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MOLECULAR AND SYNAPTIC MECHANISMS

Differential expression of axon-sorting molecules in mouse olfactory sensory neurons

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Abstract

In the mouse olfactory system, the axons of olfactory sensory neurons that express the same type of odorant receptor (OR) converge to a specific set of glomeruli in the olfactory bulb (OB). It is widely accepted that expressed OR molecules instruct glomerular segregation by regulating the expression of axon-sorting molecules. Although the relationship between the expression of axon-sorting molecules and OR types has been analyzed in detail, those between the expressions of axon-sorting molecules remain to be elucidated. Here we collected the expression profiles of four axon-sorting molecules from a large number of glomeruli in the OB. These molecules demonstrated position-independent mosaic expressions, but their patterns were not identical in the OB. Comparing their expressions identified positive and negative correlations between several pairs of genes even though they showed various expressions. Furthermore, the principal component analysis revealed that the factor loadings in the principal component 1, which explain the largest amount of variation, were most likely to reflect the degree of the cyclic nucleotide-gated (CNG) channel dependence on the expression of axon-sorting molecules. Thus, neural activity generated through the CNG channel is a major component in the generation of a wide variety of expressions of axon-sorting molecules in glomerular segregation.

Introduction

In the mouse olfactory system, olfactory receptor (OR) genes form a multigene family comprising > 1000 genes. An individual olfactory sensory neuron (OSN) expresses only one functional OR of this rich repertoire of the OR genes (Buck & Axel, 1991). Although OSNs expressing a given type of OR are randomly distributed in restricted regions of the olfactory epithelium (OE), their axons converge to a few spatially invariant glomeruli in the olfactory bulb (OB) (Mombaerts *et al.*, 1996). The development of the olfactory glomerular map comprises initial global targeting and subsequent local glomerular segregation (Mori & Sakano, 2011; Takeuchi & Sakano, 2014). The global targeting process is genetically determined and regulated by two independent mechanisms that control different axes of axonal extension in the OB. For dorsal–ventral projection, the positional information of OSNs within the OE regulates both OR gene choice and expression levels of axon guidance molecules, thereby correlating OR identity with glomerular location (Norlin *et al.*, 2001; Cho *et al.*, 2007; Takeuchi *et al.*, 2010). Furthermore, anterior–posterior (A–P) projection is dependent on the expressed OR species (Wang *et al.*, 1998). Our recent study has shown that ligand-independent basal activities of ORs regulate global targeting along the A–P axis (Nakashima *et al.*, 2013a).

After OSN axons are directed to approximate target regions, axons expressing the same OR type are locally sorted out from other axons and converge to form glomerular structures. The local sorting and convergence of OSN axons are regulated by OR proteins with remarkable precision; only a single amino acid substitution of OR sequence can generate closely located, but segregated, glomeruli (Ishii *et al.*, 2001; Feinstein & Mombaerts, 2004). How is the OR identity represented at OSN axon termini for axonal convergence? It has long been believed that OR protein functions not only as an odor detector but also as a guidance molecule, because the protein is present at OSN axon termini (Barnea *et al.*, 2004; Feinstein *et al.*, 2004). In contrast, we have previously shown that OR molecules control the expression of axon-sorting molecules, which regulate glomerular segregation through their adhesive or repulsive interactions (Serizawa *et al.*, 2006). Moreover, evidence is accumulating that OR proteins generate the neuronal identity code by a unique combination of axon-sorting molecules. To date, more than five genes have been reported to be

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involved in glomerular segregation (Serizawa *et al.*, 2006; Kaneko-Goto *et al.*, 2008; Williams *et al.*, 2011). However, no study has yet drawn a complete picture of the gene expression patterns at the single glomerular level. In this study, we collected the expression profiles of axon-sorting molecules from a large number of glomeruli and compared their expressions to identify correlations between them.

Material and methods

Animals

A total of 11 animals was used in this study. All animals were deeply anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and perfused transcardially with 4% paraformaldehyde in 0.1 M PBS. OE and OB tissues were then removed. All experimental procedures were performed with the approval of the animal experiment ethics committee at the University of Tokyo and according to the University of Tokyo guidelines for the care and use of laboratory animals. *CNG-KO* mice were purchased from the Jackson Laboratory.

In situ *hybridization and immunostaining*

In situ hybridization was performed as described (Takeuchi *et al.*, 2010). Following sequences were used to generate anti-sense RNA probes: *Kirrel2* (nt1413-2103), *OLPC* (nt1-527, 2433–2631), *Sema7A* (nt265-1058, 1037–1622, 1556–1995), *PCDH17* (nt16-821, 1884–2697) and *OMP* (nt1-2168).

Immunostaining was performed essentially as described (Serizawa *et al.*, 2006). Primary antibodies used are as follows: goat anti-Kirrel2 antibodies (1 : 1000, AF2930; R&D Systems); goat anti-Sema7A antibodies (1 : 500, AF2068; R&D Systems); rat anti-OLPC antibodies (1 : 500, MABT20; Merck Millipore); mouse VGLUT-2 antibody (1 : 500, MAB5504; Merck Millipore); goat anti-NQO1 antibody (1 : 500, ab2346; Abcam) guinea pig anti-PCDH17

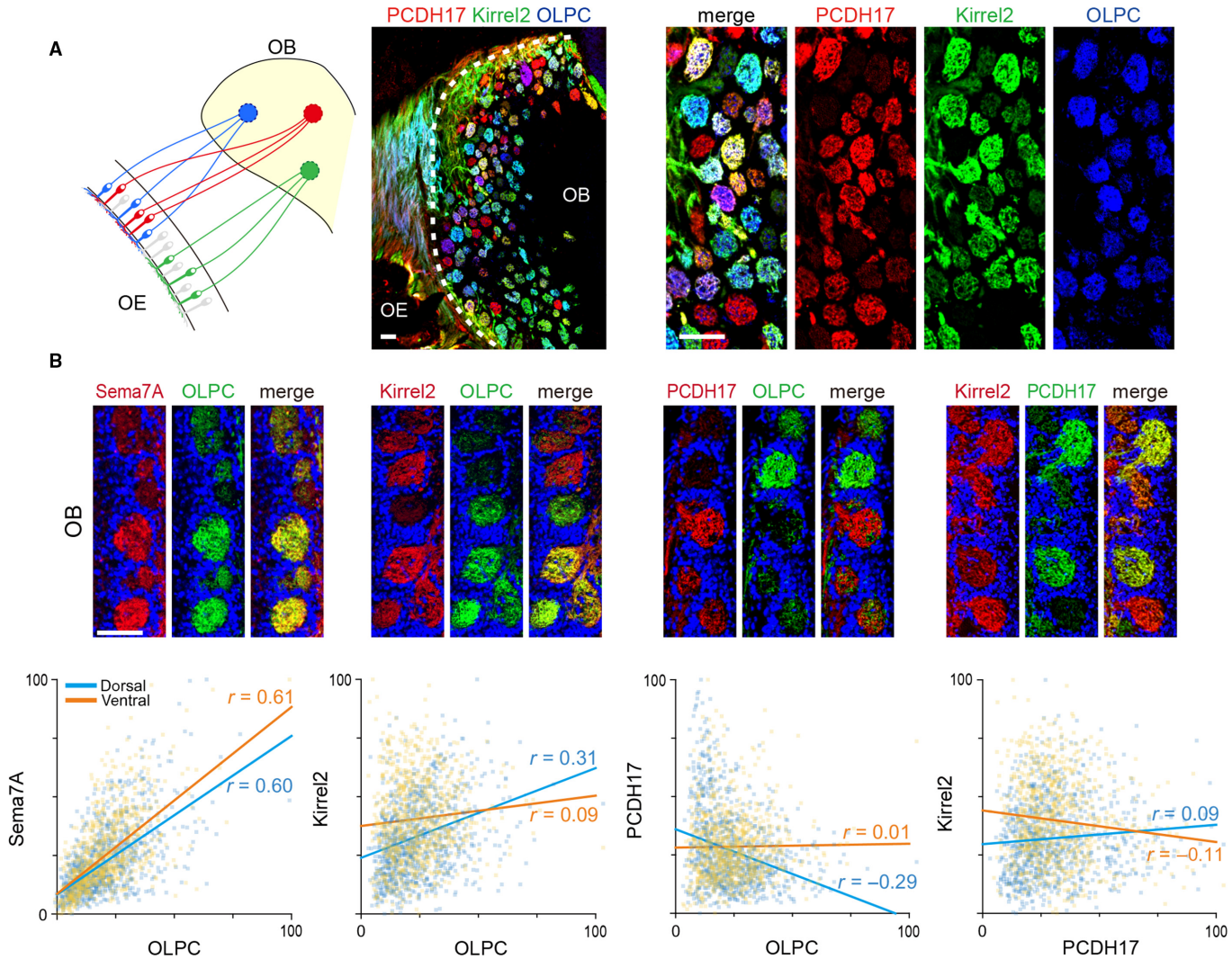

FIG. 1. Differential expression patterns of axon-sorting molecules in OB. (A) A parasagittal OB section from 2-week-old mice was immunostained with antibodies against PCDH17 (red), Kirrel2 (green) and OLPC (blue). Enlarged images are shown at right. (B) Relationships between expression levels of axon-sorting molecules. The OB sections were immunostained with antibodies against Sema7A, OLPC, Kirrel2 and PCDH17 (top). Staining intensities of Sema7A, OLPC, Kirrel2 and PCDH17 in each glomerulus were measured and plotted (bottom). Each dot represents a single glomerulus. Glomeruli were divided into two groups, dorsal (blue) and ventral (orange), according to immunohistochemistry with antibodies against the dorsal OSN marker, NQO1. The regression lines for dorsal and ventral glomeruli are shown in blue and orange, respectively. A total of 1799 glomeruli (940 from the dorsal and 859 from the ventral region) were analyzed. Scale bars, 100 μm.

antibodies (1 : 500) (Hoshina *et al.*, 2013). Anti-Kirrel2 antibodies (guinea pig) were generated by immunizing guinea pigs with KLH-conjugated synthetic peptides (644–673aa): CRLYRARAGYLTTPH-PRAFTSYMKPTSFGP (Operon Biotechnologies).

Intensity measurements and statistical analyses

Optical and fluorescent Images were photographed with BZ-X700 microscope (Keyence). Glomerulus was defined by immunofluorescence signals of vesicular glutamate transporter2. Staining intensities were measured with ImageJ (NIH). After subtracting background signals, staining intensities were normalized to 100% and correlation coefficients between all possible axon-sorting molecule pairs were calculated. Affinity propagation and Principal component analysis (PCA) were performed with MATLAB software and ORIGINPRO software, respectively.

Results

Glomerular segregation is regulated by adhesive/repulsive axonal interactions mediated by molecules that are present at OSN axon termini. As candidate molecules meeting this criterion, we selected the following genes: *Kirrel2*, *Sema7A*, *OLPC* and *PCDH17*. Kirrel2 belongs to the immunoglobulin superfamily and is involved in glomerular segregation by homophilic adhesion (Serizawa *et al.*, 2006). Sema7A is a membrane-bound semaphorin that acts as an axon guidance and pruning molecule (Pasterkamp *et al.*, 2003). OLPC and PCDH17 belong to the non-clustered delta-protocadherin subfamily and both play important roles in cell adhesion (Williams *et al.*, 2011; Hayashi *et al.*, 2014). Immunohistochemistry of the OB sections revealed that all molecules showed position-independent mosaic expressions, but their patterns were not identical (Fig. 1A, right). To identify relationships between their expressions, serial sections of the entire OB were immunostained with antibodies against these molecules (Fig. 1B, top). We collected expression profiles of these genes by measuring the staining intensities of glomeruli from the entire OBs. Correlations of the expression of axon-sorting molecules were calculated for all possible combinations. As shown in Fig. 1B and Table 1, we observed the following features. We observed a strong positive correlation between the expression of Sema7A and OLPC. We also found a weak positive correlation between the expression of Sema7A and Kirrel2. These positive correlations suggest common regulatory mechanisms underlying their expressions. However, there was no significant correlation between the expression of Kirrel2 and OLPC. PCDH17 and OLPC showed a week negative correlation. The relationship between their expression profiles differed with glomerular location, suggesting that there is a different regulatory mechanism for expressions in the dorsal and ventral OSNs.

A single glomerulus can be considered as a data unit that is represented by multiple variables. To analyze the multivariate data, we performed affinity propagation, an unsupervised clustering method that is used for multivariate data (Frey & Dueck, 2007). Affinity propagation separated 1799 glomeruli into eight subclusters (Fig 2A); each subcluster was defined by a unique expression profile

TABLE 1. Summary of correlation coefficients between the expression profiles of axon-sorting molecules

	Dorsal OB		Ventral OB		Whole OB	
	#1 (n = 940)	#2 (n = 934)	#1 (n = 859)	#2 (n = 751)	#1 (n = 1799)	#2 (n = 1685)
OLPC/Sema7A	**0.60***	**0.61***	**0.61***	**0.57***	**0.54***	**0.50***
OLPC/Kirrel2	0.31*	0.19*	0.09	0.14	0.03	0.05
OLPC/PCDH17	−0.29*	0.17*	0.01	−0.17*	−0.18*	−0.19*
Kirrel2/PCDH17	0.09	0.21*	−0.11	0.08	0.02	0.18*
Sema7A/Kirrel2	**0.45***	0.49*	0.25*	**0.45***	0.37*	**0.51***
Sema7A/PCDH17	0.03	0.09	0.14*	0.03	0.09	0.10*

Correlation coefficients of the expression profiles of axon-sorting molecules (Sema7A, OLPC, Kirrel2 and PCDH17) are separately shown according to glomerular position in the OB. Significant correlations are indicated by an asterisk ($P^* < 0.0001$). Values in bold indicate strong correlations (correlation coefficient > 0.4).

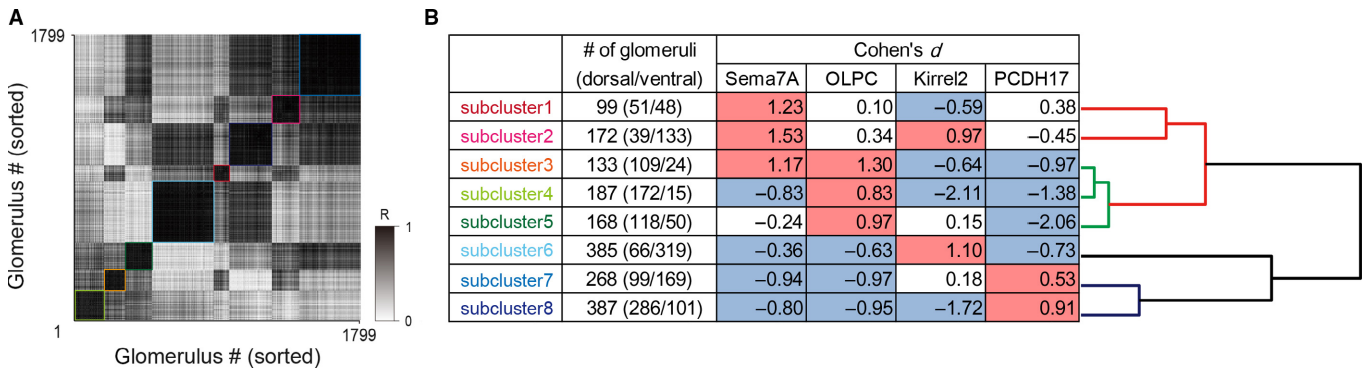

FIG. 2. Cluster analysis by affinity propagation. (A) Correlation coefficients of the expression profiles of axon-sorting molecules (Sema7A, OLPC, Kirrel2 and PCDH17) were calculated for all pairwise combinations. The correlation matrix obtained from 1799 glomeruli was sorted and separated into eight subclusters by affinity propagation. (B) Each subcluster was characterized by a unique gene expression profile. Cohen's d effect sizes of each subcluster in the entire datasets are shown in the table. Shadings indicate that the mean value of a subcluster is significantly ($P < 0.001$) higher (red) or lower (blue) than that of the entire cluster. Relationships of similarity among the subclusters are also shown as a dendrogram.

of axon-sorting molecules (Fig. 2B). It was found that the mean expression levels of Sema7A and OLPC in the subclusters 6–8 were significantly lower than the average. In addition, the mean expression levels of Sema7A and OLPC in the subcluster 3 were significantly higher than the average. These results suggest that subclusters 3, 6, 7 and 8 contribute to the strong positive correlation between Sema7A and OLPC expressions. The positive correlation between Sema7A and Kirrel2 expressions could be explained in the same manner. On the other hand, the subclusters 2, 4, 5 and 8 were consistent with the positive correlation between Sema7A and Kirrel2 expressions. Therefore, we conclude that different subclusters contributed differently to the expression correlations between axon-sorting molecules.

What are the major components involved in generating the wide variety of gene expression of these molecules? Principal component analysis (PCA) defines a new coordinate system, so that the first coordinate has the greatest variance across datasets and that each succeeding coordinate has the greatest residual variance. The expression datasets were subjected to PCA and plotted in the space of the first, second and third principal components (PC1, PC2 and PC3, respectively; Fig. 3A and B). The PC1, PC2 and PC3 accounted for 41.7, 28.2 and 22.1% of the total variance, respectively (Fig. 3C). The variables of Sema7A, OLPC and Kirrel2 were positively loaded in the PC1, but that of PCDH17 was not (Fig. 3C). Rather, the variable of PCDH17 was loaded in the PC2 and PC3. On the PC2 and PC3, the variables of Kirrel2 and OLPC were loaded in an opposite manner; Kirrel2 exhibited a positive value in the PC2 but a negative value in the PC3, whereas OLPC exhibited a positive value in the PC3 but a negative value in the PC2. The dorsal (blue dots in Fig. 3A) and ventral glomeruli (orange dots in Fig. 3A) were best separated in the PC2–PC3 space, suggesting that the PC2 and PC3 are likely associated with the positioning of OSNs.

The subclusters generated by affinity propagation were also well separated on the PCA space (Fig. 3B), validating the affinity

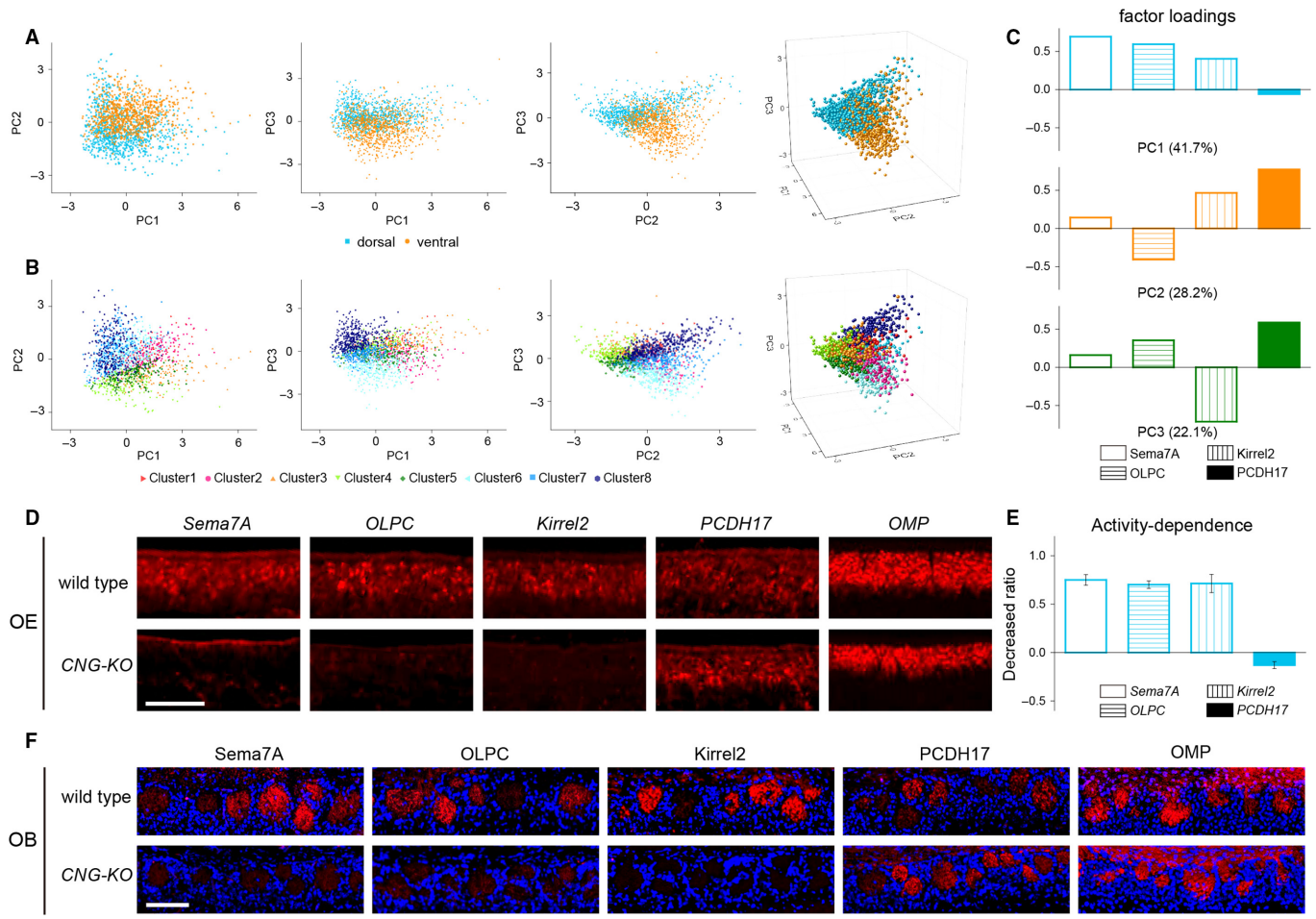

FIG. 3. PCA and activity-dependent expressions of axon-sorting molecules. (A) PCA score biplots (PC1 vs. PC2, PC1 vs. PC3 and PC2 vs. PC3) of the full dataset. Dorsal and ventral glomeruli are shown in blue and orange, respectively. The Dunn's index, which measures compactness and cluster separation, was calculated between the dorsal and ventral glomeruli (0.47 in PC1 vs. PC2, 0.47 in PC1 vs. PC3 and 0.64 in PC2 vs. PC3). A three-dimensional plot of PCA scores is also shown on the right. (B) The same as (A), but for the subclusters identified by affinity propagation in Fig. 2. (C) Factor loadings of Kirrel2, Sema7A, OLPC and PCDH17 in PC1–3. Eigenvalues of PC1, PC2 and PC3 were 1.67, 1.13 and 0.88, respectively. (D) *In situ* hybridization of the OE sections from *CNG-KO* and wild type using probes for *Sema7A*, *OLPC*, *Kirrel2*, *PCDH17* and *OMP*. (E) Activity-dependent expressions of axon-sorting molecules. Signal intensities of axon-sorting molecules were compared between *CNG-KO* and wild type. The expression of *OMP*, a marker for OSNs, was used to normalize signal intensities. The degree of dependence on CNG channel-mediated activity was calculated as decreased ratio of staining intensities. $n = 4$ animals, error bars indicate SEM. (F) The OB sections from 2-week-old *CNG-KO* and wild type mice were immunostained with antibodies against Sema7A, OLPC, Kirrel2, PCDH17 and OMP. Scale bars, 100 μm.

propagation-based clustering and the PCA-based replotting. Then, what does the PC1 reflect? It must be the major component in glomerular segregation. We reasoned that the PC1 is related to neural activity for the following two observations: First, glomerular convergence is severely impaired by the suppression of neural activity (Yu *et al.*, 2004; Nakashima *et al.*, 2013a). Second, several axon-sorting molecules are expressed in an activity-dependent manner (Serizawa *et al.*, 2006; Kaneko-Goto *et al.*, 2008; Williams *et al.*, 2011). The cyclic nucleotide-gated (CNG) channel is a component of the olfactory signal transduction (Brunet *et al.*, 1996). Mutant mice deficient for the *CNGA2* gene (*CNG-KO*), which is a key component of CNG channel function, were analyzed with respect to the expression of axon-sorting molecules by *in situ* hybridization. *CNG-KO* showed marked downregulation of the expression of *Sema7A*, *OLPC* and *Kirrel2*, but not of *PCDH17* (Fig. 3D). The expression of Sema7A, OLPC and Kirrel2 in *CNG-KO* were also diminished at a protein level, whereas the mosaic expression of PCDH17 on the OB was not affected (Fig. 3F). It is evident that neural activity via the CNG channel is a major determinant of the expression of *Kirrel2*, *Sema7A* and *OLPC*. The patterns of CNG-dependent changes in the expression levels (Fig. 3E) resembled the factor loadings in the PC1 (Fig. 3C); the variables of Sema7A, OLPC and Kirrel2 were positively loaded in the PC1. These results are consistent with our idea that the variety of expressions of these molecules is generated by CNG channel-mediated neural activity.

Discussion

It is widely accepted that glomerular segregation is regulated by neural activity. Several candidate channels such as the CNG channel (Serizawa *et al.*, 2006; Kaneko-Goto *et al.*, 2008) and the HCN channel (Mobley *et al.*, 2010; Nakashima *et al.*, 2013b) have shown to be involved in glomerular segregation. In this study, we analyzed the expression dataset using hypothesis-free data mining. Even though the emergent results were independent of a preexisting bias or model, we obtained the result that the largest amount of variation in their expressions can be generated by neural activity via the CNG channel. This result strongly suggests that the CNG channel contributes most to generate the combinatorial expression of axon-sorting molecules for glomerular segregation.

PCDH17 expression was not affected by the *CNG-KO*. How is the expression of *PCDH17* regulated? It is possible that the *PCDH17* expression is regulated by neural activity via other channels such as the HCN channel. This CNG channel independent expression of *PCDH17* could explain why the axonal convergence was differentially affected according to expressed OR types in the *CNG-KO* (Zheng *et al.*, 2000).

We also found that the expressions of *Sema7A*, *Kirrel2* and *OLPC* show differential expression patterns even though their expressions are almost entirely dependent on neural activity via the CNG channel. How is it, then, that OR proteins can differentially regulate the expressions of these axon-sorting molecules through the CNG channel? OSNs may have different decoding schemes of neuronal activity that generate the differential expression patterns of axon-sorting molecules. For example, it is possible that the expressions of axon-sorting molecules are differentially induced by different patterns of action potentials. Further analysis is required to understand how OR identity is converted into a combinatorial expression of axon-sorting molecules through neural activity.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

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Abbreviations

CNG, cyclic nucleotide-gated; OB, olfactory bulb; OE, olfactory epithelium; OR, olfactory receptor; OSN, olfactory sensory neuron; PCA, principal component analysis.

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