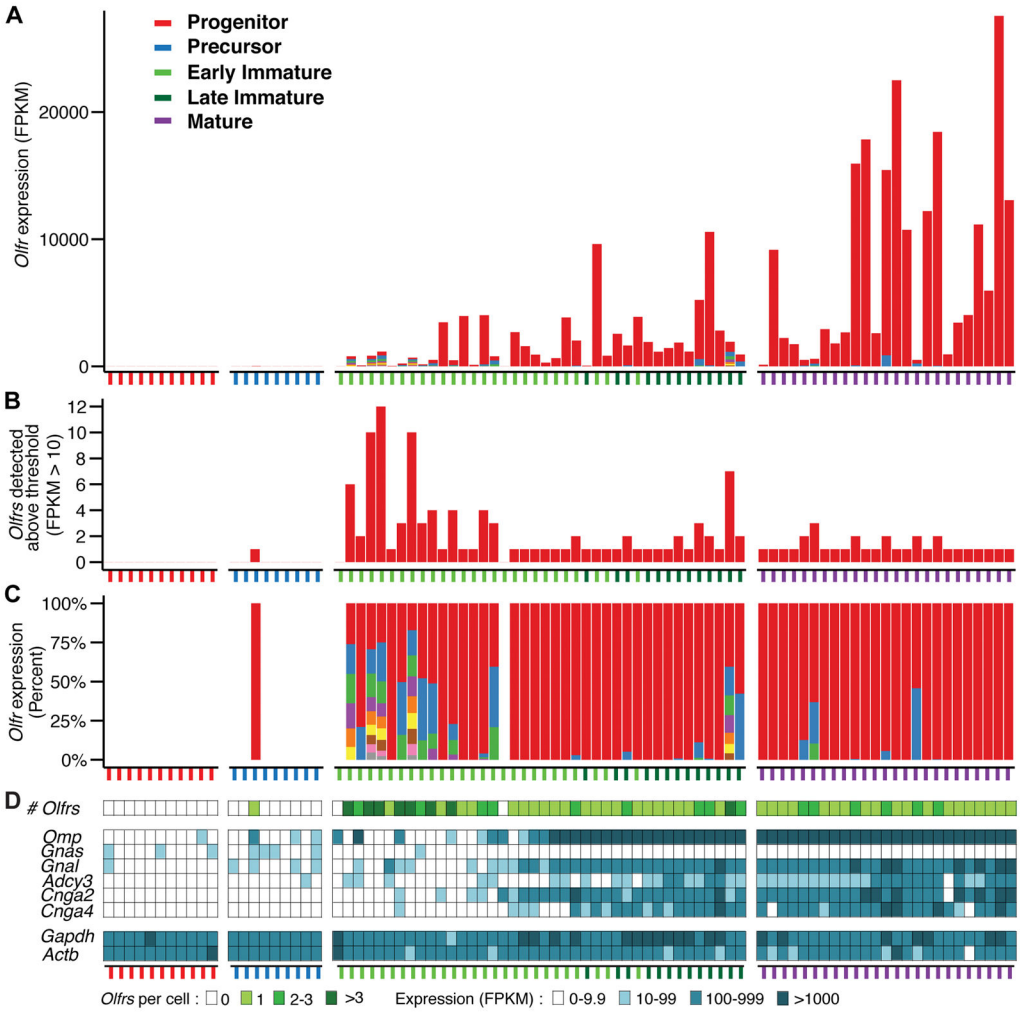

Figure 2. Immature neurons can express multiple *Olfrs*

A. Neurons assigned to different developmental stages were arranged by developmental progress, as measured in pseudotime. Early versus late immature OSNs are indicated by differently colored ticks. Different *Olfrs* are represented by different colors in the bars. The total number of *Olfr* transcripts per cell shows a steady, though variable, increase over development.

B. Multiple different *Olfr* transcripts were detected in 13/25 early immature, 5/13 late immature, and 6/25 mature OSNs.

C. The number of different *Olfr* transcripts per cell was highest in early immature OSNs and then declined over development. Early immature OSNs tended to express similar levels of different *Olfrs*. In contrast, the majority of mature OSNs expressed only one *Olfr* or high levels of one *Olfr* and low levels of one or two additional *Olfrs*. Each color in a bar represents a single *Olfr,* except gray, which represents >1 *Olfr.*

D. *Olfrs* stimulate neuronal activity via mechanisms involving sensory transduction molecules encoded by *Gnal* (or possible *Gnas* in immature OSNs), *Adcy3*, *Cnga2*, and *Cnga4*. Five immature and six mature neurons with >1 *Olfr* expressed all four genes, suggesting that neuronal activity downstream of odorant receptors is not what reduces the

number of *Olfrs* expressed per neuron. *Omp*, which is highly expressed in mature OSNs, was absent from six early immature OSNs with >1 *Olfr*, arguing against contamination from mature OSNs. *Gapdh* and *Actb* are housekeeping genes.

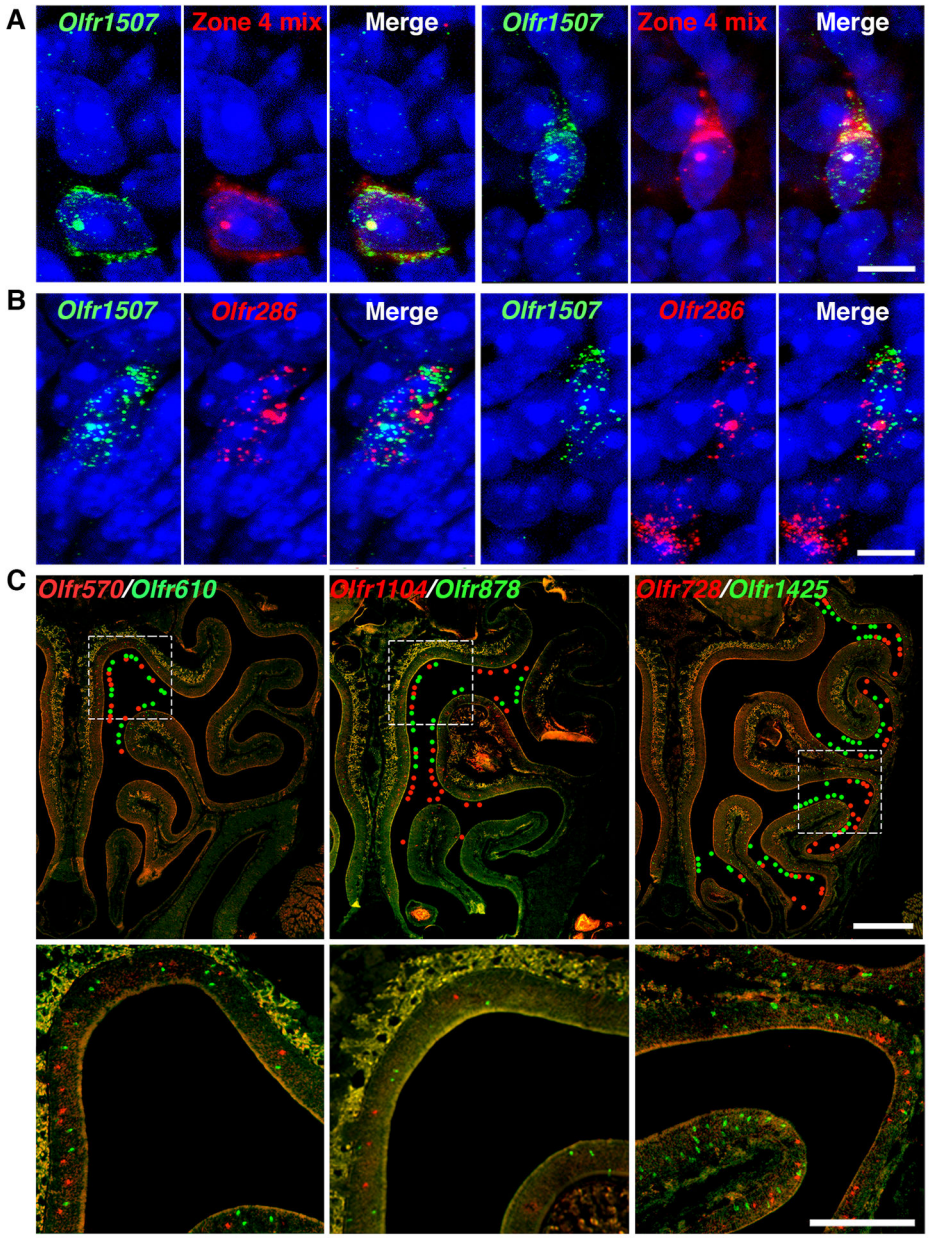

Fig. 3

Figure 3. *Olfrs* expressed in the same neuron belong to a zonal gene set

A. Dual RNA-FISH of P3 tissue sections using a conventional method showed a small percentage of OSNs colabeled with an *Olfr1507* probe and a mix of probes for other *Olfrs* expressed in the same zone (Zone 4). Cells were counterstained with DAPI (blue). Two colabeled cells are seen here, one at left and the other at right. Scale bar, 5 μm.

B. Dual RNA-FISH of P3 tissue sections using a highly sensitive method showed a small percentage of OSNs coexpressing *Olfr1507* and *Olfr286*. Two colabeled cells are seen here at left and right and a cell labeled with only one probe is also seen at right. Scale bar, 5 μm.

C. Dual RNA-FISH shows that *Olfrs* coexpressed in single immature OSNs (Cells D200, D197, or D243) are singly expressed in neurons in the same or partially overlapping zones in adult olfactory epithelium sections. This correspondence indicates that *Olfr* expression in the immature OSN is restricted to a zonally-determined set of *Olfr* genes. Colored dots indicate the locations of labeled neurons in the upper row. Boxed areas in the upper row are shown at higher magnification in the lower row. Scale bar, 500 μm (upper row), 250 μm (lower row).

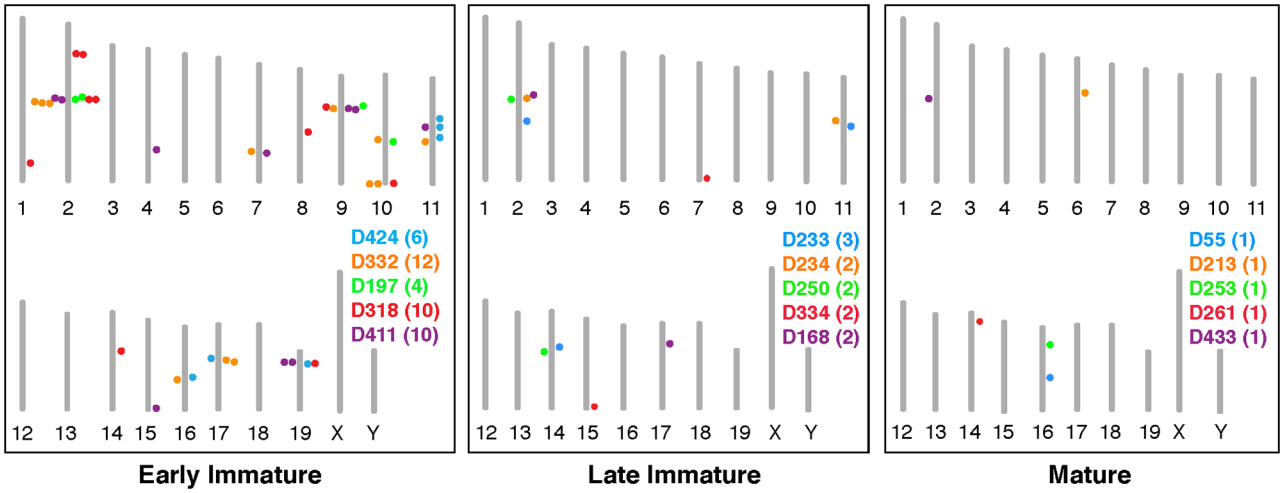

Figure 4. Immature neurons coexpress *Olfrs* from multiple chromosomal loci

Diagrams show the chromosomal locations of *Olfrs* expressed in single OSNs of different stages. Each mouse chromosome is indicated by a vertical bar with its number below. The names of neurons, parenthesized number of *Olfrs* per neuron, and dots showing the chromosomal locations of those *Olfrs* are shown in different colors for different neurons.