INTRODUCTION: The development of precise neural circuits is initially directed by genetic programming and subsequently refined by neural activity. In the mouse olfactory system, axons from various olfactory sensory neurons expressing the same olfactory receptor converge onto a few spatially invariant glomeruli, generating the olfactory glomerular map in the olfactory bulbs. During development, olfactory receptors instruct axon sorting to form discrete glomeruli. Olfactory receptors generate a combinatorial code of axon-sorting molecules whose expression is regulated by neural activity. However, it remains unclear how neural activity induces olfactory receptor–specific expression patterns of axon-sorting molecules.

RATIONALE: The prevailing model for the activity-dependent development of neural circuits postulates an interaction between pre-pattern-dependent gene regulation may also expand beyond development to plastic changes in neural circuits throughout the lifetime.

CONCLUSION: We have demonstrated an instructive role of neural activity in olfactory map formation. We propose an activity-dependent mechanism, different from Hebbian plasticity theory, in which specific patterns of spontaneous neuronal spikes were not spatially organized, but rather were correlated with the olfactory receptor types. Receptor substitution experiments demonstrated that olfactory receptors determine spontaneous activity patterns. Moreover, optogenetically differentiated patterns of neuronal activity induced expression of corresponding axon-sorting molecules and regulated glomerular segregation.

RESULTS: We performed calcium imaging experiments and optogenetic stimulation to address how neural activity generates olfactory receptor–specific expression patterns of axon-sorting molecules. Calcium imaging of olfactory sensory neurons revealed that the temporal patterns of spontaneous neuronal spikes were not spatially organized, but rather were correlated with the olfactory receptor types. Receptor substitution experiments demonstrated that olfactory receptors determine spontaneous activity patterns. Moreover, optogenetically differentiated patterns of neuronal activity induced expression of corresponding axon-sorting molecules and regulated glomerular segregation.
Structured spike series specify gene expression patterns for olfactory circuit formation

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Neural circuits emerge through the interplay of genetic programming and activity-dependent processes. During the development of the mouse olfactory map, axons segregate into distinct glomeruli in an olfactory receptor (OR)-dependent manner. ORs generate a combinatorial code of axon-sorting molecules whose expression is regulated by neural activity. However, it remains unclear how neural activity induces OR-specific expression patterns of axon-sorting molecules. We found that the temporal patterns of spontaneous neuronal spikes were not spatially organized but were correlated with the OR types. Receptor substitution experiments demonstrated that ORs determine spontaneous activity patterns. Moreover, optogenetically differentiated patterns of neuronal activity induced specific expression of the corresponding axon-sorting molecules and regulated axonal segregation. Thus, OR-dependent temporal patterns of spontaneous activity play instructive roles in generating the combinatorial code of axon-sorting molecules during olfactory map formation.

In the mammalian brain, the development of precise neural circuits is initially directed by intrinsic genetic programming and subsequently refined by neural activity (1–3). In the mouse olfactory system, individual olfactory sensory neurons (OSNs) express a single gene of a functional olfactory receptor (OR) out of >1000 OR genes (4–6). Axons from various OSNs expressing the same OR converge onto a few spatially invariant glomeruli, generating the olfactory glomerular map in the olfactory bulbs (OBS) (7–9). The olfactory glomerular map forms through two processes: global targeting and glomerular segregation (10, 11). OSN axons are first guided to approximate target regions according to gradients of axon guidance molecules (12, 13) and are subsequently sorted into specific glomerular structures in an activity-dependent manner (11, 14–16). In glomerular segregation, OSN axons discriminate expressed OR types. Substitution of an OR-coding DNA sequence with another result in glomerular segregation that are distinct from the original ones (17, 18). Further, suppression of spontaneous neural activity disrupts the glomerular segregation (15). However, it remains a mystery how neural activity mediates the OR-specific glomerular segregation.

The most prevailing model for the activity-dependent development of neural circuits postulates the interaction between pre- and postsynaptic neurons. In Hebbian plasticity (19), the correlated activity of pre- and postsynaptic neurons strengthens synaptic connections, whereas uncorrelated activity or lack of activity weakens them. However, this theory does not explain activity-dependent mechanisms for axon sorting before the formation of synapses. OSN axons are capable of converging to form glomerular-like structures even in mutant mice lacking synaptic partners (20, 21). These findings suggest another activity-dependent mechanism for glomerular segregation.

OR molecules control the expression of various axon-sorting molecules, which serve to regulate glomerular segregation through their adhesive or repulsive interactions (22–26). These molecules exhibit both mosaic and glomerular-specific expression patterns (27). However, their spatial patterns are not identical in the OB. As a result, OR identity is represented as a unique combinatorial code of axon-sorting molecules at the axon termini, which provides the self-identification tags for OR-specific glomerular segregation. Because the expression of axon-sorting molecules is activity-dependent (22, 25), neural activity is expected to bridge between the OR types and the combinatorial expression of the axon-sorting molecules. Previous studies reported that spontaneous firing rates are different among OSN classes in several species (28–32). However, it remains unclear whether neural activity functions in an instructive way or a permissive way; it is not clear whether specific features of neural activity patterns are important or whether the presence of neural activity is sufficient to trigger a predetermined gene expression. Here, we applied genetic approaches to address how neural activity generates OR-specific expression patterns of axon-sorting molecules.

Activity-dependent expression of axon-sorting molecules

On the basis of our previous study (27), we focused on Kirrel2, Sema7A, and PCDH10 as the axon-sorting molecules for glomerular segregation. Genetic knockout (KO) experiments demonstrated that these molecules are involved in neural activity is sufficient to trigger a predetermined gene expression. Here, we applied genetic approaches to address how neural activity generates OR-specific expression patterns of axon-sorting molecules.

Visualizing OSN spontaneous activity

To examine the nature of OSN spontaneous activity, we used a Cre/loxP approach (pGadgh-Cre: pCAG-LSL-GeCaMP6f (Ai953D) mice) to express the genetically encoded calcium indicator GCaMP6f (33) specifically in OSNs (Fig. 2A). In the OSN-specific GCaMP6f mice, action potentials were reliably detected as transient increases in the fluorescence intensities of GCaMP6f (fig. S3). Correlation analyses revealed that spontaneous calcium events were not spatially or temporally correlated between OSNs in ex vivo acute olfactory epithelium (OE) slices from neonatal mice (Fig. 2, A and B; see also movie S1), which suggested that spontaneous calcium transients are sporadic events and occur independent of neighboring OSNs. These results are in contrast to the spontaneous activity patterns observed in the developing retina, which generates temporally correlated activity among neighboring retinal
ganglion cells and regulates precise circuit formation (10, 36–39). In the CNG-KO mice, spontaneous activity was still present (34) (fig. S3E, see also movie S2) but in altered patterns; the event frequencies of calcium spikes and the inter-event intervals (IEIs) (frequency = 0.89 ± 1.02 times/min, IEI = 59.09 ± 61.52 s; mean ± SD, N = 230 cells) were lower and longer, respectively, relative to those in wild-type mice (frequency = 0.90 ± 1.02 times/min, IEI = 59.09 ± 61.52 s; mean ± SD, N = 230 cells). Because CNG channel-mediated activity is essential for the expression of Kirrel2, Sem7A, and PCDH10 (27), these observations indicate that specific features of neural activity (e.g., frequency, IEI), rather than the presence of neural activity, are important for the expression of axon-sorting molecules, leading to the hypothesis that neural activity is instructive for glomerular segregation.

**ORs determine spontaneous activity patterns**

We next compared the temporal features of spontaneous calcium transients between OSNs expressing different OR types. We selected two types of OR-defined neurons based on the expression profiles of the axon-sorting molecules: (i) I7-expressing OSNs, in which expression levels of Kirrel2 are high, and (ii) MOR28-expressing OSNs, in which expression levels of Kirrel2 are low (Fig. 1, A and B, and fig. S4). We generated the transgenic mice H-I7-Cre-mcherry and H-MOR28-Cre-mcherry (hereafter abbreviated as H-OR-Cre lines), in which Cre recombinase was coexpressed with I7 or MOR28 (Fig. 2C and fig. S4). The forced expression of OR from a transgene suppresses endogenous OR expression (6, 40, 41), the monogenic OR expression is maintained in OSNs expressing the transgenes. The H-OR-Cre lines were then crossed with Ai95D mice to visualize spontaneous calcium transients of OSNs expressing specific ORs. We compared the features of calcium spikes, including baseline fluorescence, total event number, IEI, peak amplitude, coefficient of variation (CV), and rise and fall times of calcium spikes between I7- and MOR28-expressing neurons (N = 59 cells for I7, N = 61 cells for MOR28). We found that calcium transient patterns were significantly different between I7- and MOR28-expressing OSNs (Fig. 2C), even though both ORs were expressed under the control of the same MOR23 promoter (42) (Fig. 2C and fig. S4A). I7-expressing OSNs exhibited calcium transients of higher amplitudes and higher frequencies than those of MOR28-expressing OSNs (Fig. 2, C and D). We conducted multidimensional scaling (MDS) to plot the datasets of I7- and MOR28-expressing OSNs according to relative distance (Fig. 2E). In the MDS space, OSNs expressing different ORs were significantly separated by a single line determined by the linear support vector machine (SVM) classifier (gray dashed line in Fig. 2E, F1 = 0.775, P < 0.0001), indicating that different ORs are sufficient to induce differing patterns of spontaneous calcium transients.

To overview a variety of calcium transient patterns in OSNs, we next collected the features of calcium transients from a large number of OSNs (N = 3214 cells; N = 59 for I7 (red), N = 61 for MOR28 (green), N = 45 for M71 (blue), and N = 3049 for randomly selected OSNs (gray)). The dataset was dimension-reduced by principal component analysis (PCA). In two-dimensional PC space, OSNs were clustered depending on the expressed OR types (Fig. 2F and fig. S5). Taken together, this result indicates that OSNs expressing the same OR type exhibited similar temporal profiles of calcium transients.
To assess the causal links between the neural activity and optogenetic stimulation protocols that increased Kirrel2 expression (Fig. 3F, a to d) were plotted onto the Kirrel2-high side of the graph, whereas those that did not change Kirrel2 expression (Fig. 3F, a to g) were plotted onto the Kirrel2-low side. Further, OSNs in the I7 cluster frequently exhibited calcium transients with high amplitude that resembled phasic burst stimulation (Fig. 3F, right). Calcium event frequency = 0.047 ± 0.031 Hz, calcium event amplitude = 10.25 ± 4.06%, mean ± SD). In contrast, such high-amplitude calcium transients were not frequently observed in the MOR28-expressing OSNs, where the endogenous Kirrel2 expression levels are low (Fig. 3D, right), indicating that the change in the expression levels of Kirrel2 was not attributable to a difference in the OSN population. In addition, the induced spike numbers within bursts affected Kirrel2 expression (Fig. 3E).

To find the relationship between endogenous spontaneous activity and optogenetically induced firing, we conducted SVM classification in terms of Kirrel2 expression levels. The nonlinear SVM classifier based on endogenous activity patterns of Kirrel2-high (I7) and Kirrel2-low (MOR28 and M71) OSNs could predict the light-stimulation-induced Kirrel2 up-regulation (Fig. 3F, left). The optogenetic stimulation protocols that increased Kirrel2 expression (Fig. 3F, a to d) were plotted onto the Kirrel2-high side of the graph, whereas those that did not change Kirrel2 expression (Fig. 3F, a to g) were plotted onto the Kirrel2-low side. Further, OSNs in the I7 cluster frequently exhibited calcium transients with high amplitude that resembled phasic burst stimulation (Fig. 3F, right). Calcium event frequency = 0.047 ± 0.031 Hz, calcium event amplitude = 10.25 ± 4.06%, mean ± SD). In contrast, such high-amplitude calcium transients were not frequently observed in the MOR28-expressing OSNs, where the endogenous Kirrel2 expression levels are low (Fig. 3D, right), indicating that the change in the expression levels of Kirrel2 was not attributable to a difference in the OSN population. In addition, the induced spike numbers within bursts affected Kirrel2 expression (Fig. 3E).

Spike bursts increase Kirrel2 expression

To assess the causal links between the neural activity patterns and the expression of axonsorting molecules, we used an optogenetic approach to manipulate the neural activity of OSNs. OSN-specific channelrhodopsin-2 (ChR2) mice were generated by crossing pGoofy-Cre mice with pCAG-LSL-ChR2 (Ai32) mice (43) (Fig. 3A, top). Blue light pulses (3 ms at ≤40 Hz) could precisely control the neural activity of OSNs at a single spike level, irrespective of the light power (Fig. S6). The OSN-specific ChR2 mice were stimulated by blue light pulses with two different protocols, namely phasic burst stimulation (intraburst frequency, 10 Hz; action potentials in burst, 15; interburst interval, 15 s) and tonic stimulation (Fig. 3B). In both protocols, light-induced action potentials were maintained at the overall firing rate of 1 Hz because the median firing rate of the spontaneous activity of OSNs was 1.1 Hz (fig. S7). We illuminated the unilateraldorsal lateral side of the OE with blue light pulses for 12 hours according to the protocols and compared the changes in the expression of axonsorting molecules between the light-illuminated and shaded sides of the OE (Fig. 3A, bottom). We found that in the phasic burst protocol, Kirrel2 was up-regulated in the light-stimulated side relative to the shaded side (Fig. 3B). However, no significant change was observed in the tonic protocol. Kirrel2 expression was up-regulated in the phasic burst protocols when the interspike intervals (ISIs) of the induced spikes within bursts were less than 200 ms (Fig. 3C). Single-cell quantification further revealed that the intraburst frequency of the induced spikes had different impacts on Kirrel2 up-regulation (ISI = 50 ms, d = 0.74; ISI = 100 ms, d = 1.54; ISI = 200 ms, d = 1.09) (Fig. 3D, left). Kirrel2 expression increased even in MOR28-expressing OSNs, where the endogenous Kirrel2 expression levels are low (Fig. 3D, right), indicating that the change in the expression levels of Kirrel2 was not attributable to a difference in the OSN population. In addition, the induced spike numbers within bursts affected Kirrel2 expression (Fig. 3E).

To find the relationship between endogenous spontaneous activity and optogenetically induced firing, we conducted SVM classification in terms of Kirrel2 expression levels. The nonlinear SVM classifier based on endogenous activity patterns of Kirrel2-high (I7) and Kirrel2-low (MOR28 and M71) OSNs could predict the light-stimulation-induced Kirrel2 up-regulation (Fig. 3F, left). The optogenetic stimulation protocols that increased Kirrel2 expression (Fig. 3F, a to d) were plotted onto the Kirrel2-high side of the graph, whereas those that did not change Kirrel2 expression (Fig. 3F, a to g) were plotted onto the Kirrel2-low side. Further, OSNs in the I7 cluster frequently exhibited calcium transients with high amplitude that resembled phasic burst stimulation (Fig. 3F, right). Calcium event frequency = 0.047 ± 0.031 Hz, calcium event amplitude = 10.25 ± 4.06%, mean ± SD). In contrast, such high-amplitude calcium transients were not frequently observed in the MOR28-expressing OSNs, where the endogenous Kirrel2 expression levels are low (Fig. 3D, right), indicating that the change in the expression levels of Kirrel2 was not attributable to a difference in the OSN population. In addition, the induced spike numbers within bursts affected Kirrel2 expression (Fig. 3E).
endogenous calcium transients were more static (fig. S8). These results demonstrate that the spike bursts specifically up-regulate Kirrel2 expression.

**Prolonged burst stimulation increases Sema7A and PCDH10**

We next sought to determine which pattern of neural activity was necessary for the expression of Sema7A and PCDH10. Because they showed similar protein distribution patterns at the OSN axon termini (27), there must be a common regulatory mechanism underlying their expression. We stimulated OSNs with various burst duration protocols ranging from 1.5 to 720 s (Fig. 4A). We kept the overall firing rate at 10 Hz. Optogenetic stimulation with various burst duration protocols revealed that Sema7A and PCDH10 were up-regulated by burst durations for more than 10 s and 20 s, respectively (Fig. 4B). It is notable that the 360-s and 720-s burst duration protocols increased the expression of Sema7A and PCDH10, but not that of Kirrel2 (Fig. 4, B and C).
To investigate the minimum duration of bursts required for the expression of Sema7A and PCDH10, we further tested the effects of various burst units by changing the burst duration irrespective of the firing rate of the induced spikes (Fig. 4D). To this end, OSNs were stimulated hourly with various types of single burst units (intraburst frequency, 10 Hz; burst duration, 10 to 360 s) for 12 hours. We found that both genes were up-regulated by prolonged bursts. The sensitivities to the burst duration differed between Sema7A and PCDH10; a 20-s burst duration induced the expression of PCDH10, but not that of Sema7A (Fig. 4E). The differential sensitivities of Sema7A and PCDH10 to the burst durations may...
optogenetic stimulation affects axonal segregation.

(A) Schematic for generating OSN-specific ChR2 mosaic mice. (B) Double staining of an OE section from the ChR2 mosaic mouse. An OE section was subjected to in situ hybridization using an OMP probe (red) and immunostaining with antibodies to GFP and Kirrel2 after chronic light stimulation (intrapulse frequency, 10 Hz; APs within burst, 15 s). Left: Kirrel2 up-regulation upon light stimulation is indicated by arrowheads. Right: Quantification of protein levels of axon-sorting molecules. *P < 0.05, **P < 0.01 (one-way ANOVA with post hoc Tukey test). See also data S5. (C) Immunostaining of an OB section with antibodies to GFP and Kirrel2 after chronic light stimulation (intraburst frequency, 10 Hz; APs within burst, 15 s; interburst interval, 15 s). Left: Kirrel2 up-regulation upon light stimulation is indicated by arrowheads. Right: Quantification of protein levels of axon-sorting molecules. *P < 0.05, **P < 0.01 (one-way ANOVA with post hoc Tukey test). See also data S5. (D) Immunostaining of OB sections using antibodies to Kirrel2, GFP, and MOR28. Kirrel2-positive and -negative MOR28-positive axons are indicated by arrowheads and arrows, respectively. (E) Numbers of MOR28 glomeruli per OB in ChR2 mosaic mice with and without light stimulation. A glomerular structure was semiautomatically defined by immunofluorescence signals of vesicular glutamate transporter2 (VGLUT2). Box and whiskers in all graphs represent 25th to 75th and 10th to 90th percentiles, respectively. Scale bars, 50 μm. N = 6 glomeruli. **P < 0.01 (Student t test).

We also performed long-term calcium imaging for OSNs expressing M71 and MOR28 in which PCDH10 expression levels are high and low, respectively (Fig. 1, B and C). As shown in Fig. 4F, both types of OSNs exhibited stationary states during the majority of the recorded time. However, we observed events of prolonged calcium elevation several times per hour in M71-expressing OSNs (Fig. 4F). We defined the active state according to the duration when OSNs showed a higher amplitude of calcium transients above average for >20 s. The active state in M71-expressing OSNs lasted for 34.14 ± 13.47 s with an IEI of 445.85 ± 376.03 s (mean ± SD), which is enough to induce PCDH10 expression. In contrast, the active state was hardly observed in MOR28-expressing OSNs (0.09 ± 0.30 times/hour (N = 11 cells) versus 2.4 ± 1.90 times/hour for M71-expressing OSNs (N = 10 cells), means ± SD). These results suggest that prolonged burst stimulations and their impact on gene expression mimicked physiological conditions.

Discussion

Hebbian plasticity explains the activity-dependent development of many neural circuits (39). Synchronous firing of presynaptic neurons, such as retinal waves in the visual system (2, 36, 37, 39, 44, 45), is a prerequisite for the Hebbian plastic changes. In the present study, we did not observe spatial or temporal correlations for the spontaneous activities among OSNs (Fig. 2, A and B). Rather,
spontaneous activity patterns of OSNs were uniquely correlated with OR types. We also showed that differing neural activity patterns induced the expression of different axon-sorting molecules regulating axonal segregation. On the basis of these results, we propose an activity-dependent mechanism, different from the Hebbian plasticity theory, in which specific patterns of spontaneous activity determined by the expressed ORs contribute to generate the combinatorial code of axon-sorting molecules for OR-specific glomerular segregation.

Our calcium imaging analyses in OR substitution experiments demonstrated that ORs determine spontaneous activity patterns. How are spontaneous activity patterns regulated by ORs? It has been reported that G protein–coupled receptors (GPCRs), including ORs, produce unique levels of basal cAMP (cyclic adenosine monophosphate) in a ligand-independent manner (12, 46, 47). A plausible explanation is that the OR-derived basal activity contributes to the pattern of spontaneous activity. However, mutations of a GPCR that result in altered basal cAMP levels do not affect the expression of axon-sorting molecules in OSNs (12). Therefore, the OR-derived basal activity is unlikely to be a determinant of spontaneous activity patterns. In this study, we found that individual OSNs show different firing patterns in response to continuous optogenetic stimulation (fig. S6D). Moreover, several voltage-dependent calcium channels and transient receptor potential channels are expressed in a subset of OSNs (48–51). It is possible that the intrinsic membrane properties vary among OSNs expressing different OR types for OR-correlated patterns of spontaneous activity. However, the mechanisms linking the membrane properties to expressed ORs remain unknown.

In this study, we demonstrated the firing pattern–dependent expression of axon-sorting molecules by optogenetic stimulation. The SVM classification indicated that optogenetic stimulation experiments mimicked physiological conditions (Fig. 3F and fig. S11). It was found that phasic, repetitive short bursts of neural activity induced Kirrel2 expression, whereas prolonged durations of bursting activity induced the expression of Sema7A and PCDH10. Increased levels of Kirrel2 were dependent on spike numbers and ISIs within bursts in the phasic burst stimulation (Fig. 3, D and E). The sensitivities to the burst duration might differ between PCDH10 and Sema7A. We observed statistically significant up-regulation of PCDH10 and Sema7A by burst durations of >20 s and >60 s, respectively (Fig. 4E). This firing pattern–dependent expression allows OSNs to separately regulate the types and levels of axon-sorting molecules to generate the combinatorial code for glomerular segregation (fig. S12).

How do OSNs decode the temporal patterns of their own neural activity into diverse expression of axon-sorting molecules? Previous studies have identified transcription factors that are activated by different patterns of calcium transients (52–54). For example, CaMKII (calcium/calmodulin-dependent protein kinase II) is activated by high-frequency calcium oscillations, whereas NFATs (nuclear factors activated T cells) are activated by low-frequency oscillations (55). The differential properties (e.g., sensitivity, affinity, and kinetics) of calcium-responsive factors may provide a molecular basis for the differential readouts of neural activity patterns.

In the nervous system, neural activity is involved in various aspects of the neural development and plasticity—including cell type specification, dendritic branching, synaptic maturation, and learning and memory—through a complex program of gene regulation (56–59). Although several hundreds of genes have been identified as activity-dependent genes (60), their regulatory mechanisms and functions are not fully understood. In this study, we demonstrated firing pattern–dependent gene expression regulating neural circuit formation. With this strategy, neurons can generate variation through diversifying gene expression with only a single second messenger. The pattern-dependent regulation may also expand beyond development to the plasticity of neural circuits, which is the basis for learning and adapting to environmental changes throughout the lifetime.

**Materials and methods**

**Mutant mice**

All experimental procedures were performed with the approval of the animal experiment ethics committee of the University of Tokyo and Institutional Animal Care and Use Committee (IACUC) of RIKEN Kobe Branch, and in accordance with the guidelines for the care and use of laboratory animals of the University of Tokyo and RIKEN Kobe Branch. H-Cre mice were as described (13). AilSD, Ail2, M77-ires-Cre, and CNG-KO mice were purchased from the Jackson Laboratory, pGoofy-Cre and Kirrel2 conditional KO (RBRC06340) mice were generous gifts provided by Y. Yoshihara and H. Sakano, respectively. To generate the H-17 (accession no. CD0537T: www2.clst.riken.jp/arg/TG%20mutant%20mice%20list.html) and H-MOR28 (accession no. CD0536T: www2.clst.riken.jp/arg/TG%20mutant%20mice%20list.html) minigene constructs, a 10.1-kb SacI fragment containing the MOR23 gene from a BAC clone of a C57BL/6 mouse (RP23-306I18) was cloned into the pBluescript II SK(+) (accession no. CDB0536T: www2.clst.riken.jp/arg/TG%20mutant%20mice%20list.html) and M77-ires-Cre and Kirrel2 conditional KO (RBRC06340) mice were used to obtain higher intensity in the immunostaining of ORs. In situ hybridization was performed as described (13). The sequences of the RNA probes that were used are listed in data S7. The full-length coding sequence for mcherry was subcloned into the pGEM-T vector (Promega) and used as a template.

**Signal intensity measurement and statistical analysis**

Optical and fluorescent images of sections were obtained using a BX-710 microscope (Keyence). A glomerular structure was defined by immunofluorescence signals of vesicular glutamate transporter-2 (VGlut2), and staining intensities of Kirrel2, Sema7A, and PCDH10 within glomeruli were measured using ImageJ (NIH). After subtracting the background signals, staining intensities were divided by the highest staining intensity and were normalized to 100% (Fig. 1C). In Fig. 3D, signal intensities were rescaled such that the rescaled mean in the shaded side is 1. The data are presented as means ± SEM.

**Optogenetic stimulation**

Mice were placed on ice to anesthetize them by hypothermia and a skin incision was made over the dorsal surface of the head. Xylocaine jelly (2%, AstraZeneca) was applied to the bone overlying the OE as a local anesthetic, and a cover slip was placed over the bone to prevent the olfactory tissue from drying out. The olfactory sensory neurons (OSNs) were stimulated using 470-nm LEDs (Lex2; Brain Vision LLC). To stimulate the OE unilaterally, LEDs were placed directly outside the right side of the OE, whereas the other side was covered with thin aluminum to avoid light stimulation. After 12 hours of stimulation, the glass cover was removed from the skull and olfactory tissues were collected for reverse transcription polymerase chain reaction (RT-PCR) or in situ hybridization. For chronic stimulation, pups were wrapped tightly in gauze bandage.
before stimulation and placed in an acryl chamber. Blue LEDs were placed directly outside the dorsal side of the OE. After stimulation, pups were placed on a temperature-controlled heating pad before they were returned to their mothers.

In all protocols, the light pulse duration was 3 ms with a power of 3 mW mm⁻². The timing, duration, and cycle of LED light stimulation were controlled with a pulse stimulator (Nihon Kohden).

**RT-PCR**

Quantitative PCR was performed as described (13). The expression levels of Kira2L2, Sena7A, and PCDH10 were normalized to that of CNG. Primer sets and TaqMan probes used in this study were designed by Universal Probe Library (Roche) and Nihon Gene Research Laboratories Inc. (Miyagi). The primers and probe sequences used for RT-PCR are listed in data S7.

**Slice preparation**

Acute slices were prepared from neonatal mice (P3-D6). Mice were anesthetized and decapitated, and the olfactory tissue was embedded in 5% low-melting agar in Ringer’s solution (125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM NaHCO₃, 10 mM HEPES, and 10 mM glucose) and cut into 200- to 300-μm-thick slices using a vibratome (Leica VT1200S) in ice-cold oxygenated artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, and 10 mM glucose). Slices were briefly transferred to an interface chamber containing oxygenated artificial cerebrospinal fluid (aCSF) at RT.

**Ex vivo culture**

For ex vivo culture experiments, mice were anesthetized by hypothermia and were quickly decapitated. The nose was dissected en bloc to prepare intact septal nasal cartilage with epithelia. Samples were briefly transferred to an interface chamber containing oxygenated aCSF and were incubated at 35°C for 6 hours. After the incubation, OE samples were fixed overnight with 4% paraformaldehyde in PBS.

**Electrophysiological recording**

Loose-patch recording was performed largely as described (60). Briefly, after anesthesia was achieved by hypothermia, mice were quickly decapitated, and the nose was dissected en bloc to prepare intact OE. The septal nasal cartilage with epithelia was placed in a submerged recording chamber with the mucus layer facing up and was continuously perfused with oxygenated aCSF at 32°C. For extracellular recording, pipettes with resistance of 4 to 8 MΩ were fabricated using the P-1000 micropipette puller (Sutter Instrument) and filled with aCSF. All recordings were performed using the Olympus BX51WI-1 microscope. A 40× objective lens (0.8 numerical aperture, LUMPlan FN; Olympus) was used to guide a patch pipette to individual OSNs. Recordings were performed in a loose patch configuration. A loose seal (resistance, 40 to 200 MΩ) was formed by applying gentle suction when an electrode tip contacted an OSN. Signals were band-passed between 200 Hz and 5 kHz.

**Calcium imaging and data analysis**

Calcium imaging was conducted using acute slices of OE from mice expressing GCaMP6f. OE slices were placed in a recording chamber perfused with aCSF at 3 to 4 m/s. Fluorophores were excited at 488 nm and visualized using a 507-nm long-pass emission filter. Videos were recorded at 10 frames/s for 10 min or 5 frames/s for 1 hour using a 4× water immersion objective lens (0.8 numerical aperture, CFI Apo 40XW NIR; Nikon), a Nikpovisk confiscal microscope (SXU-Xi; Yokogawa Electric), and a cooled electron multiplying charge-coupled device camera (Ixon DU897; Andor Technologies). Image acquisition was controlled by Andor Solis (Andor Technologies). The fluorescence intensity was measured as F₁ (F₂ – F₀)/F₀, where F₁ is the fluorescence intensity at a given time point and F₀ is the baseline. Automated quantification of the features of calcium transients was performed using FluoroSNNAP software (61). Principal components analyses were performed with Origin Pro (OriginLab). Multidimensional scaling (MDS) and support vector machine (SVM) classifications were performed with MATLAB software. R1 score was calculated as a measure of the accuracy in the SVM classification.

**Correlation analysis**

To quantify the correlation between pairs of recorded cells, the correlation index (CI) was calculated as described (33, 40). The formula was as follows:

\[
CI = \frac{N_{AB} - M_{-1.0}}{N_{0.0}R_{0.0} - M_{-2.0}T}
\]

where \(N_{AB}\) is the number of spikes from cell B that fall within ±\(\Delta t\) of any spike from cell A, \(\Delta t\) is the duration of the recording in seconds, and \(N_{0.0}\) and \(N_{0.0}R_{0.0}\) are the total numbers of spikes detected from cells A and B during the recording. To evaluate the significance of deviations from the “random” baseline of the CI, surrogate data were generated through the random shuffling of real data. The spike coincidence time window in Fig. 2 was ±\(\Delta t\) = 200 ms.

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38. T. W. Chen et al., A toolbox of Cre-dependent optogenetic materials. All materials can be requested from H.T. Authors declare no competing interests. Funding: Supported by the Mitsubishi Foundation, the Takeda Science Foundation, the Japan Foundation for Applied Enzymology, the Kato Memorial Bioscience Foundation, the Japan Science and Technology Agency, PRESTO, JST ERATO grant JPMJER1810, the Human Frontier Science Program (RGP0019/2016), JSPS Grants-in-Aid for Scientific Research grant 18H05525, and JSPS KAKENHI grants 20H05202, 19K02939 and 18K04712. The work was conducted partially as a program at the International Research Center for Neurointelligence (WPI-IRCN) of the University of Tokyo Institutes for Advanced Study. Author contributions: A.N., N.I., Y.I., and H.T. conceived the experiments; A.N., N.I., M.S., H.K., and H.T. performed the experiments and analyzed the data; all authors contributed to the writing of the manuscript and provided helpful comments; and all authors read and approved the final manuscript. Competing interests: Authors declare no competing interests. Data and materials availability: All data are available in the manuscript or supplementary materials. All materials can be requested from H.T.

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Structured spike series specify gene expression patterns for olfactory circuit formation
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Temporal code underlies circuit formation
Olfactory neurons respond to various odorants according to which olfactory receptors, of many, they express. During development, axons from olfactory neurons that express the same olfactory receptor converge to share the same glomeruli. Nakashima et al. now show that, in mice, the neurons build these connections according to shared patterns of activity. When the olfactory receptor is triggered, it causes its cell not simply to fire but to fire in specific patterns. Neurons that speak the same code end up connected at the same glomerulus.

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