Doctorate Dissertation
博士論文

Studies on a role for prior activity in olfactory behavior of *Drosophila* larvae

(ショウジョウバエ幼虫の嗅覚行動における神経活動履歴の役割に関する研究)

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Abstract

Animals can detect novel external stimuli and change their behavior in response to the external world. During this process, neurons are activated by the novel stimuli. In reality, however, neurons are active prior to the detection of such novel stimuli. For example, in olfactory system, neurons are always exposed to environmental odors and are activated by them. Additionally, neurons are spontaneously active in the absence of such external stimuli. These prior activities might affect the detection of the novel stimuli and could thus lead to changes in animal behavior. However, the impact of prior activity on animal behavior remains elusive because of the difficulty in controlling the prior activity and assessing its impact on animal behavior.

In this thesis, to investigate the effects of prior activity on animal behavior, I focused on the olfactory receptor neurons (ORNs) of *Drosophila* larvae. Stimulation of a single ORN can induce attractive or aversive stereotypic behaviors. Thus, this relationship between neural activity and behavior allowed assessment of how changes in prior activity are reflected in the neural response and the stereotypical behavior.

To define the prior activity of ORNs, I characterized the neural activity of ORNs before and after odor stimulation, by calcium imaging (Fig. 1). The neural activity prior to the stimulation is termed prior activity in this thesis. To genetically regulate the prior activity, I also confirmed Orco, which is co-receptor of olfactory receptor (OR), was essential for the prior activity of ORNs (Fig. 2, 3). To estimate the functional importance of the prior activity, I compared the behavior of *Orco* mutant, which exhibited reduced prior activity of ORNs, with that of wild-type (WT) larvae (Fig. 5, 6, 7). I found that *Orco* mutant behaved differently from WT larvae when their ORNs were optogenetically stimulated. In Fig. 8 and 9, to investigate the reason for the behavioral changes in *Orco* mutants, I observed the neural responses of ORNs to the stimulation. I demonstrated that *Orco* mutants exhibited a large amplitude and distinct temporal pattern of neural response. I also found that projection neurons (PNs) and local neurons (LNs), which are synaptically coupled with ORNs, changed their responses to the optogenetic stimulation of ORNs (Fig. 13), suggesting that the reduction of prior activity in ORNs can affect sensory processing globally. Finally, I developed a novel tool to increase prior activity by modifying Orco-OR complex (Fig. 14). This approach

successfully increased prior activity of PNs, suggesting that Orco-OR complex can be available for a tool to modulate prior activity of various neurons besides ORNs.

Taken together, I here demonstrated the possible impacts of prior activity on sensory processing and animal behavior. My findings in the present thesis will shed light on the functional importance of prior activity and contribute to elucidating new principles of sensory processing and animal behavior.

Abbreviations

AHL .....adult hemolymph-like

Brp .....Bruchpilot

Ca^{2+} .....calcium

CHO .....Chinese hamster ovary

ChR2 .....channelrhodopsin-2

EM-CCD .....electron-multiplying charge-coupled device

fMRI .....functional magnetic resonance imaging

LNs .....local neurons

ORs .....olfactory receptors

Orco di .....Orco dimer

ORNs .....olfactory receptor neurons

PBS .....phosphate buffered saline

PI .....performance index

PNs .....projection neurons

RGECO .....red fluorescent genetically-encoded Ca^{2+} indicator

ROI .....region of interest

SCN1B .....single transmembrane protein sodium channel subunit beta-1

WT .....wild-type

Introduction

Neurons are active prior to detection of novel external stimuli. In many sensory systems, such neural activity prior to stimulation, or prior activity, affects the neural response to the stimulus. For example, in olfactory system of locusts, the prior activity related to background odorants can enhance or reduce the response to a freshly introduced odorant (Saha et al., 2013). In addition, even in the absence of such background stimuli, neurons are spontaneously active. This spontaneous activity can also be regarded as a type of prior activity. Indeed, in cat striate cortex, the response magnitude to visual stimuli correlates with the fluctuation of the spontaneous membrane potential preceding the onset of the stimuli (Azouz & Gray, 1999), indicating that the neural response to the stimuli may be affected by spontaneous activity. These neural activities prior to stimuli are generally observed in many sensory systems and cortical regions (Arieli, Shoham, Hildesheim, & Grinvald, 1995; Hallem, Ho, & Carlson, 2004; Luczak, Bartho, & Harris, 2013; Romano et al., 2015). In addition, these neural activities synchronize within a region and between regions of the brain (Raichle et al., 2010), suggesting that prior activity might be propagated to synaptically coupled

neurons and have a global effect on sensory processing. Therefore, understanding the impact of prior activity on the neural response to stimuli provides insights into the general principles underlying sensory processing and the consequent animal behavior. However, little is known about functional significance of prior neural activity in sensory processing and animal behavior, because of the difficulty in controlling prior activity and assessing its effect on output behavior.

Olfactory receptor neurons (ORNs) of *Drosophila melanogaster* larvae are an attractive model for studying the link between prior neural activity and output behavior. Activation of a single ORN can successfully induce attractive or aversive behavior (Bellmann et al., 2010; Fishilevich et al., 2005; Semmelhack & Wang, 2009), indicating that the neural activity of each neuron is strongly related to stereotypical output behavior. This relationship allows the evaluation of the effect of prior activity on the stereotypical behavior. Furthermore, adult fly ORNs exhibited cell-specific frequencies of spontaneous and evoked activities (Hallem & Carlson, 2006; Hallem et al., 2004). These characteristics of the neural activities of ORNs depend on the ORN-expressing olfactory receptor complex, which consists of a cell-specific olfactory receptor (OR)

and co-receptor, Orco (Neuhaus et al., 2005; Sato et al., 2008; Wicher et al., 2008). Notably, ORNs of *Orco* mutants exhibit less spontaneous activity (Larsson et al., 2004), suggesting that Orco may also contribute to the prior neural activity of ORNs.

In the present thesis, by taking advantage of the reduction of prior activity of ORNs in *Orco* mutants, I investigated the effect of prior activity on sensory processing and behavioral output. In Fig. 1, I firstly characterized the prior activity of ORNs. I also confirmed the impact of *Orco* expression on the prior activity of ORNs in *Orco* mutants (Fig. 2, 3). In Fig. 5, to examine whether prior activity affects animal behavior, I compared the behaviors of *Orco* mutants with that of wild-type (WT) larvae. By optogenetic stimulation of ORNs, I demonstrated that the attractive behavior of *Orco* mutants was reduced compared to that of WT larvae (Fig. 5). This behavior change in *Orco* mutant was caused by a reduction of turning behavior when larvae detected decreased light stimulation (Fig. 7). In Fig. 8, 9 and 13, to understand how the reduction of the prior activity altered the sensory processing at the circuit level, I observed the neural responses of ORNs and their post synaptic neurons, projection neurons (PNs) and local neurons (LNs), to the optogenetic stimulation of ORNs. A combination of the

optogenetic stimulation and *ex vivo* calcium imaging revealed that ORNs in *Orco* mutants changed the temporal pattern of the responses to the stimulation (Fig. 9). This change in neural responses of ORNs were transferred to PN and LN responses (Fig. 13). Considering the results of behavioral assay and calcium imaging, the data further indicated that *Orco* mutant larvae were defective in detecting the reduction in stimulation. Finally, in Fig. 14, I developed a novel tool for increasing prior activity. I generated an Orco-OR fusion protein and assessed its effects on prior activity. This fusion protein successfully increased the prior activity of PNs. Interestingly, larvae expressing the Orco-OR fusion protein in their PNs exhibited odor-seeking behavior more frequently than the control. Taken together, my findings suggested the possibility that the prior neural activity of ORNs might carry information that determines animal behavior.

Materials and Methods

Drosophila strains

Fly strains used in the present study were as follows: *Orco-Gal4* (second, Bloomington #26818), *UAS-GCaMP6f* (second, Bloomington #42747), $w^{-};;\ w^{+},orco^{1}$ (null allele, Bloomington #23129), *Or42a-Gal4* (second, Bloomington #9970), *Or42b-Gal4* (second, Bloomington #9972), *UAS-Orco* (second, this study), *UAS-CsChrimson* (second, Bloomington #55135), *Tsh-Gal80* (Gift from Gero Miesenböck), *UAS-RGECO1* (second, Kanamori et al., 2013), *UAS-hChR2[H134R]* (second, Kanamori et al., 2013), *GH146-Gal4* (second, Bloomington #30026), *LN2-Gal4* (X, DGRC NP2426), *Orco-ChR2* (second, Bloomington #63041), *UAS-Orco::SCN1B::Or47a* (second, present study), *UAS-mCD8::RFP* (second, Bloomington #27398), *UAS-Brp::GFP* (first, Bloomington #35848), $w^{-};;w^{+},orco^{2}$ (null allele, Bloomington #23130), *Or13a-Gal4* (second, Bloomington #9945), *Or1a-Gal4* (second, Bloomington #9949), *Or1a-Gal4* (third, Bloomington #9950), *Or33b-Gal4* (third, Bloomington #9963), *Or33b-Gal4* (X, Bloomington #9964), *Or35a-Gal4* (second, Bloomington #9967), *Or42a-Gal4* (third, Bloomington #9969), *Or45b-Gal4* (third, Bloomington #9977), *Or47a-Gal4* (second, Bloomington #9981), *Or59a-Gal4*

(third, Bloomington #9990), *Or67b-Gal4* (third, Bloomington #9995), *Or74a-Gal4* (second, Bloomington #23123), *Or82a-Gal4* (third, Bloomington #23126), *Or83a-Gal4* (third, Bloomington #23127), *Or85c-Gal4* (X, Bloomington #23914), *Or94b-Gal4* (second, Bloomington #23916).

Transgenic strain

UAS-Orco. The complementary DNA of *Orco* was amplified from a plasmid (kindly gifted by Dr. Vosshall, The Rockefeller University) by PCR and then subcloned into the pJFRC-MUH-20xUAS vector (Kanamori et al., 2013). The plasmid was injected into flies carrying an attP docking site, attP40 (BestGene Inc.).

UAS-Orco::SCN1B::Or47a. A complementary DNA of *Orco::SCN1B* was amplified by PCR from *Orco::SCN1B::Orco* plasmid (kindly gifted by Dr. Wicher, Max Planck Institute) and then subcloned into the pJFRC-MUH-20xUAS vector (Kanamori et al., 2013). Or47a was also amplified from a plasmid (Addgene #59659). The amplicon was subcloned into pJFRC-MUH-20xUAS-Orco::SCN1B. The plasmid was injected into flies carrying an attP docking site, attP40 (BestGene Inc.).

Calcium imaging

Ex vivo calcium imaging was performed as previously described (Asahina, Louis, Piccinotti, & Vosshall, 2009). Larvae were raised with the standard fly food at 25℃ under 12-h light/dark conditions. Third instar larvae were washed in 1 × PBS and transferred to adult hemolymph-like (AHL) saline (Wang et al., 2003). To expose the larval brain, each larva was dissected, and the larval head was collected by removing the fat body, the salivary gland, and the digestive system. The nose tip of the larval head was inserted into the hole of 22 mm × 22mm plastic cover slip (Thermo Fisher Scientific) that was punched with 23G needle (Terumo Corporation). To fix the brain position, the brain was covered with low-melting agarose (1.0%; LowMelt Agarose, GeneMate) in AHL and coverglass. For odor stimulation, 10 μl ethyl acetate at a concentration of 10^{-3} was applied manually.

Calcium imaging was performed using an Olympus BX51WI (UPlanSApo objective, 40x NA = 0.75, Olympus), with a Yokogawa CSU10 (Yokogawa, Tokyo, Japan) spinning disk confocal system and an electron-multiplying charge-coupled device (EM-CCD) digital camera (Evolve, Photometrics) (Kanamori et al., 2013). The GCaMP6f signal was obtained at about 20 frames s^{-1} (exposure time, 50 msec). Motion

correction of the obtained movies was performed using Metamorph (Molecular Devices). For calculation of the signal intensities, a region of interest (ROI) was manually selected using Image J software (http://rsb.info.nih.gov/ij/). F_0 was computed as the mean of the 25th percentile of signal intensities in each ROI. $\Delta F/F_0$ was calculated according to $(F-F_0)/F_0$. For peak detection, σ was calculated from a $\Delta F/F_0$ time series. Peaks were then detected as events that deviated 2σ from the baseline. Peaks were detected and the power spectrum was calculated using MATLAB (http://www.mathworks.com/). For statistical analysis of the frequency of prior activities, Wilcoxon's rank-sum test, followed by Bonferroni correction, was applied.

Optogenetic behavioral assay

Larvae were raised with the standard fly food containing 1 mM all-trans-retinal at 25℃ in the dark. Third instar larvae were washed in water and transferred into 10-cm diameter agarose gel (Nippon gene) plates. Ten larvae were placed at the center of the plate. This plate was put in the dark experimental chamber. In this chamber, the agarose plate was illuminated by red light. Larvae expressing CsChrimson were allowed to move between two dark quadrants and two illuminated

quadrants on the plate. For optogenetic stimulation, larvae on the plate were exposed to light (617 nm, ~35 μW/mm^2). The larval behaviors were recorded at about 1 frame s^{-1} for 5 min by means of a CCD Camera (1500M-GE, THORLABS). The positions of larvae on the plate were analyzed using Image J software. The performance index (PI) was calculated as the number of larval positions detected in the illuminated area, minus the number of larval positions detected in the dark area, divided by the total number of larvae, over a period of 5 min. For statistical analysis of PIs, Wilcoxon's rank-sum test, followed by Bonferroni correction, was applied. Entering and turning behaviors at the boundary were counted manually. The probability of entering illuminated quadrants from dark quadrants was calculated as the number of larvae that entered illuminated quadrants, divided by the total number of larvae that crossed the boundary from dark quadrants into illuminated quadrants. The probability of turning back into illuminated quadrants was calculated as the number of larvae that turned back into the illuminated quadrants, divided by the total number of larvae that crossed the boundary from the illuminated quadrants into the dark quadrants. For statistical analysis of these probabilities, Fisher's exact test, followed by Benjamini–Hochberg correction, was

applied (Benjamini and Hochberg, 1995).

Optical stimulation

Larvae were raised with the standard fly food containing 1 mM all-trans-retinal at 25℃ in the dark. The dissection of larvae and the sample preparation were described in the calcium imaging section. The stimulation and recording were performed with an Olympus BX51WI upright microscope (UPlanSApo objective, 40x NA=0.75, Olympus). For stimulation of ORNs, blue light was illuminated by pE-100 (CoolLED). GCaMP6f and RGECO1 signals were obtained at about 5 frames s^{-1} (exposure time, 200 ms).

Obtained images were essentially analyzed as described in the calcium imaging section. In addition, background correction was performed to reduce the background noise by light stimulation. The images were mean-filtered (radius: 2 pixels) and then the background was subtracted using the rolling ball method (rolling ball radius: 50 pixels). For calculation of the fluorescence intensities, a region of interest (ROI) was manually selected using Image J software (http://rsb.info.nih.gov/ij/). F_0 was computed as the mean intensity of nine frames from stimulation onset in each ROI.

$\Delta F/F_0$ was calculated as $(F-F_0)/F_0$.

Quantification of basal locomotion

Larvae were raised with the standard fly food at 25℃ under 12-h light/dark conditions. Third instar larvae were washed in water and transferred into 10-cm diameter agarose gel (Nippon gene) plates. A larva was placed at the center of the plate. This plate was put in experimental chamber. Larvae expressing Orco-OR fusion protein in their PNs were allowed to move freely on the plate. The larval behaviors were recorded at about 1 frame s^{-1} for 5 min using a CCD Camera (1500M-GE, THORLABS). The head casting and turning behaviors were counted manually. For statistical analysis of them, Wilcoxon's rank-sum test, followed by Bonferroni correction, was applied.

RNA-Seq Library Preparations

Four control and six *Orco* mutant samples with 20 dorsal organs each were isolated and subjected to mRNA-Seq analysis. Thoracic segments containing chemosensory organs were dissected from third instar larvae expressing

UAS-mCD8-GFP in ORNs under control of *Orco-Gal4*. Experimental genotypes were: *w*^{1118}*;Orco-Gal4,UAS-mCD8-GFP/Orco-Gal4,UAS-mCD8-GFP* (control); *w*^{1118}*;Orco-Gal4,UAS-mCD8-GFP/Orco-Gal4,UAS-mCD8-GFP;orco*^{1}*/orco*^{1} (*Orco* mutant). Dissected tissue was enzymatically treated with type I collagenase (Fisher), GFP-positive dorsal organs were manually separated from neighboring tissue and snap frozen in RNAqueous lysis buffer (Fisher). RNA was isolated using the RNAqueous Micro Kit, diluted to 0.2ng/ul, and converted to pre-amplified cDNA libraries using Smart-seq2 template-switching reverse transcription(Picelli et al., 2014). cDNA libraries were fragmented, barcoded, and amplified using the Nextera XT DNA kit, and all libraries were pooled and purified using AMPure XP beads. Quality was confirmed on an Agilent Bioanalyzer and libraries were sequenced on a HiSeq 4000 at the UCSF Center for Advanced Technology as 51 base single-end reads.

RNA-Seq Data Analysis

Reads were demultiplexed with CASAVA (Illumina) and read quality was assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). One *Orco* sample was excluded from analysis due to low read count and the remaining

samples had read counts ranging from five to eleven million reads. Reads containing adapters were removed using cutadapt (version 1.9.1)(Martin, 2011). To generate high recall of differentially expressed genes(Williams, Baccarella, Parrish, & Kim, 2017), reads were aligned to the *D. melanogaster* transcriptome, FlyBase genome release 6.10, using Kallisto (version 0.43.0)(Bray, Pimentel, Melsted, & Pachter, 2016) with default parameters. Transcript-level counts were condensed to gene-level counts using tximport (Soneson, Love, & Robinson, 2015) and differential expression analysis was performed using DESeq2 (version 1.14.1)(Love, Huber, & Anders, 2014). A Benjamini & Hochberg multiple comparison adjustment (Benjamini & Hochberg, 1995) was run on the original p-values to specifically assess differential expression for ion channel genes (identified as members of the Flybase Ion channel gene group, FBgg0000582). Genes with an adjusted p-value of under 0.05 were considered significantly different. The raw sequencing reads and gene expression estimates are available in the NCBI Sequence Read Archive (SRA) and in the Gene Expression Omnibus (GEO), accession number GSE111334.

Confocal imaging

Larvae were raised with the standard fly food at 25 °C under dark conditions. For dorsal organ imaging, third instar larvae were washed and dissected in PBS, and dorsal organs were mounted in Vectashield mounting medium (Vector Laboratories). Images were obtained by confocal microscopy (Leica TCS SP8). For larval brain imaging, larval brains were fixed by 4% formaldehyde/PBS for 30 min at room temperature. After fixation, the brains were transferred in PBS containing 0.1% Triton X-100 and incubated at 4°C for four hours. These samples were mounted in Vectashield mounting medium. Images were obtained by confocal microscopy (Leica TCS SP8).

Immunostaining

For normalization of CsChrimson::Venus signal, 3rd instar larvae were dissected and fixed in 4% formaldehyde/PBS and then stained with nc82 antibody (1:50; Developmental Studies Hybridoma Bank). As a secondary antibody, goat anti-mouse IgG Alexa Fluor 635 (1:500; Life technologies) was used. The samples were mounted in Vectashield mounting medium (Vector Laboratories).

Results

1. Olfactory receptor neurons are active prior to stimulation

Olfactory receptor neurons (ORNs) are sensory neurons that first receive odor information from the environment. ORNs express an olfactory receptor complex that consists of cell-specific olfactory receptor (OR) and co-receptor Orco (Larsson et al., 2004). To change the prior activity of ORNs genetically, I investigated the molecule affecting the prior activity of ORNs. Adult ORNs exhibited spontaneous activity with cell-specific frequency (Hallem & Carlson, 2006), suggesting that a cell-specific molecule in each ORN contributes to spontaneous activity. I selected the Orco-OR complex, which functions as a ligand-gated cation channel (Nakagawa, Pellegrino, Sato, Vosshall, & Touhara, 2012; Sato et al., 2008; Wicher et al., 2008), as a candidate. Indeed, ectopic expression of ORs in the ab3A ORN of Δhalo mutant (Dobritsa, van der Goes van Naters, Warr, Steinbrecht, & Carlson, 2003), which lacks the endogenous OR expression, transformed the endogenous spontaneous and odor-evoked activities into exogenous OR-dependent activities (Hallem et al., 2004). This observation demonstrated that Orco-OR channels determine the electrophysiological properties of ORNs. Importantly, in *Orco* mutants (Larsson et al., 2004), ORNs exhibited lower

spontaneous activity than in wild-type (WT) larvae. This result also suggested that Orco-OR channels may be required for spontaneous activity of ORNs because, in *Orco* mutants, the OR cannot form an ion channel with Orco. Given that spontaneous activity is a type of prior activity, I expected that Orco-OR channels would also affect the prior activity of ORNs. Accordingly, I assessed the impact of Orco on the prior activity of ORNs.

To characterize neural activity in larval olfactory receptor neurons (ORNs), I performed calcium (Ca^{2+}) imaging of ORNs. Since neuronal activity is tightly coupled to calcium influx (Smetters, Majewska, & Yuste, 1999), calcium imaging allowed monitoring of the neural activity of ORNs. To that end, I expressed the Ca^{2+} indicator GCaMP6f (Chen et al., 2013) in ORNs using a Gal4/UAS system (Brand & Perrimon, 1993) and monitored the Ca^{2+} signal in ORN axonal terminals in the larval brain. Consistent with previous reports (Asahina et al., 2009), I detected a robust Ca^{2+} response when the larval head was exposed to ethyl acetate, a strong attractant to larvae (Kreher, Mathew, Kim, & Carlson, 2008) (Fig. 1B: left). In addition to the odor-evoked Ca^{2+} response, Ca^{2+} fluctuations were observed prior to the odor stimulation in ORNs

(Fig. 1B: right). These fluctuations in ORNs had a smaller amplitude than odor-evoked activities. For example, in Or42a-expressing ORNs, the maximum amplitude of the fluctuation was 61.1% of the evoked activity on average to ethyl acetate (Fig. 1B: Or42a, n = 11). To characterize these fluctuations further, I checked the frequency of them. Consistent with previous reports of the spontaneous activity of adult ORNs (Hallem & Carlson, 2006), the frequencies of the fluctuations in larval ORNs differed from one another. To confirm whether these fluctuations occurred rhythmically or randomly, I calculated the power spectrum of the fluctuation (Fig. 2D: blue). The power spectrum showed a characteristic peak, reflecting rhythmicity in the fluctuation of ORNs.

This fluctuation of ORN neural activity prior to the stimulation was treated as spontaneous activity in previous studies. However, it was not possible to remove the effect of background odorant in behaving animals and the experimental environment completely. Therefore, in the present study, I refer to this fluctuation as prior activity, which includes both the background stimuli-based evoked activity and genuine spontaneous activity.

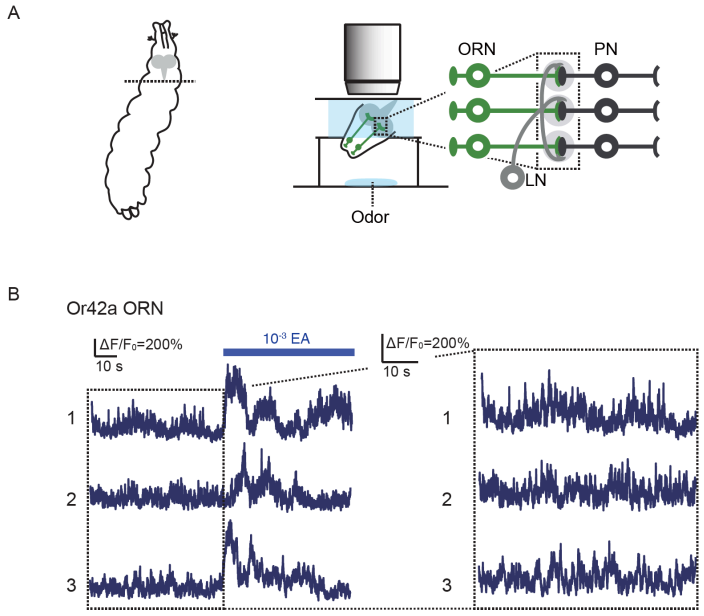

Figure 1 Calcium imaging of larval olfactory receptor neurons

(A) Schematic of the *ex vivo* calcium imaging preparation and the larval olfactory circuit. ORN, olfactory receptor neuron; LN, local neuron; PN, projection neuron. (B) Representative fluorescence changes from baseline ($\Delta F/F_0$) of Or42a ORN in three different animals. 10^{-3} EA, 10^{-3} concentration of ethyl acetate. Genotype: w^{1118};*Or42a-Gal4,UAS-GCaMP6f/+*

2. Expression of Orco in each ORN is essential for the prior activity of ORNs

To examine the potential roles for the Orco-OR complex in the prior activity of ORNs, I compared the prior activity of ORNs between WT and *Orco* mutants (Fig. 2A, B). In *Orco* mutant ORNs, the prior activity was rarely detectable (Fig. 2B: red). Quantitative analysis indicated that the frequency of the prior activity in *Orco* mutant ORNs was significantly reduced compared to WT (Fig. 2C: WT, median 0.90, 1st quartile 0.48, 3rd 1.47; *Orco* mutant, median 0.060, 1st quartile 0.017, 3rd 0.083). I then calculated the power spectrum of the prior activity and found that the characteristic peak in WT almost disappeared in *Orco* mutant (Fig. 2D), indicating that not only the amplitude, but also the rhythmicity of the prior activity was extinguished in *Orco* mutant ORNs. I also recorded the prior activity of a single ORN, Or42a and Or42b ORN, and found that, in *Orco* mutant, the average frequency was reduced at the single-neuron level (Fig. 3A, B). These data indicated that the prior activity of ORNs was significantly reduced in *Orco* mutant.

To confirm that the reduction of prior activity was due to the deletion of *Orco*, I rescued the expression of Orco in ORNs of *Orco* mutant, by means of *Orco-Gal4* that

drives the expression of *UAS*-Orco. By recovering *Orco* expression (Fig. 2C: black), the average frequency of the prior activity was restored to the WT level. Furthermore, restricted induction of Orco in a single ORN also restored the prior activity in *Orco* mutant (Fig. 3A, B: black). Therefore, the expression of Orco in each ORN was essential for the prior activity of ORNs (Fig. 2A).

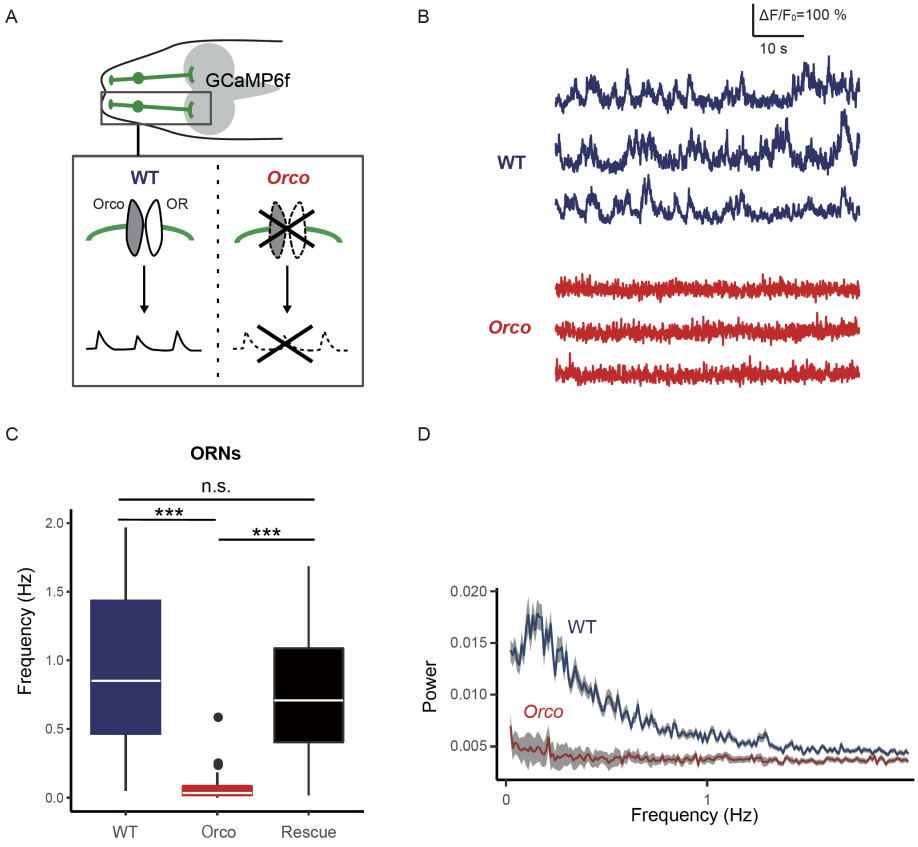

Figure 2 Orco is required for prior activity in larval ORNs

(A) Schematic of Orco-OR channel of ORNs in wild-type (WT) and *Orco* mutant larvae. The channel contributes to the prior activity of ORNs. (B) Representative fluorescence changes from baseline ($\Delta F/F_0$) of ORNs in wild-type (WT) and *Orco* mutant (*Orco*) larvae. (C) Quantification of the frequency of the prior activity of ORNs in the WT (WT, blue; n = 57), *Orco* mutant (Orco, red; n = 57) and *Orco* expression-rescued strain (Rescue, black; n = 43). Box plots show the median (white line), 25th, and 75th percentiles (box); the data range (whiskers); and outliers (circles). Outliers are data points located outside the whisker range. (D) Average power spectra of the prior activity of ORNs in WT (blue; n =132) and *Orco* mutant (red; n = 118). Means ± SEM. Genotype: WT, w^{1118};*Orco-Gal4,UAS-GCaMP6f/+*. Orco, w^{1118};*Orco-Gal4,UAS-GCaMP6f/+;orco*^{1}/*orco*^{1}. Rescue, w^{1118};*Orco-Gal4,UAS-GCaMP6f/UAS-Orco;orco*^{1}/*orco*^{1}. ***P < 0.001, Wilcoxon's rank-sum test with Bonferroni correction; n.s., not significant.

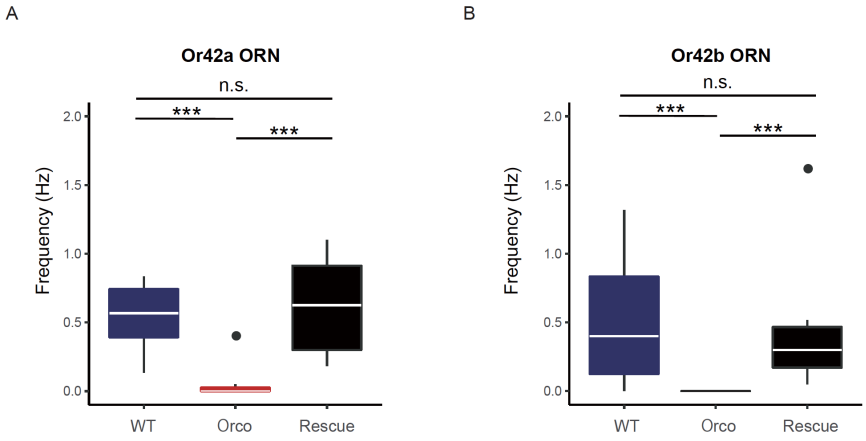

Figure 3 Orco in each ORN is essential for the prior activity of ORNs

(A and B) Quantification of the frequency of prior activity of a single ORN, Or42a (A) and Or42b (B). Genotype: WT, *w^{1118};Or42a/b-Gal4/UAS-GCaMP6f*. Orco, *w^{1118};Or42a/b-Gal4/UAS-GCaMP6f;$orco^{1}$/$orco^{1}$*. Rescue, *w^{1118};Or42a/b-Gal4,UAS-Orco/UAS-GCaMP6f;$orco^{1}$/$orco^{1}$*. ***P < 0.001, Wilcoxon's rank-sum test with Bonferroni correction; n.s., not significant.

3. *Orco* mutant larvae exhibit reduced attractive behavior in response to optogenetic stimulation of ORNs

In Fig. 2 and 3, I showed that the prior activity of ORNs was reduced in *Orco* mutant. This feature of *Orco* mutants allowed assessment of the contribution of the prior activity to behavior. Previous studies have reported that larvae exhibited stereotypic attractive or aversive behavior to an odor, even when only a single cell was stimulated (Bellmann et al., 2010; Fishilevich et al., 2005; Semmelhack & Wang, 2009). For this reason, I expected that the contribution of the prior activity could be estimated by comparing the stereotypic behavior of *Orco* mutant larvae with that of WT larvae. However, *Orco* mutant larvae lack their olfactory receptor complexes, indicating that their ORNs cannot be stimulated by odor (Larsson et al., 2004).

To overcome this difficulty and stimulate the ORNs in *Orco* mutant, I took advantage of optogenetics. To minimize the influence of phototaxis on behavior, I used red-light gated cation channel, CsChrimson (Klapoetke et al., 2014) to stimulate ORNs, because *Drosophila* larvae are reported to be blind to red light (Xiang et al., 2010). To examine whether larval behavior would change in the absence of prior activity, I

stimulated ORNs and compared the behavioral output of *Orco* mutant with that of WT larvae. I expressed the red light gated cation channel, CsChrimson (Klapoetke et al., 2014), in ORNs to induce evoked activity (Fig.5A). To activate ORNs in free-moving larvae, I used the agarose gel plates, which consisted of two illuminated quadrants and two dark quadrants, in a dark chamber (Fig. 5A). Larvae were placed on the plate and monitored for 5 minutes. I then calculated the performance index (PI) by using larval locations on the agarose plate (Fig. 5B).

I first checked CsChrimson expression and found no significant difference in CsChrimson expression levels in wild-type and *Orco* mutant ORNs (Fig. 4). WT larvae were strongly attracted to the red light by the expression of CsChrimson in ORNs (Fig. 5C: blue, PI, median 0.54, 1st quartile 0.42, 3rd quartile 0.67). In contrast, *Orco* mutant did not show significant attraction (Fig. 5C: red, PI, median 0.17, 1st quartile 0.10, 3rd quartile 0.25). To confirm these results in single ORN level, I expressed CsChrimson in each ORNs and observed larval behavior (Fig. 6). I chose Or42a and Or42b for sing-cell analysis (Fig. 5D, E), since larvae expressing CsChrimson in their Or42a or Or42b showed strong attractive behaviors to the red light. Similarly, by expressing

CsChrimson only in Or42a or Or42b, WT larvae were attracted to the light stimulation, like odor-induced behavior, while the attraction behavior of the *Orco* mutant larvae was weaker than that of the WT larvae (Fig. 5D, E). These results suggested that the reduction of prior activity might contribute to reducing the attractive behavior induced by optogenetic activation of ORNs.

Rescue experiments further support this notion. By rescuing the expression of Orco in ORNs, *Orco* mutant larvae gathered in the illuminated quadrants (Fig. 5C: black, PI, median 0.55, 1st quartile 0.47, 3rd quartile 0.59). Similar results were obtained when the rescue of *Orco* expression was restricted to a single ORN, Or42a or Or42b ORN (Fig. 5D, E). Thus, the prior activity might be required for the induction of attractive behavior to optogenetic activation of ORNs.

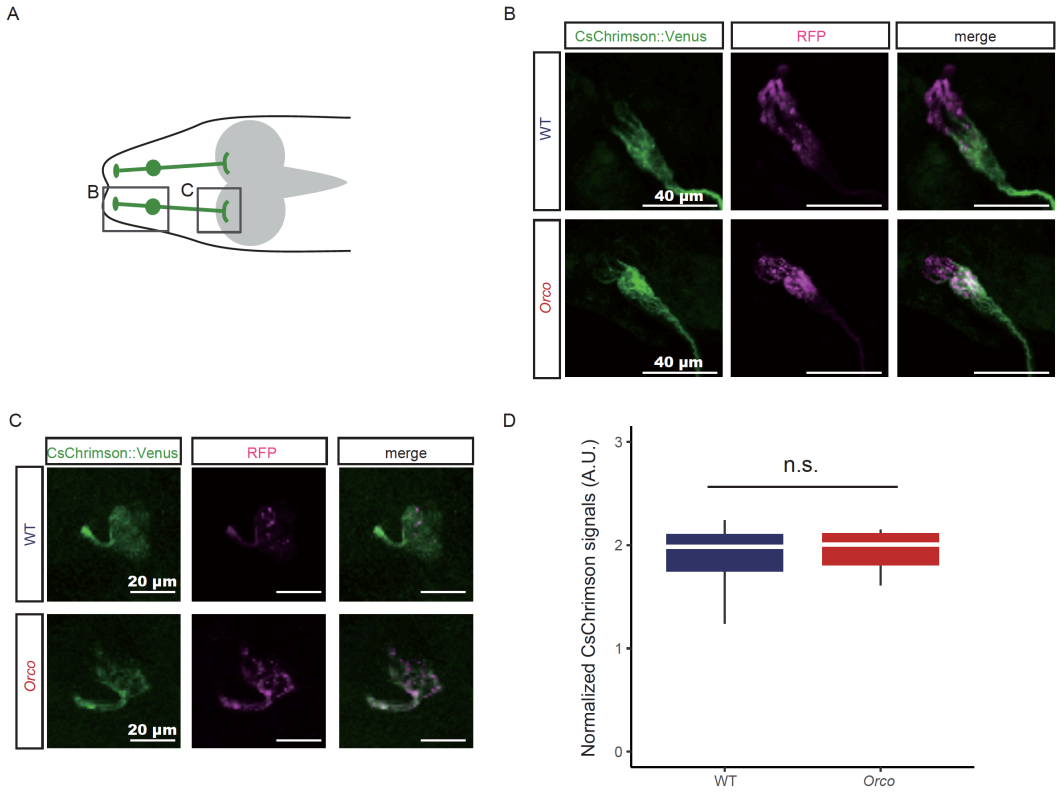

Figure 4 Expression levels of CsChrimson in ORNs

(A) Schematic shows ORNs in larval head. (B and C) CsChrimson::Venus (green) expression in dendrites (B) or axonal terminals (C) of ORNs. ORNs were visualized by mCD8::RFP (magenta). (D) Quantification of CsChrimson::Venus signals normalized to nc82 staining at the axonal terminals. Wilcoxon's rank-sum test; n.s. not significant.

Genotypes: WT, w^{1118};*Orco-Gal4,UAS-CD8::RFP/UAS-CsChrimson::Venus,Tsh-Gal80*.

Orco, w^{1118};*Orco-Gal4,UAS-CD8::RFP/UAS-CsChrimson,Tsh-Gal80;orco*^{1}/*orco*^{1}.

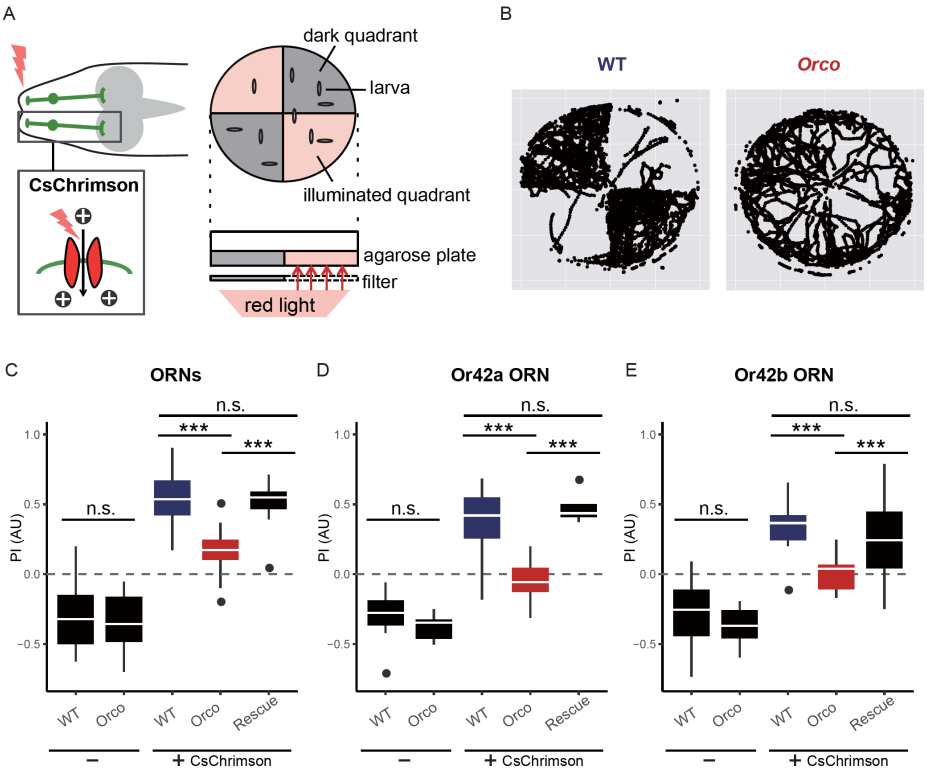

Figure 5 *Orco* mutant larvae exhibit reduced response to ORN activation

(A) Right, expression of CsChrimson in ORNs. Left, agarose gel arena for optogenetic behavioral assay. (B) Representative plots of WT (left) and *Orco* mutant (right) larval positions over a period of 5min. (C) Performance index (PI) of larvae expressing CsChrimson in their ORNs. Genotype: WT, w^{1118};*Orco-Gal4/UAS-CsChrimson,Tsh-Gal80*. Orco, w^{1118};*Orco-Gal4/UAS-CsChrimson,Tsh-Gal80;orco*^{1}/*orco*^{1}. Rescue, w^{1118};*Orco-Gal4,UAS-Orco/UAS-CsChrimson,Tsh-Gal80;orco*^{1}/*orco*^{1}. (D and E) Performance index (PI) of larvae expressing CsChrimson in a single ORN, Or42a (E) and Or42b (F). Genotype: WT, w^{1118};*Or42a/b-Gal4/UAS-CsChrimson,Tsh-Gal80*. Orco, w^{1118};*Or42a/b-Gal4/UAS-CsChrimson,Tsh-Gal80;orco*^{1}/*orco*^{1}. Rescue, w^{1118};*Or42a/b-Gal4,UAS-Orco/UAS-CsChrimson,Tsh-Gal80;orco*^{1}/*orco*^{1}. ***P < 0.001, Wilcoxon's rank-sum test with Bonferroni correction; n.s. not significant.

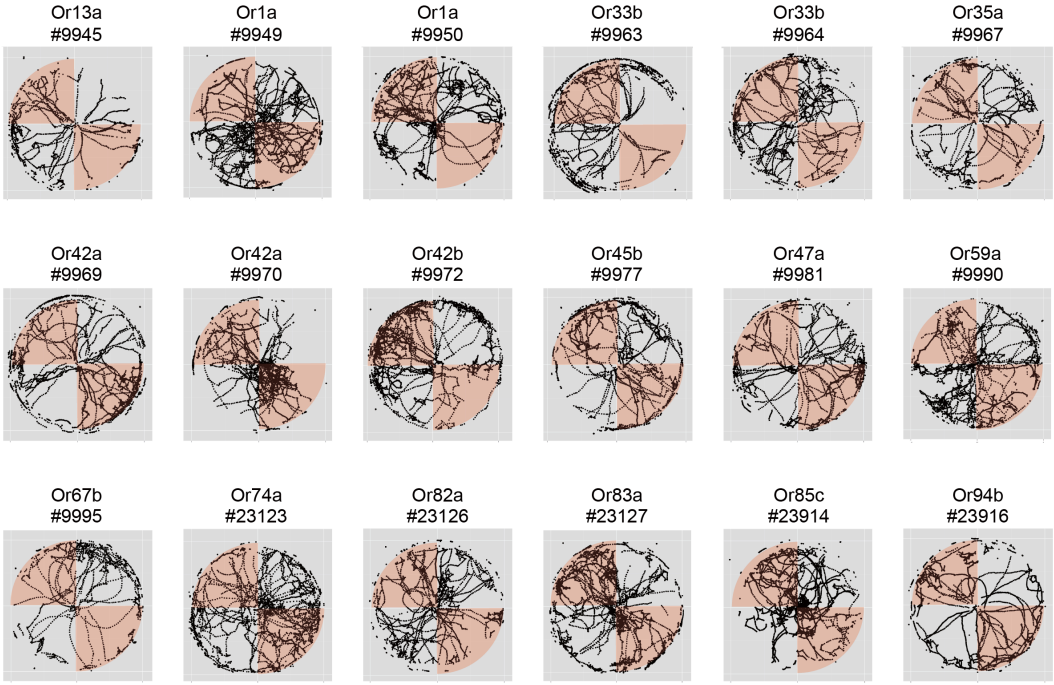

Figure 6 Larval behaviors induced by optogenetic stimulation of single ORNs

Representative plots of positions of larvae expressing CsChrimson in each ORNs over a period of 5min. Red shadow indicates illuminated area (Fig.5 A).

Genotype: w^{1118};*OrX-Gal4/UAS-CsChrimson,Tsh-Gal80*.

4. Reduced attractive behavior of Orco mutant is attributable to the reduction of turning behavior at the light/dark boundary

What behavioral changes cause *Orco* mutant larvae to spend less time in the illuminated areas? To address this, I focused on the behavior at the boundary of the quadrants and explored the following possibilities. The first possibility is that WT larvae enter the illuminated quadrants more frequently than *Orco* mutants (Fig. 7A: left, a) and do not go back into the dark quadrants. The second possibility is that, when WT larvae go out from illuminated quadrants, they more frequently turn back into the illuminated quadrants than *Orco* mutants (Fig. 7B: left, c) and do not enter the dark quadrants. To distinguish these possibilities, I calculated the probabilities of the behavior at the boundary.

I firstly assessed the movement of larvae from dark to illuminated quadrants (Fig. 7A). The larvae expressing CsChrimson in their ORNs were more likely to enter the illuminated quadrants (Fig. 7A: WT, 79.3%; *Orco* mutant, 75.3%) than the controls that did not express CsChrimson (Fig. 7A: WT, 51.0%; *Orco* mutant, 41.2%). Regarding the CsChrimson-expressing larvae, the probabilities of entrance were not

significantly different between WT and *Orco* mutant larvae (Fig.7A, right), suggesting that both WT and *Orco* mutant larvae expressing CsChrimson were attracted to enter the illuminated quadrants from the dark quadrants. Next, I focused on the larvae moving out from illuminated quadrants toward dark quadrants, and calculated the probability of turning back into illuminated quadrants at the boundary (Fig. 7B). Notably, *Orco* mutant larvae showed significantly lower probability of turning back into illuminated quadrants than WT larvae (Fig. 7B: WT, 95.5%; *Orco* mutant, 50.4%), indicating that *Orco* mutants did not go back into illuminated quadrants, but they did cross the boundary to enter dark quadrants. Thus, the reduction of attractive behavior of *Orco* mutant larvae could be attributed to a reduction of turning behavior at the boundary between illuminated and dark quadrants. Hence, these observations suggested that the prior activity of ORNs might contribute to inducing turning behavior when the attractive stimuli were reduced.

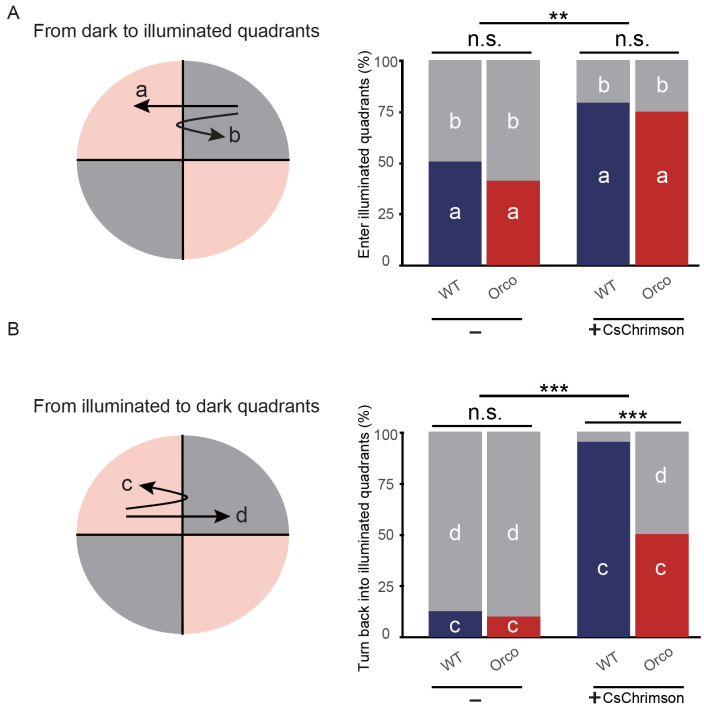

Figure 7 *Orco* mutant larvae are defective in turning behavior at the light/dark boundary

(A) Probability of larvae entering illuminated quadrants from dark quadrants. (B) The probability of turning back into illuminated quadrants. Genotype: WT, w^{1118};*Orco-Gal4/UAS-CsChrimson, Tsh-Gal80*. Orco, w^{1118};*Orco-Gal4/UAS-CsChrimson,Tsh-Gal80;orco*^{1}/*orco*^{1}. ***P < 0.001, **P < 0.01, Fisher's exact test with Benjamini–Hochberg correction (Ref); n.s. not significant.

5. Temporal dynamics of the neural response was altered in *Orco* mutant

In this section, from the perspective of the neural response, I investigated the reason for the difference in behavior between WT and *Orco* mutant larvae. In vertebrate brain, spontaneous activity may affect the neural response to the stimulation. For example, in cat striate cortex, the magnitude of the evoked response was linearly correlated with the spontaneous fluctuation of the membrane potential preceding the stimulation onset (Azouz & Gray, 1999). This observation suggested that the spontaneous fluctuation might cause variability in the evoked response. Moreover, the variability in the evoked response correlates with animal behavior (Pessoa, Gutierrez, Bandettini, & Ungerleider, 2002; Ress & Heeger, 2003). Taken together, spontaneous activity might change the behavioral output by affecting the evoked activity. Therefore, I expected that the evoked neural responses of ORNs to the stimulation differed between WT and *Orco* mutant larvae. To assess this notion, I examined the neural responses of ORNs evoked by optogenetic stimulation.

I first tested whether ORNs in *Orco* mutants could respond to optogenetic stimulation, by using Ca^{2+} imaging. I expressed the channelrhodopsin-2 (ChR2), a

cation channel gated by blue light (Zhang, Wang, Boyden, & Deisseroth, 2006), and GCaMP6f in ORNs and measured the Ca^{2+} responses of ORNs to blue light stimulation (Fig. 8A). ORNs in both WT and *Orco* mutant larvae were successfully activated by the transient blue light stimulation (Fig. 8B). Intriguingly, the average amplitude of the response was higher in *Orco* mutant rather than in WT larvae (Fig. 8C). When the stimulation was repeated, the responses of ORNs in WT larvae exhibited a large variability in amplitude (Fig. 8D). Conversely, in *Orco* mutant, the responses exhibited a fixed amplitude (Fig. 8E). Hence, the behavioral change in *Orco* mutant was not caused by a failure in the response of ORNs to the optogenetic stimulation, though the response amplitude in *Orco* mutant was different from that in WT larvae (Fig. 8).

Next, I aim to obtain insights into the actual neural responses of ORNs evoked during the behavioral assay. In my behavioral assay (Fig. 5, Fig. 7), larvae detected the stimulation light when they entered the illuminated quadrants (Fig. 9A, 1), kept detecting the stimulation when they remained within the quadrant (Fig. 9A, 2), and detected the reduction in the stimulation when they go out from that quadrant (Fig. 9A, 3). Thus, by continuously stimulating the ORNs and monitoring their activity from the

onset of the stimulation until its termination, I could estimate the neural response of ORNs when larvae entered, remained within, and went out from the illuminated quadrants during the behavioral assay (Fig. 9A).

I expressed ChR2 and the Ca^{2+} indicator RGECO1 (Zhao et al., 2011) in ORNs and measured the calcium responses of ORNs to continuous light stimulation (Fig. 9B). Similar to pulse stimulation (Fig. 8B, C), ORNs in *Orco* mutant responded to the stimulation more strongly than those in WT larvae at the onset of the stimulation (Fig. 9C), indicating that ORNs in both WT and *Orco* mutant larvae successfully detected the stimulation onset. This observation was consistent with my behavioral assay showing that both WT and *Orco* mutant larvae entered the illuminated quadrants with similarly high probabilities (Fig. 7A). During the stimulation, the response in WT larvae fluctuated but was sustained at a certain level (Fig. 9D). After termination of the stimulation, the neural activity of ORNs was suppressed below baseline in WT larvae. On the other hand, the response in *Orco* mutants gradually decreased during stimulation (Fig. 9E). After termination of the stimulation, the response in *Orco* mutants did not return to the baseline for a period of time. These observations revealed that ORNs in

Orco mutants were defective in both the maintenance of the response amplitude during stimulation and the suppression of the response after stimulation (Fig. 9D, E). I found that these different neural responses in *Orco* mutant ORNs were not caused by changes in expression levels of ion channels (Fig. 10). We performed RNA-Seq experiments in ORNs and did not detect significant differences in the expression levels of ion channels between the wild-type and *Orco* mutant ORNs.

Since a reduction in the turning behavior of larvae was observed when they exited from illuminated quadrants and detected a reduction in the optogenetic stimulation, these characteristics of the neural response, the maintenance and suppression of the neural activity, might contribute to inducing the turning behavior when optogenetic stimulation was reduced.

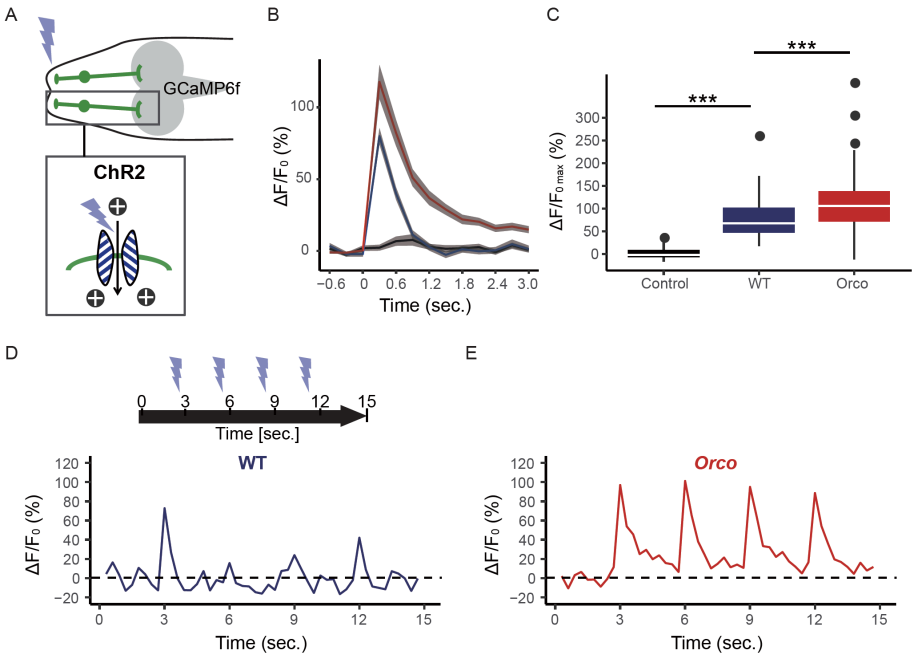

Figure 8 Temporal dynamics of ORN response to pulse stimulations

(A) Expression of channelrhodopsin-2 (ChR2) and GCaMP6f in ORNs. (B) Average fluorescence changes ($\Delta F/F_0$) to blue light stimulation in WT (blue; n = 64), Orco mutant (red; n = 40), and control larvae that did not carry UAS-ChR2 (black; n = 50). Means ± SEM. (C) Quantification of maximum $\Delta F/F_0$ ($\Delta F/F_{0max}$) of ORNs in the WT and *Orco* mutant. (D and E) Representative traces of $\Delta F/F_0$ of a single ORN in WT (D) and *Orco* mutant (E). ORNs are stimulated with the blue light pulses at four time points. Genotype: Control, *w^{1118};Orco-Gal4,UAS-GCaMP6f/Orco-Gal4*. WT, *w^{1118};Orco-Gal4,UAS-GCaMP6f/Orco-Gal4, UAS-hChR2[H134R]*. Orco, *Orco-Gal4,UAS-GCaMP6f/Orco-Gal4,UAS-hChR2[H134R];$orco^1$/$orco^1$*. ***P < 0.001, Wilcoxon's rank- sum test with Bonferroni correction; n.s. not significant.

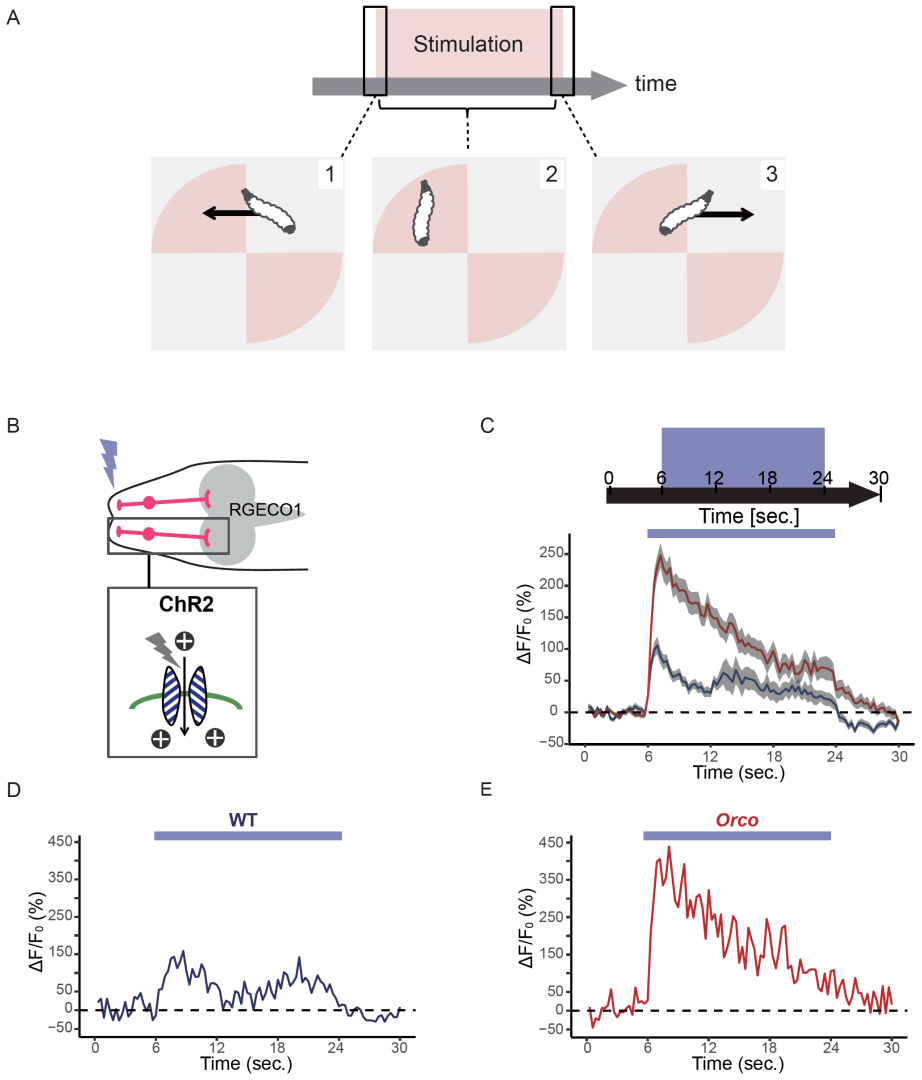

Figure 9 Temporal dynamics of ORN response to continuous stimulations

(A) Schematic of the stimulation that larvae experience during optogenetic behavioral assay (Fig. 5). (B) Expression of ChR2 and RGECO1.0 in ORNs. (C) Average $\Delta F/F_0$ to blue light stimulation in WT (blue; n = 31) and Orco mutant (red; n = 33). Means ± SEM. (D and E) Representative traces of $\Delta F/F_0$ of a single ORN in WT (D) and *Orco* mutant (E). ORNs are stimulated with the blue light for 18 s.

Genotype: WT, w^{1118};*Orco-Gal4,UAS-RGECO1/Orco-Gal4,UAS-hChR2[H134R]*.

Orco, *Orco-Gal4,UAS- RGECO1/Orco-Gal4,UAS-hChR2[H134R];orco*^{1}/*orco*^{1}.

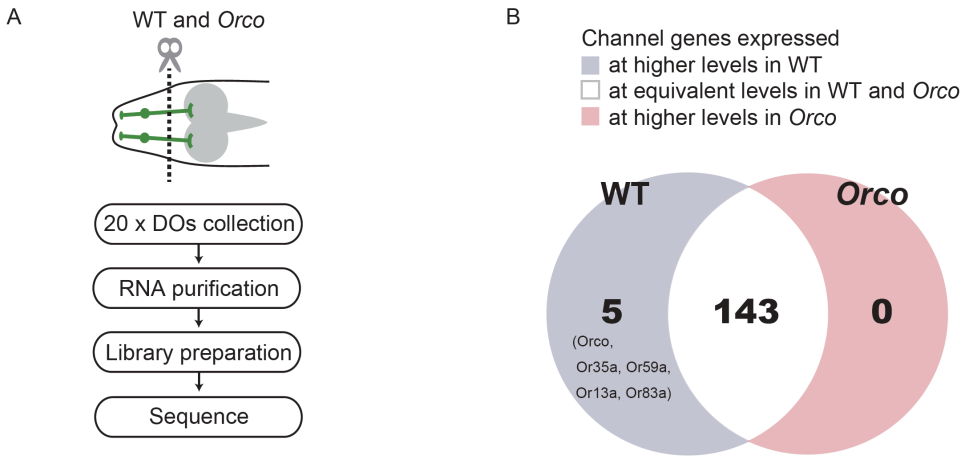

Figure 10 Expression levels of ion channels in ORNs

(a) Schematic diagram of RNA-seq analysis. DOs indicates dorsal organs. A detailed description of the library preparation protocol is provided in the methods. (b) Differential expression analysis of ion channel gene expression in wild-type and Orco mutant dorsal organs. Venn diagram shows the number of ion channel genes that were expressed at significantly higher levels in WT (Blue), at higher levels in Orco mutants (Red), or at equivalent levels in WT and Orco mutant dorsal organs (White).

Genotypes: WT, w^{1118};*Orco-Gal4,UAS-CD8::GFP/Orco-Gal4,UAS-CD8::GFP*.

Orco, w^{1118};*Orco-Gal4,UAS-CD8::GFP/Orco-Gal4,UAS-CD8::GFP;*$orco^{1}/orco^{1}$.

6. Prior activity of ORNs affects prior and evoked activity of PNs and LNs

Given that spontaneous activities often correlate among regions in the brain (Fox, Corbetta, Snyder, Vincent, & Raichle, 2006), I expected that the prior activity of ORNs might affect the neural activities of synaptically coupled neurons. In the *Drosophila* olfactory system, projection neurons (PNs) transfer olfactory information from ORNs to higher-order brain areas (Berck et al., 2016; Masuda-Nakagawa, Gendre, O'Kane, & Stocker, 2009; Stocker, Lienhard, Borst, & Fischbach, 1990). Additionally, the signal transduction between ORNs and PNs is modulated by local neurons (LNs) (Liang et al., 2013). I therefore investigated whether the prior activity of ORNs affects prior and evoked activities in PNs and LNs.

I firstly compared the prior activity of PNs and LNs in *Orco* mutant larvae to those in WT larvae (Fig. 11A, D). Both in PNs and LNs, the frequencies of the prior activities were not significantly different between WT and *Orco* mutant larvae (Fig. 11B, E). However, the power spectrum of the prior activity of PNs in *Orco* mutants had a smaller peak than in WT larvae (Fig. 11C), indicating that rhythmic activity was decreased. In contrast, the power spectrum of the prior activity of LNs in *Orco* mutant

showed a larger peak than in WT (Fig. 11F), indicating that rhythmic activity was increased. Thus, the prior activity of ORNs conversely affected the spontaneous activity of PNs and LNs (Fig. 11C, F). These observations revealed that the prior activity of ORNs affects the prior activity of synaptically coupled neurons.

To investigate whether PNs and LNs could respond to continuous stimulations of ORNs, I confirmed that synapses between ORNs and PNs would be formed in *Orco* mutants. I expressed the presynaptic marker, Bruchpilot::GFP (Brp::GFP) (Wagh et al., 2006), in Or42a and observed the localization of the marker signals. I found that the Brp signals were localized at the axonal terminals in both wild-type and *Orco* mutant ORNs, though the signals in *Orco* mutant ORNs were condensed compared to wild-type (Fig. 12). This result suggests that the synapses between ORNs and PNs would be formed in *Orco* mutants.

I then continuously activated ORNs and monitored the calcium responses in PNs and LNs (Fig. 13A, C). At the onset of the ORN activation, the response of PNs was larger in *Orco* mutant than in WT larvae (Fig. 13B). However, the response in *Orco* mutant was gradually decreased and finally became weaker than in WT larvae. I noticed

that this response in PNs resembled that of ORNs from the point of view of temporal dynamics. In contrast, the response dynamics of LNs was markedly different (Fig. 13D). The response of LNs was the same between WT and *Orco* mutant at the beginning of the ORN activation. During the activation, however, the response of LNs in *Orco* mutants was sustained, in contrast to the reduction of the response in WT larvae. These data suggested that, in *Orco* mutant, the temporal dynamics of the response was changed in both PNs and LNs (Fig. 13B and D). Therefore, the changes in the ORN responses were possibly reflected in the neural activities of synaptically connected neurons.

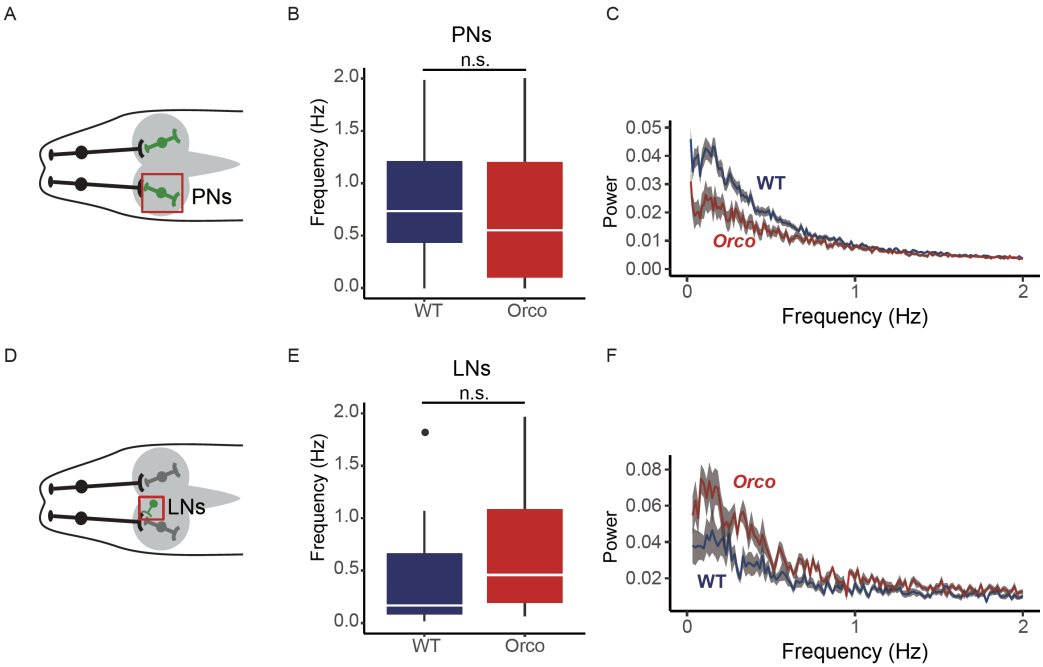

Figure 11 Prior activities of PNs and LNs

(A) Expression of GCaMP6f in PNs. (B) Quantification of the frequency of the prior activity of PNs in the WT (WT, blue; n = 139), and *Orco* mutant (Orco, red; n = 81). (C) Average power spectrum of the prior activity of PNs in WT (blue; n =139), and Orco mutant (red; n = 81). Means ± SEM. (D) Expression of GCaMP6f in LNs. (E) Quantification of the frequency of the prior activity of LNs in the WT (WT, blue; n = 18), and *Orco* mutant (Orco, red; n = 15). (F) Average power spectrum of the prior activity of LNs in WT (blue; n =18), and Orco mutant (red; n = 15). Means ± SEM. Genotype: PNs, WT, w^{1118}*;GH146-Gal4/UAS-GCaMP6f.* Orco, w^{1118}*; GH146-Gal4/UAS-GCaMP6f;orco*^{1}*/orco*^{1}. LNs, WT, *LN2-Gal4/*w^{1118}*;+/UAS-GCaMP6f.* Orco, *LN2-Gal4/*w^{1118}*;+/UAS-GCaMP6f;orco*^{1}*/orco*^{1}.

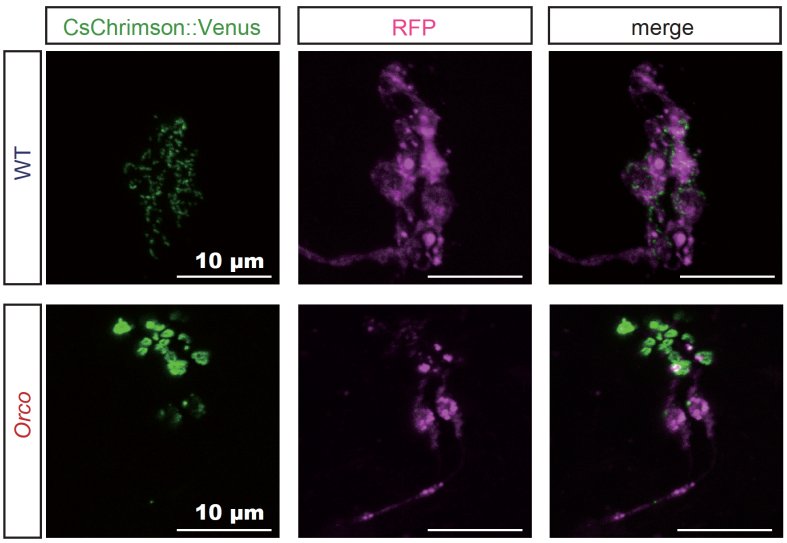

Figure 12 Localization of presynaptic protein in axonal terminal of Or42a

Brp::GFP (green) expression in axonal terminals of Or42a ORNs that additionally express *mCD8::RFP* (magenta). Genotypes: WT, w^{1118}*;Or42a-Gal4,UAS-CD8::RFP/UAS-Brp::GFP*. Orco, w^{1118}*;Or42a-Gal4,UAS-CD8::RFP/UAS-Brp::GFP;*$orco^{1}$/$orco^{2}$.

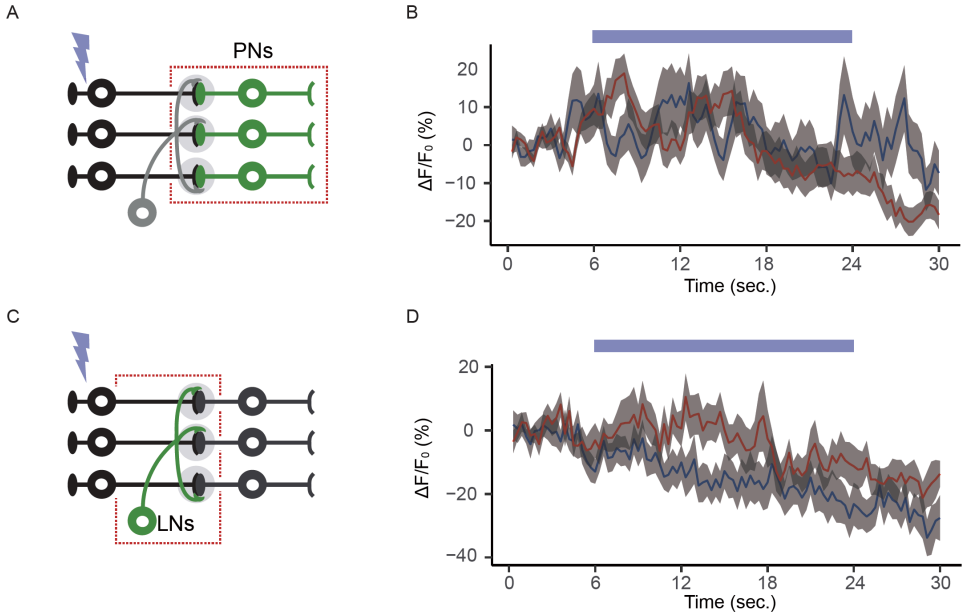

Figure 13 PN and LN responses to continuous stimulations of ORNs

(A) Expression of Channelrhodopsin-2 (ChR2) in ORNs and RGECO1.0 in PNs. (B) Average $\Delta F/F_0$ of PNs to blue light stimulation of ORNs in WT (blue; n = 56), and Orco mutant (red; n = 70). Means ± SEM. (C) Expression of Channelrhodopsin-2 (ChR2) in ORNs and RGECO1.0 in LNs. (D) Average $\Delta F/F_0$ of LNs to blue light stimulation of ORNs in WT (blue; n = 12), and Orco mutant (red; n = 14). Means ± SEM.

Genotype: PNs, WT, *w^{1118};GH146-Gal4,UAS-RGECO1/GH146-Gal4,Orco-hChR2*. Orco, *w^{1118};GH146-Gal4,Orco-hChR2/UAS-RGECO1,Orco-hChR2;$orco^1$/$orco^1$*.

LNs, WT, *LN2-Gal4/w^{1118};UAS-RGECO1,Orco-hChR2/+*.

Orco, *LN2-Gal4/w^{1118}; UAS-RGECO1,Orco-hChR2/+;$orco^1$/$orco^1$*.

7. Orco-OR fusion protein has a potential to increase prior activity

So far, I demonstrated the behavior and neural response changes in *Orco* mutant larvae, which exhibited the reduced prior activity in their ORNs. However, the impact of increased prior activity remains elusive. To increase the prior activity, I have developed a novel tool for regulating the prior activity. In Fig.2, I revealed that Orco-OR complex was essential for the prior activity of ORNs. Therefore, I hypothesized that the Orco-OR complex could be applied to a tool for modulating prior activity of not only ORNs, but also other cells.

To apply Orco-OR complex to a tool for modulating prior activity, I generated the Orco-OR fusion protein and assessed its effects on the prior activity (Fig. 14A). Both Orco and OR are seven-transmembrane proteins and have intracellular N termini and extracellular C termini (Benton, Sachse, Michnick, & Vosshall, 2006). For these reasons, the C terminal of Orco and the N terminal of OR could not be linked directly. To overcome this problem, I employed a single transmembrane protein, sodium channel subunit beta-1 (SCN1B) as a linker (Fig. 11A). In a previous functional study about the Orco dimer (Mukunda, Lavista-Llanos, Hansson, & Wicher, 2014), SCN1B was

employed as a linker to generate an Orco dimer fusion protein (Orco di). This dimer protein, expressed in Chinese hamster ovary (CHO) cells, was successfully activated by a synthetic agonist of Orco, VUAA1 (Jones, Pask, Rinker, & Zwiebel, 2011), suggesting that Orco di preserves its structure and ion channel function. Thus, SCNB1 might be suitable for linking Orco and OR. I chose Or47a as an OR domain and then generated Orco::SCN1B::Or47a (Fig. 14A). To examine whether the Orco-OR fusion protein could increase prior activity, I performed calcium imaging. To avoid interaction with the endogenous olfactory complexes, I expressed the Orco-OR fusion protein in PNs (Fig. 14A). As hypothesized, the prior activity was significantly increased in PNs expressing the Orco-OR fusion protein (Fig. 14B). I also calculated the power spectrum of the prior activity of PNs expressing the fusion protein (Fig. 14C). This power spectrum exhibited a larger characteristic peak than the control, suggesting that the increased prior activity is similar in characteristics to the intrinsic prior activity of PNs.

Interestingly, larvae expressing Orco-OR fusion protein in their PNs seemed to be hyperactive and to behave differently from the control larvae. Indeed, the larvae expressing the Orco-OR fusion protein showed head casting and turning behavior more

frequently compared to control larvae (Fig. 14D, E, F), indicating that Orco-OR fusion protein expression in PNs increased odor-seeking behavior.

These data suggested that Orco-OR fusion protein has the potential to increase prior activity and can be available to a novel tool for increasing prior activity.

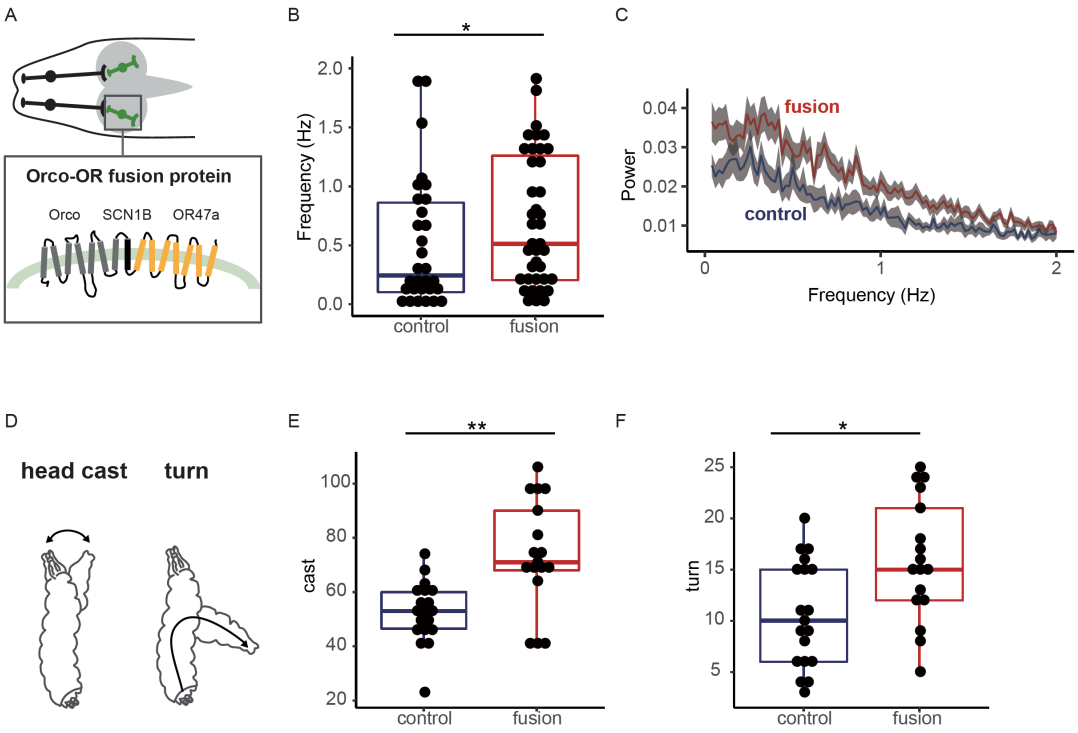

Figure 14 Effects of Orco-OR fusion protein on prior activity and basal locomotion

(A) Schematic of Orco-OR fusion protein. Expression of the fusion protein and GCaMP6f in PNs. (B) Quantification of the frequency of the prior activity of the control PNs (control, blue; n = 30), and PNs expressing the fusion protein (fusion, red; n = 41). (C) Average power spectra of the prior activity of PNs in WT (blue; n =30) and Orco mutant (red; n = 41). Means ± SEM. Genotype: control, *w^{1118};GH146-Gal4,UAS-GCaMP6f/+*. fusion, *w^{1118};GH146-Gal4,UAS-GCaMP6f/UAS-Orco::SCN1B::Or47a.* (D) Schematic of odor-seeking behaviors. (E) Quantification of the frequency of the head casting of the controls (control, blue; n = 19), and the larvae expressing the fusion protein (fusion, red; n = 18). (F) Quantification of the frequency of the turning of the controls (control, blue; n = 19), and the larvae expressing the fusion protein (fusion, red; n = 18). Genotype: control, *w^{1118};GH146-Gal4/+*. fusion, *w^{1118};GH146-Gal4/UAS-Orco::SCN1B::Or47a.*

Discussion

Mechanisms underlying the effects of Orco on the prior activity of ORNs

Animals rely on their sensory neurons to detect external stimuli and change their behavior in response to the external world. However, sensory neurons are often activated by background stimuli prior to experiencing external stimuli. In addition, even in the absence of such background stimuli, many types of neurons exhibit spontaneous activity. These evoked or spontaneous neural activities prior to stimuli, or prior activity, are observed in many sensory systems and cortical regions, yet their impacts on animal behavior remain unclear.

I demonstrated, by Ca^{2+} imaging, that ORNs were active prior to the odor stimulation (Fig. 1B). This prior activity was reduced at the population level and a single-cell level in *Orco* mutants (Fig. 2, 3). I also showed that the reduced prior activity was rescued by recovering the expression of *Orco* (Fig. 2C, 3A, B). Taken together, I revealed that *Orco* expression was cell-intrinsically essential for the prior activity of ORNs (Fig. 2, 3).

How does Orco affect the prior activity of ORNs? One possibility is that Orco is required for the activation of ORNs by background odor. In ORNs of *Orco* mutants,

neither Orco nor OR localize to the dendrites (Larsson et al., 2004). Because odorant molecules bind to the OR on the dendrite, background odorants cannot activate ORNs in *Orco* mutants. Therefore, the lack of evoked activity by background odorants likely led to the reduced prior activity in *Orco* mutant. However, this possibility does not fully explain the effect of Orco on the prior activity of ORNs. Since the power spectrum of the prior activity showed a characteristic peak (Fig. 2D, blue), the prior activity might include rhythmic activity. Assuming that external background odor randomly activates ORNs, rhythmic internal activity might be required for prior activity. In many systems, spontaneous activity is spatiotemporally organized (Arieli et al., 1995; Ikegaya et al., 2004; Romano et al., 2015). Therefore, the decrease in the peak of the power spectrum of ORNs may imply a decrease in spontaneous activity in *Orco* mutant. Indeed, even in the absence of odor ligands, intracellular Ca^{2+} levels were increased in HeLa cells expressing Orco and OR (Sato et al., 2008), indicating that Orco-OR complex may mediate spontaneous calcium influx. In this case, considering that Orco and OR form a non-selective cation channel, spontaneous opening of the channel likely triggered Ca^{2+} influx. This calcium influx might contribute to prior activity of ORNs. The frequency of

the spontaneous activity in adult ORNs differed depending on the OR (Hallem & Carlson, 2006; Hallem et al., 2004), suggesting that the OR also contributes to ion permeability. Therefore, the difference in the interaction between Orco and cell-specific OR may characterize ion permeability of each channel and thereby determine the cell-specific frequency of spontaneous activity. Taken together, Orco possibly triggers the prior activity of ORNs by contributing to both the background stimuli-based evoked activity and genuine spontaneous activity.

Inhibitory effect of prior activity on evoked activity in ORNs

In Fig. 8 and 9, I confirmed that ORNs in *Orco* mutants successfully responded to the optogenetic stimulation. Interestingly, ORNs in *Orco* mutants exhibited larger and less variable responses to the stimulation, while ORNs in WT larvae exhibited lower and highly variable responses (Fig. 8).

Although the causal effect of spontaneous activity on evoked activity is largely unknown, recent studies demonstrated that spontaneous activity preceding the onset of stimulation correlated with trial-to-trial variability in the neural response (Arieli, Sterkin, Grinvald, & Aertsen, 1996; Azouz & Gray, 1999), suggesting that spontaneous activity might contribute to the neural response variability. Assuming that prior activity

also contributes to the variability in the neural response, the reduction in prior activity might result in a reduction in the neural response variability. Consistently, I showed that ORNs in *Orco* mutants showed less variable response than those in WT larvae (Fig. 8D, E), indicating that the prior activity of ORNs might be involved in the trial-to-trial variability in evoked activity. In some systems, spontaneous activity is supposed to cause variable evoked responses by additively modulating the evoked activity (Arieli, Sterkin, Grinvald, & Aertsen, 1996; Azouz & Gray, 1999). However, in the present thesis, the prior activity of ORNs seemed to inhibit the evoked activity, since the average response in WT larvae was smaller than that in *Orco* mutants (Fig. 8B, C). This result was similar to the effects of the spontaneous activity in the somatosensory cortex of rodents (Petersen, Hahn, Mehta, Grinvald, & Sakmann, 2003).

How does the prior activity exert inhibitory effects on the evoked activity? Theoretical and experimental studies of spontaneous activity reported that excitatory currents should be balanced by inhibitory currents to maintain spontaneous activity (Denève & Machens, 2016). In other words, spontaneous activity reflects intracellular depolarizing force and repolarizing force. Considering that spontaneous activity is one

type of prior activity, prior activity might also reflect the balance between excitatory currents and inhibitory currents. Since the balance between depolarizing force and repolarizing force is different depending on cell types or circuits, prior activity might have various effects on evoked activity according to the cell types or circuits. Given that, in the *Drosophila* olfactory circuit, the prior activity of ORNs might have inhibitory effects on evoked activity, the difference in neural responses between WT and *Orco* mutant ORNs might be partly explained by the effect of the repolarizing force reflected by the prior activity.

As the inhibitory source of the prior activity in WT ORNs, I raised the following possibilities: First, inhibitory neurons, such as GABAergic LNs, exert an inhibitory effect. Second, prior activity may change expression level of ion channel proteins and increase inhibitory currents through ion channels in a cell-intrinsic manner. In the present thesis, I examined the first possibility by assessing the neural activity of GABAergic LNs. I expected that, in *Orco* mutants, prior activity in LNs would be lower than in WT larvae, if the recruitment of LNs was different in *Orco* mutants. Unexpectedly, however, the prior activity of LNs in *Orco* mutants was not different

from those in WT and seemed to be more rhythmic than those in WT larvae (Fig. 11E, F), suggesting that the prior activity of ORNs could indirectly affect the activity of LNs. LNs receive the different strength inputs from ORNs and are divided into various subsets (Berck et al., 2016), suggesting that different subsets of LNs might be differently affected by the prior activity of ORNs. Because LNs mutually inhibit one another in a hierarchical fashion (Berck et al., 2016), a reduction in the prior activity of ORNs might alter the balance of mutual inhibition between LNs. This imbalance of mutual inhibition might result in the activation of some LNs. In the present thesis, I examined only one subset of LNs that were labeled by the available Gal4 line, LN2-*Gal4*. To fully elucidate the influence of the prior activity of ORNs on the recruitment of LNs, identification of Gal4 lines that can label specific subsets of LNs will be required in future studies.

I examined the second possibility by assessing expression levels of ion channels. My RNA-Seq analysis showed that the expression levels of ion channels were not significantly different between wild-type and *Orco* mutant ORNs (Fig. 10). Thus, one possibility is that prior activity might regulate post-transcriptional control of

channel expression or modulate ion channel function by another mechanism, such as post-translational modification and/or trafficking, and hence change the neural responses.

In addition to suppression of the evoked response of ORNs, I found that the temporal pattern of evoked activity changed in *Orco* mutants (Fig. 9). With continuous activation, the response of ORNs in WT larvae fluctuated with the fixed amplitude of peaks during ORN activation, whereas the response was constantly decreased in *Orco* mutants. How is the response amplitude maintained in wild-type ORNs? One possibility is that the repolarization force reflected by the prior activity is necessary to maintain the response. Generally, prolonged depolarization inactivates ion channels that are required for firings (Bean, 2007). To recover from the inactivation state, repolarization is necessary. The fluctuating response of ORNs in WT larvae indicates that ion channels can switch between an inactivated state and an activated state, implying the presence of a repolarizing force. In contrast, the amplitude of the response was gradually decreased in *Orco* mutant, suggesting that ion channels may be inactivated by prolonged depolarization and cannot open anymore by further depolarization, due to the lack of

repolarizing force. Thus, the repolarizing force reflected by the prior activity might be one important factor for the maintenance of the response amplitude.

Potential roles for the prior activity of ORNs in larval behavior

In Fig. 5, I firstly showed that WT larvae expressing CsChrimson gathered in the illuminated quadrants, while *Orco* mutant did not. These results indicated that *Orco* mutant larvae exhibited reduced attractive responses to the optogenetic stimulation.

Although the effect of the prior activity on animal behavior remains unknown, recent studies using functional magnetic resonance imaging (fMRI) have suggested that spontaneous activity might account for trial-to-trial variability in neural responses and thus variability of behavior to the same stimulation (Fox, Snyder, Vincent, & Raichle, 2007). Indeed, the fMRI signal magnitude can predict the behavioral variability in visual perception and working memory performance (Pessoa et al., 2002; Ress & Heeger, 2003). Assuming that prior activity may also affect evoked responses and behavior similar to spontaneous activity, I expected that *Orco* mutant larvae could respond to the stimulus and exhibit more robust behavior than WT larvae, since ORNs of *Orco* mutant exhibited reduced prior activity in their ORNs. However, *Orco* mutants

failed to gather in illuminated quadrants and exhibited the reduced attractive behavior to the stimulation, while WT larvae exhibited the robust attractive behavior (Fig. 5).

Why did *Orco* mutant larvae show less attractive behavior to optogenetic stimulation than WT larvae? One possibility is that neural circuit formation was impaired in *Orco* mutants. Recent studies on mammalian brain development have reported that spontaneous activity is observed in many developmental neural circuits, including the visual, the auditory, the cerebellar and the hippocampal neural circuits, and have suggested that the spontaneous activity is involved in neural circuit formation (Blankenship & Feller, 2009). Furthermore, blocking neural activity of ORNs led to alterations in the morphology of ORN axon terminals in *Drosophila* embryos(Prieto-Godino, Diegelmann, & Bate, 2012). These observations suggest that prior activity in ORNs might contribute to the neural circuit formation. Although, in the *Drosophila* olfactory system, the projection patterns of ORNs were not affected both in ether *Orco* mutant or OR mutant (Dobritsa et al., 2003; Larsson et al., 2004), I found that the presynaptic marker signals of the axon terminals were more condensed in *Orco* mutant ORNs compared to wild-type ORNs (Fig. 12). I, therefore, cannot exclude the

possibility that the connectivity between ORNs and PNs might be modified. However, *Orco* mutant PNs responded to continuous ORN activation (Fig. 13), suggesting the functional connectivity between ORNs and PNs in *Orco* mutants is intact. Importantly, I showed that, when larvae entered the illuminated quadrants from the dark quadrants, both WT and *Orco* mutant larvae entered the illuminated quadrants more frequently than the controls that did not express CsChrimson (Fig. 7A). These data suggest that *Orco* mutant larvae likely detect the stimulation and induce attractive behavior at the onset of the stimulation, similar to WT larvae. On the other hand, *Orco* mutant larvae went out from the illuminated quadrants to the dark quadrants significantly more often (Fig. 7B), suggesting that *Orco* mutants failed to detect the reduction of the light stimulation at the boundary. Taken together, *Orco* mutant larvae were defective in induction of turning behavior when the stimulation was reduced, leading to the reduced attractive behavior. Given that spontaneous activity might affect evoked response (Fox, Corbetta, Snyder, Vincent, & Raichle, 2006; Fox, Snyder, Vincent, & Raichle, 2007), the behavioral changes in *Orco* mutant might be attributed to the changes in neural response to the stimulation.

To explore the reason why *Orco* mutant larvae failed to induce turning behavior during the behavioral assay (Fig. 7B), I compared the temporal dynamics of the neural response between WT and *Orco* mutant larvae by continuous stimulation of ORNs (Fig. 9). As expected from the behavioral assay (Fig. 7B), I demonstrated that *Orco* mutant might be defective in detecting the termination of the stimulation (Fig. 9C, D). During continuous activation of ORNs (Fig. 9), the response of ORNs in WT larvae fluctuated with the fixed amplitude of peaks, whereas, after the stimulation, the GCaMP signal seemed to be suppressed below the baseline. On the other hand, the response of ORNs in *Orco* mutant was decreased gradually during the continuous stimulation. In addition, the neural response in *Orco* mutant ORNs remained above the baseline after stimulation. The temporal pattern of neural activity during stimulation or its suppression after stimulation might be important for the induction of turning behavior when the stimulation was reduced. Consistently, the larvae expressing CsChrimson in Or42a ORN demonstrated increased run-to-turn transitions when optogenetic activation was reduced (Hernandez-Nunez et al., 2015).

Additionally, in the present thesis, I also suggested that the prior activity might propagate to synaptically coupled neurons (Fig. 11, 13). Similar to the power spectrum of the prior activity of ORNs (Fig. 2), that of the prior activity of PNs showed a decreased peak in *Orco* mutants (Fig. 11C), indicating that the prior activity of ORNs directly affects the activity of PNs. Moreover, in contrast to ORNs and PNs, the power spectrum of the prior activity of LNs exhibited an increased peak in *Orco* null mutants (Fig. 11F), indicating the indirect effect of the prior activity of ORNs via the neural circuit. These propagations of prior activity might largely alter behavioral output. Indeed, only by a reduction in the prior activity of ORNs, the behavioral output was altered, as shown in Fig. 5. Taken together, the change in prior activity of ORNs might affect the neural activity at the circuit level, implying the mechanism that change of the prior activity of some neurons alters the global sensory processing in the circuit and thereby output behavior.

Possible effects of prior activity on basal locomotion

To investigate the effects of increased prior activity, I developed a novel tool for increasing prior activity. I examined whether Orco-OR fusion proteins enhance prior

activity in PNs (Fig. 14). Calcium imaging in PNs revealed that the prior activity was significantly increased in PNs expressing Orco-OR fusion protein (Fig. 14B). This increased prior activity of PNs exhibited a larger characteristic peak in the power spectrum than that in the controls (Fig. 14C). Furthermore, calculation of basal locomotion demonstrated that the larvae expressing Orco-OR fusion protein increased their odor-seeking behavior (Fig. 14E, F). These observations suggested that Orco-OR fusion protein enhanced the prior activity of PNs and changed the basal locomotion of the larvae.

Although further investigation will be required to elucidate the mechanism by which the Orco-OR fusion protein enhanced the prior activity of PNs, a recent study about Orco dimer linked with SCN1B (Orco di) showed that CHO cells expressing Orco di exhibited higher intracellular calcium levels than control cells (Mukunda et al., 2014), indicating that Orco di can trigger a calcium influx similar to Orco-OR complex (Sato et al., 2008). Assuming that the Orco-OR fusion protein linked by SCN1B preserved the ion channel property of original Orco-OR complex, Orco-OR fusion protein might also spontaneously open and thus enhance the prior activity of PNs.

Indeed, power spectrum analysis showed that Orco-OR fusion protein increased the characteristic peak that was decreased in *Orco* mutant, implying that Orco-OR fusion protein in PNs had an effect similar to Orco-OR complex in ORNs. Taken together, Orco-OR fusion protein might increase prior activity in PNs in the same manner as olfactory receptor complex.

The larvae expressing Orco-OR fusion protein showed head casting and turning behavior frequently (Fig. 14E, F). Given that GH146-*Gal4*, which was used to express CsChrimson in PNs, can label many neurons other than PNs (Flybase, expression data, http://flybase.org/reports/FBti0016783.html), the other neurons possibly affect the larval behavior by expressing Orco-OR fusion protein. Another possibility is that prior activity of PNs might be involved in regulation of basal locomotion. Head casting and turning behavior are often observed when larvae are seeking the odor source (Gomez-Marin, Stephens, & Louis, 2011). Larvae can follow the odor by casting their head and turning their bodies towards the source. When ORNs that sense attractive odors were optogenetically activated, larvae switch their behavior from running to turning upon a decrease in the stimulation. In contrast, when ORNs that

sense aversive odors were optogenetically activated, larvae switch their behavior from runnig to turning upon an increase in the stimulation (Hernandez-Nunez et al., 2015). Taken together, such behavioral switching between running to turning might be induced by a change in the ORN neural activity. The change in the ORN activity might be encoded by the spike rate of PNs (Kim, Lazar, & Slutskiy, 2015). Therefore, the prior activity of PNs might affect the basal locomotion, especially head casting and turning behavior. Since GH146 line labeled many neurons in addition to PNs, further studies by other Gal4 lines that can label PNs will be required for elucidating the effects of the prior activity in PNs on larval basal locomotion.

Conclusion

To realize appropriate sensory processing, neural circuits in the brain should decode an evoked response based on neural activity prior to the stimulation. Such prior activity is generally observed in olfactory, auditory, visual, and cortical neurons (Arieli et al., 1996; Hallem et al., 2004; Luczak, Barthó, & Harris, 2009; Petersen, Hahn, Mehta, Grinvald, & Sakmann, 2003; Romano et al., 2015). In some cases, the prior activity is thought to modify responses evoked by stimuli (Arieli, Sterkin, Grinvald, & Aertsen, 1996; Azouz & Gray, 1999; Fox, Snyder, Vincent, & Raichle, 2007; Petersen, Hahn, Mehta, Grinvald, & Sakmann, 2003). However, the contribution of the prior activity to output behavior is largely unknown, because of the difficulty in manipulating the prior activity and the lack of a suitable model for assessing the effect of prior activity from circuit to behavior. In the present thesis, I have developed a model system to assess potential roles for prior activity in animal behavior and revealed the potential roles in sensory processing and animal behavior.

In the present study, I investigated the functional significance of prior activity of ORNs in *Drosophila* larvae. In Fig. 2, I firstly confirmed that the prior

activity of ORNs depends on Orco. Surprisingly, I found that *Orco* mutant larvae, which had the decreased prior activity of ORNs, showed less attractive behavior to the optogenetic stimulation light than WT larvae (Fig. 5). This behavioral change in *Orco* mutant larvae was attributed to a reduction in turning behavior when larvae went out from the illuminated area to the dark area (Fig. 7). In Fig. 9, the combination of the optogenetic stimulation and *ex vivo* calcium imaging also revealed that the temporal pattern of the neural response of ORNs differed between WT and *Orco* mutant larvae, providing insights into the induction of turning behavior upon a decrease in stimulation. In Fig. 14, I developed the Orco-OR fusion protein for modulating prior activity and found that the prior activity was enhanced in PNs expressing the fusion protein. Interestingly, the larvae expressing the Orco-OR fusion protein exhibited hyperactive basal locomotion different from the control larvae. Taken together, these data indicate the possibility that Orco-mediated prior activity is involved in shaping the neural response and inducing output behavior.

Recent studies reported that spontaneous activity was affected by ion channels, such as nicotinic acetylcholine receptors (Bansal et al., 2000), GABAA receptors

(Leinekugel et al., 2002), and hyperpolarization-activated cyclic nucleotide-gated channels (Neuhoff, Neu, Liss, & Roeper, 2002). In the present thesis, the prior activity of ORNs was also shown to be Orco-dependent (Fig. 2, 3). Taken together, the properties of ion channels, such as their expression levels or post-translational modifications, could change the prior activity. In the mammalian brain, inner states, such as starvation or circadian rhythm, regulate the protein synthesis and the endocrine system (Ko, Shi, & Ko, 2009; Sohn, 2013), indicating that the properties of ion channels are affected by inner states. Thus, it is possible that inner states tune the prior activity of each neuron and changes sensory processing at the circuit level, thereby leading to the induction of suitable behavior towards the external world. This possible link among inner state, prior activity, and output behavior might be beneficial for optimizing behavioral output depending on the inner state. In Fig. 14, larvae that showed increased prior activity in their PNs exhibited odor-seeking behavior more frequently than the controls. This increased odor-seeking behavior might enable larvae to search odor source effectively. Taken together, inner states might also optimize basal locomotion by adjusting prior activity, in addition to output behavior evoked by

external stimuli. Therefore, it would be of interest to investigate how inner states affect the relationship between prior activity and basal locomotion in future studies.

My findings in the present thesis highlighted the functional importance of prior activity and thus encourage a reevaluation of prior activity in order to elucidate new principles of sensory processing and animal behavior.

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