Neural Activation in the Olfactory Epithelium of East African Cichlid in Response to Odorant Exposure

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Abstract

Fish use olfaction to gain various information. To know what they receive and how they receive is vital for understanding the diversity of fish. However, studies on fish olfactory or pheromone receptors are still insufficient, and most of them are on a model organism. Here, we established an experimental system to detect a biological-derived neural response from the olfactory epithelium of East African cichlid, the most diversified fish lineage, by *in situ* hybridizations of *c-fos*. We first tested the response of microvillous neurons, which are expected to be dominated by *V2R*-expressing neurons, to several odorants. We showed that microvillous neurons responded to amino acids and food extraction, suggesting that fish receive amino acids via V2R receptors as food-related odorants. We next tested the response of each of the four subfamilies of V2R receptors, showing that two of four subfamilies responded to proteinogenic amino acids. Notably, individual paralogs in subfamily 14, which responded to arginine, had variability in receptivity to arginine, indicating the ligand differentiation in the cichlid-specifically expanded subfamily. Finally, we established a noninvasive method to collect urine and tested the response of putative pheromone receptor V1R to male urine, and we showed two V1R receptors responded to male urine. Furthermore, we showed V1R2 receptor responded to 4-hydroxyphenyl acetate and lithocholic acid. This study provides an experimental basis for the study of olfaction in East African cichlids related to its ecology and speciation.

Keyword

c-fos, V1R, V2R, amino acid, urine collection

Introduction

Animals use olfaction in several situations, such as finding food, finding and judging mates, recognizing territories, migration, or detecting predators. Many olfactory or pheromone receptor genes in their genomes play a vital role in these behaviors. Hence, to understand their specific behavior, the receptor function must be revealed.

In fish, several soluble chemicals are detected by the olfactory epithelium (OE) as odor. Amino acids drive feeding behavior in several species (Valentinčič *et al*., 1999; Hara, 2006; Koide *et al*., 2009). Amino acids also work as migration pheromone or sex pheromone in salmonid species (Shoji *et al*., 2003; Yambe *et al*., 2006; Yamamoto *et al*., 2010, 2013). Polyamines, which are released from carrion, also drive feeding behavior in goldfish (Rolen *et al*., 2003), whereas they drive aversive behavior in zebrafish (Hussain *et al*., 2013). Nucleotides also drive feeding behavior (Wakisaka *et al*., 2017). Sex steroids, such as 17,20β-dihydroxy-4-pregnen-3-one (17, 20β -P) or androstenedione, strongly induce EOG response and have a primer effect in goldfish (Sorensen *et al*., 1987, 2005; Stacey *et al*., 1989). Prostaglandins also work as a pheromone in goldfish and zebrafish (Sorensen *et al*., 1988; Yabuki *et al*., 2016). Moreover, bile acids are detected by several species, and they drive migration behavior in lamprey, although the functions in other fishes remain controversial (Li *et al*., 1995; Michel and Lubomudrov, 1995; Zhang *et al*., 2001; Huertas *et al*., 2010).

These odorants are detected by olfactory or pheromone receptors, which are encoded by four G protein-coupled receptor multigene families: odorant receptor (OR; Buck and Axel, 1991), trace amine-associated receptor (TAAR; Liberles and Buck, 2006), vomeronasal type-1 receptor (V1R, also termed as ORA; Dulac and Axel, 1995), and vomeronasal type-2 receptor (V2R, also termed as OlfC; Herrada and Dulac, 1997), in vertebrates including fishes. Respectively, *OR* or *TAAR* are

expressed in ciliated neurons (Hansen *et al*., 2004; Sorensen and Sato, 2005), and *V1R* and *V2R* are expressed in microvillous neurons (Hansen *et al*., 2004; Sorensen and Sato, 2005), except single *V1R* (*V1R3*), which is expressed in crypt neurons (Oka *et al*., 2012). Ciliated neurons detect a broad range of odorants, such as amino acids, sex steroids, prostaglandins, and bile acids in several fishes (Sato and Suzuki, 2001; Hansen *et al*., 2003; Sato and Sorensen, 2018). Furthermore, studies on zebrafish deorphanized OR113 receptor as fish pheromone prostaglandin $F_{2\alpha}$ receptor (Yabuki *et al*., 2016), and several TAAR receptors as polyamine receptors (Hussain *et al*., 2009, 2013). Alternatively, microvillous neurons, which are expected to be dominated by V2R expressing neurons, specifically detect amino acids (Sato and Suzuki, 2001; Hansen *et al*., 2003; Sato and Sorensen, 2018). Many studies indicate that V2R receptors detect amino acids (Koide *et al*., 2009; DeMaria *et al*., 2013). Also, several heterologous expression experiments show that zebrafish V1R receptors bind 4-hydroxyphenyl acetate (4HPAA) and bile acids (Behrens *et al*., 2014; Cong *et al*., 2019).

These studies are limited to the model organism zebrafish and several nonmodel organisms such as Salmon. Alternatively, Neoteleostei, which makes up approximately 60% of species within fish, are not focused on studies on olfaction. Studying these nonmodel organism species is crucial for understanding fish evolution.

In this study, we focused on cichlid, one of the most diversified lineages in vertebrates. Especially, cichlids in the East African Great Lakes are the most striking example of adaptive radiation (Kocher, 2004). Although visual sense was kept focused because of the highly diversified nuptial colouration, cichlid also uses olfaction in many different contexts (Keller-Costa *et al*., 2015). For example, *Pseudotropheus emmiltos* use olfaction for conspecific recognition (Plenderleith *et al*., 2005), and olfaction contribute to the sexual imprinting of *Pundamilia* species (Verzijden and Ten Cate, 2007). Other studies show that male tilapia evaluates the sexual status from female urine, and glucuronidated steroid in male urine works as a priming pheromone (Miranda *et al*., 2005; Keller-Costa *et al*., 2014). Moreover, we previously found several highly diverse polymorphic alleles in the V1R receptors of East African cichlid (Nikaido *et al*., 2014), and the copy number of V2R receptors

increased in East African cichlid (Nikaido *et al*., 2013).

Here, we have established an experimental system to detect a biological-derived neural response from olfactory sensory neurons of East African cichlid *Haplochromis chilotes* by *in situ* hybridizations using *c-fos* riboprobe (Figure 1). This experimental system tested the response of microvillous neurons, *V2R*-expression neurons, and *V1R*-expressing neurons to several odorants. Compared with previous studies on heterologous expression experiments, our experimental system can be tested on the biological-derived and can avoid difficulty in translocation of receptors to the cell surface. This study may provide a fundamental basis for the study of olfaction in East African cichlids.

Materials and Methods

Fish

Cichlid (*Haplochromis chilotes*) were maintained at 27°C on a 12 h light/12 h dark cycle. Six to twelve individuals were kept in a plastic tank (40 cm × 25 cm × 36 cm). Mature adults were used for the experiment.

Odorant solutions

Twenty proteinogenic amino acids (arginine, histidine, lysine, aspartate, glutamate, serine, threonine, asparagine, glutamine, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine), 4HPAA, lithocholic acid (LCA) were purchased from Wako Pure Chemical Industries, Sigma Chemical Co. Each amino acid (except tyrosine) and 4HPAA was dissolved in ultrapure water to 12 mM. Tyrosine and LCA were dissolved in 6 mM NaOH to 12 mM. Three conjugated steroids dehydroepiandrosterone 3-sulfate (DHEA-s), β-estradiol 17-(β-D-glucuronide) (E_2-17g), and β-estradiol 3,17-disulfate (E_2-3, 17s) were respectively purchased from Tokyo Chemical Industry, Cayman Chemical Co., and Santa Cruz Biotechnology and dissolved in DMSO to 10 mM. Food extractions were prepared by adding ultrapure water up to 14 mL to 2 g of crushed fish food Otohime EP1 (Marubeni Nisshin Feed Co.). After waiting for 5 min, the extraction liquid was centrifuged at 8,000 × g for 5 min, and the supernatant was collected as food extraction stock. The stock was stored at 4°C until the exposure experiment.

Urine collection

Urine was collected from mature male cichlid. Although several studies had collected nondiluted urine from fish, they were limited to some larger fish such as masu salmon, rainbow trout, Mozambique tilapia, and Senegalese sole (Yambe *et al*., 1999; Sato and Suzuki, 2001; Keller-Costa *et al*., 2014; Fatsini *et al*., 2017). Here, by referring to a method to collect urine from Masu salmon

used in Yambe *et al*., 1999, we developed a noninvasive method to collect urine directly from cichlid, whose size is approximately 6–9 cm under swimming conditions (Figure 5B). We used a dental root canal cleaning probe needle (28G, 490703, BSA Sakurai Co.) to construct a sampling catheter (Figure 5A). This needle has a hole in the side near the needle's tip to prevent being clogged. Approximately 0.5–1.2 cm from the tip of this probe needle was gently bent approximately 90° so that the hollow is not collapsed (Figure 5A). In the case of the female catheter, a silicone plug was further attached to the tip of the catheter (Figure 5A). This bent needle was connected to 15 mL centrifuge tube with a silicon tube (OD: 10 mm, ID: 0.5 mm) fixed with adhesive (Aron Alpha EXTRA Fast-Acting Versatile, Konishi) to trap the urine. The centrifuge tube was further connected to the aspirator (DAS-01, As one) to aspirate the urine.

Cichlid was anesthetized with ice water for 1 min. The catheter was inserted via the urogenital papilla into the urinary bladder. The silicon tube connecting the catheter was fixed to the anal fin by wire and clip to be held in place. Catheterized cichlid was placed in the net chamber made from a pot bottom net to restrict the movement. Cichlid could accumulate for 15 min. After aspirating for 3–5 h, approximately 500–1000 μL of urine was trapped into the centrifuge tube placed on ice. Urine collected during the first 30 min was discarded to prevent contamination by coelomic fluid. To ensure that the sample collected was urine, 10 μL of the collected sample was used to verify the existence of ammonia by indophenol assay (Tetra Test Ammonia Reagent, Tetra).

All experimental studies using the animals were approved by the Institutional Animal Experiment Committee of the Tokyo Institute of Technology and conducted according to the institutional and governmental ARRIVE guidelines.

Exposure and tissue preparation

Adult cichlid was isolated to a glass tank (40 cm × 25 cm × 36 cm) the day before the exposure and was not fed. The next day, the fish was transferred to the exposure tank (30 cm × 11 cm × 9 cm, 3 L), covered with black paper to make the inside dark, and served with clean dechlorinated water. The

fish was kept in this tank for 1.5–3 h before exposure. These procedures were performed to reduce the background expression of *c-fos* in the OE. The water supply was then stopped, and 15 mL of odorant solution was delivered to the tank using a peristaltic pump (SJ-1211II-H, Atto). Each solution was diluted with ultrapure water (the mixture of 20 amino acids/amino acids group A-D: 400 µM each, the mixture of conjugated steroids: 6.6 µM each, arginine/4HPAA: 2 mM, LCA: 4 mM, male urine: 30-fold dilution, food extraction: 75-fold dilution). Water was exposed in the control. After exposing the fish for 1 min, the water was resupplied, and the fish was kept in the tank for 20 min for the expression of *c-fos*. The fish was then quickly decapitated, and the olfactory epithelia were dissected out in 4% paraformaldehyde (PFA, Wako)/0.7× phosphate-buffered saline (PBS). Dissected tissues were fixed in 4% PFA/0.7× PBS at 4°C for 7.5 h. Fixed tissues were treated with 20% sucrose/0.7× PBS at 4°C overnight for cryoprotection before embedding. Tissues were embedded in the Tissue Tek O.C.T. compound (Sakura) and frozen using liquid nitrogen. Embedded tissues were sliced into a 10 µm section in a horizontal direction and loaded on a glass slide (MAS-01, Matsunami). Sections were kept at −80°C until use for the following experiments.

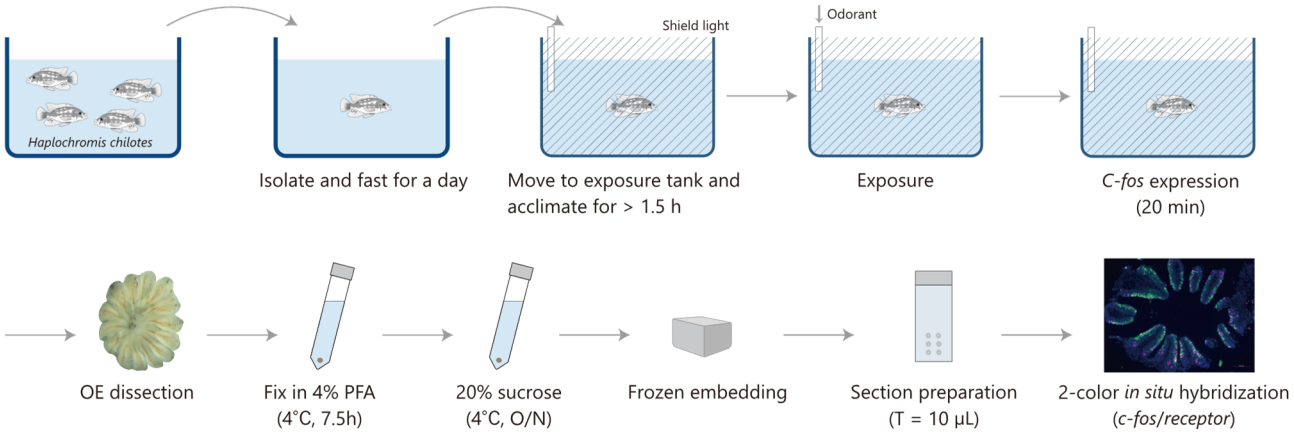

Figure 1. Schematic flow of the experiment. Cichlid was exposed to an odorant. Olfactory epithelia were then isolated, fixed, and frozen embedded. The section was prepared and used for *in situ* hybridization.

Preparation of riboprobes

Riboprobes for *in situ* hybridization (ISH) were designed in the coding region or untranslated region. Each sequence was amplified from cDNA of OE by Ex-Taq (Takara) with primers listed in Supplementary Table 1. PCR products were ligated to pGEM-T (Promega) or pBluescript SKII (−) plasmid and sequenced. Plasmids were extracted with the QIAfilter Plasmid Midi Kit (QIAGEN) and then linearized using the appropriate restriction enzyme (Takara). Digoxigenin (DIG)-labeled or fluorescein (FITC)-labeled riboprobes were synthesized with T7 or T3 or SP6 RNA polymerase (Roche) from the linearized plasmids with DIG or FITC RNA labeling mix (Roche), respectively.

In situ hybridization

Single-colour and two-colour ISH were performed according to the method that is modified in a previous study (Suzuki *et al*., 2015). Briefly, in single-colour ISH, sections were hybridized with DIG-labeled riboprobes (5 ng/μg) at 60°C overnight and treated with POD-conjugated anti-DIG antibody (1:100, Roche). Signals were amplified by Tyramide Signal Amplification (TSA) Plus Biotin kit (PerkinElmer) and detected with Alexa Fluor 488-conjugated streptavidin (1:200, Thermo Fisher Scientific). Sections were enclosed with VECTASHIELD mounting medium with DAPI (Vector Laboratories). Double ISH sections were hybridized with DIG-labeled riboprobes and FITC-labeled riboprobe (2.5 ng/μg at each) at 60°C overnight and treated with peroxidase-conjugated anti-DIG antibody (1:100). Signals from DIG-riboprobes were amplified using TSA Plus DIG kit (PerkinElmer) and detected with DyLight 594-conjugated anti-DIG antibody (1:500, Vector Laboratories). Sections were treated with 15% H_2O_2 in TBS for 30 min to inactivate peroxidase. A signal from FITC-labeled riboprobes was detected with an HRP-conjugated anti-FITC antibody (1:500, PerkinElmer). Signals were then amplified using TSA Plus Biotin kit (PerkinElmer) and detected with Alexa Fluor 488-conjugated streptavidin (1:200, Thermo Fisher Scientific). Sections were enclosed with VECTASHIELD mounting medium with DAPI (Vector Laboratories). All images were digitally captured using a fluorescence microscope (Carl Zeiss).

Quantification of neuron number

The image was first integrated into a single section. The tone of the image was corrected by "Curves." Expressing neurons were determined using the threshold of brightness (85) and the shape and marked using the "Count Tool." The area measured by the DAPI image was used to correct the number of neurons. Colocalization was also tested on the marked neuron. These procedures were performed using Adobe Photoshop CC 2018.

Results

Cichlid *c-fos* has characteristics of an immediate-early gene

In initial experiments, we exposed cichlid to food extraction and assessed the upregulation of five immediate-early genes *c-fos*, *egr1*, *c-jun*, *fra1*, and *junb* by *in situ* hybridizations in the OE. By comparing the expression of nonexposed and exposed OE, we concluded that *c-fos* is the most suitable neural activity marker for cichlid OE (Figure 2A).

To quantitatively confirm the upregulation of *c-fos* in the cichlid OE, we exposed cichlid to food extraction and tested whether the number of *c-fos*-expressing neurons would increase with time (control/10/20/30 min) since exposure (Figure 2B, C, Supplementary Table 2). We compared the number of *c-fos*-expressing neurons in five sections that were evenly selected and found that the number in 20 and 30 min was significantly greater than that in control (n = 3; $p = 0.029$, $p = 0.043$; Tukey–Kramer test, Figure 1B). Since this experiment, we have set the time length between odorant exposure and ice anesthetizing as 20 min.

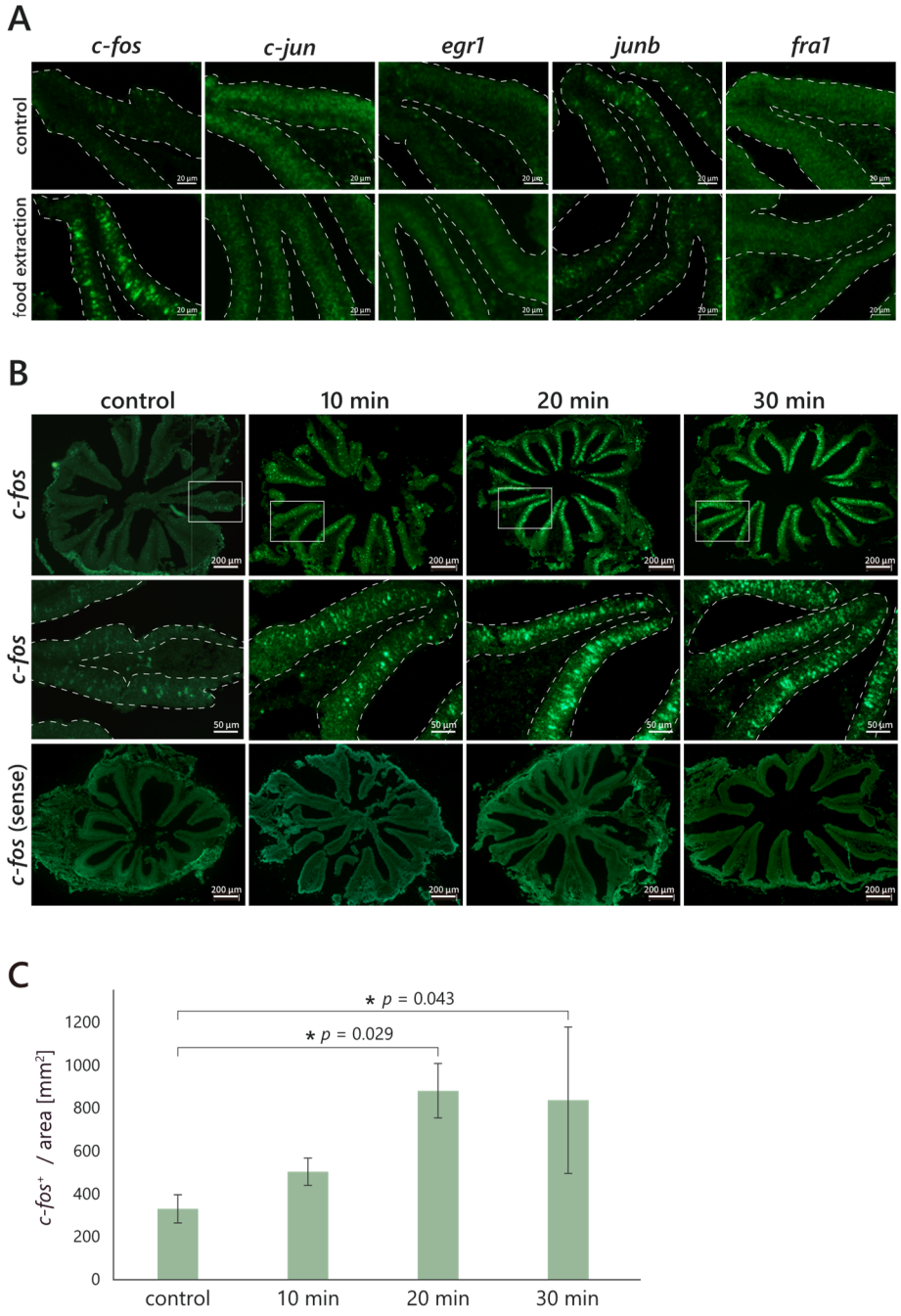

Figure 2. *C-fos* expression is robustly induced in olfactory epithelium (OE) by exposure to food extraction. Cichlid was exposed to water (control) or food extraction (final concentration: 9.5 mg/L). **(A)** *In situ* hybridization with cRNA probes for *c-fos*, *c-jun*, *egr1*, *junb*, or *fra1*. The dotted line represents the outline of the OE. **(B)** *In situ* hybridization with cRNA probes for *c-fos*. The vertical columns represent the length of time between odorant exposure and ice anesthesia. The middle panels represent the magnified image of the box in the upper panel. **(C)** Bar plot of the density of *c-fos*$^+$ (three individuals each, five sections per individual; Tukey–Kramer test). *$p < 0.05$. All data are shown as mean ± SEM.

Neural response of microvillous neurons

Next, we tested the neural response of microvillous neurons, which are expected to be dominated by *V2R*-expressing neurons (Supplementary Figure 1; Hansen *et al*., 2004; Sato *et al*., 2005), against four odorants: the mixture of 20 proteinogenic amino acids (final concentration at 2 μM each), food extractions, male urine, and the mixture of three conjugated steroids (final concentration at 33 nM each). Exposure to each of the four odorants significantly increased the number of *c-fos*-expressing neurons compared with the control with stronger intensity (n = 4; $p = 0.047$, $p = 0.035$, $p = 0.024$, $p = 0.043$; Welch's *t*-test, Figure 3A, B, Supplementary Table 2). We also calculate the colocalization rate of *Trpc2*, which is a marker gene of microvillous neurons (Sato *et al*., 2005), among *c-fos*-expressing neurons. It became the highest when exposed to amino acids (55%) and was significantly higher than when exposed to male urine or conjugated steroids ($p = 0.0080$, $p = 6.1 \times 10^{-4}$; Student's *t*-test; Figure 3C, Supplementary Table 2). It also became high when exposed to food extraction (Figure 3C). Alternatively, it became the lowest when exposed to conjugated steroids, a putative pheromone in cichlid (Cole and Stacey, 2006; Keller-Costa *et al*., 2014) and was significantly lower than when exposed to amino acids, food extraction, or male urine ($p = 6.1 \times 10^{-4}$, $p = 0.0095$, $p = 0.022$; Student's *t*-test; Figure 3C).

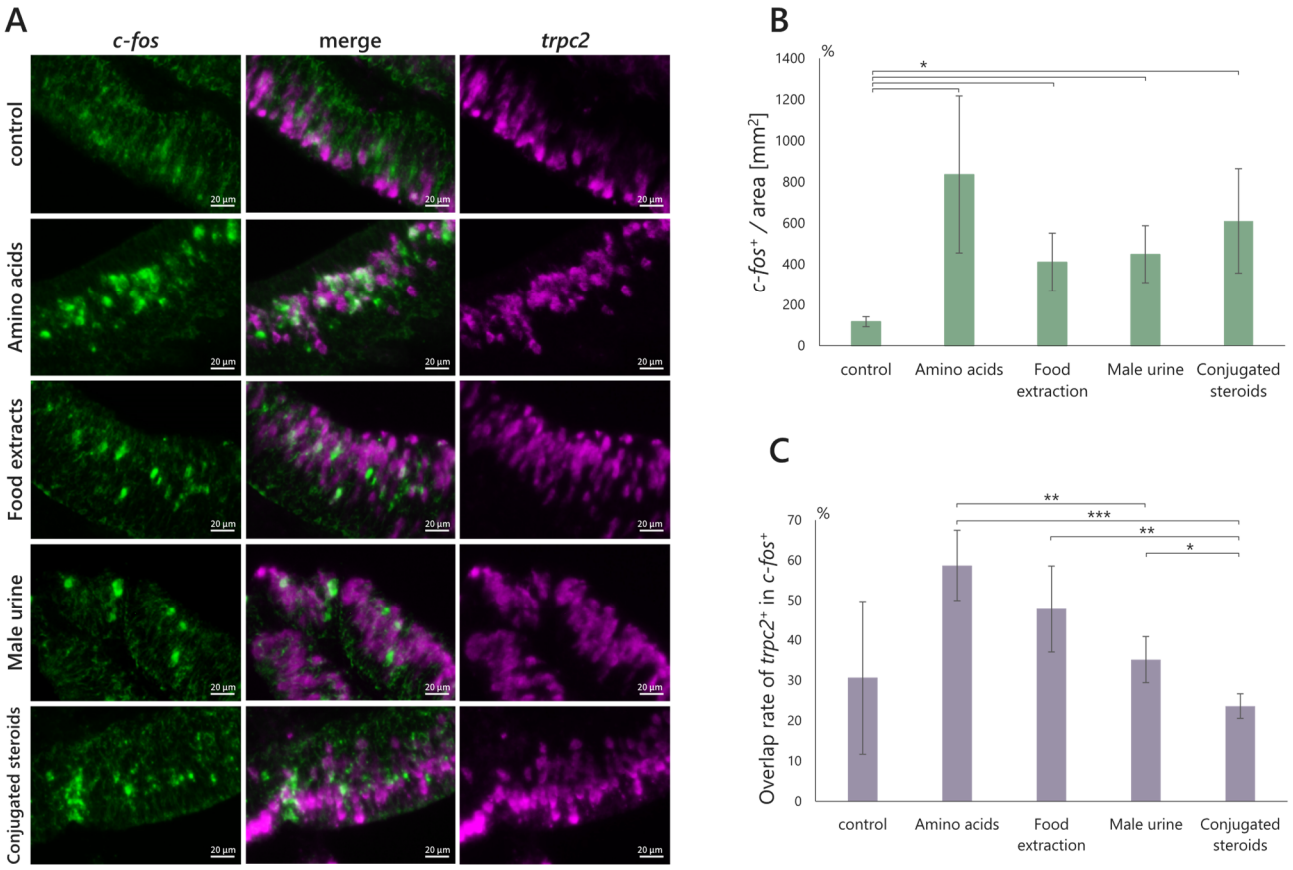

Figure 3. Response of microvillous neurons. (A–C) Double *in situ* hybridization with cRNA probes for *c-fos* (green) and *TrpC2* (magenta) of olfactory epithelium sections of cichlid exposed to water (control), amino acids (final concentration: 2 µM each), food extraction (final concentration: 9.5 mg/L), male urine (final concentration: 6000-fold dilution), or conjugated steroids (final concentration: 33 nM). **(B)** Bar plot of the density of *c-fos*$^+$ (four individuals each, five sections per individual; Welch's *t*-test). **(C)** Bar plot of overlap rate of *TrpC2*$^+$ in *c-fos*$^+$ (four individuals each, five sections per individual; Welch's *t*-test, Student's *t*-test). **p* < 0.05, ***p* < 0.01, ****p* < 0.001. All data are shown as mean ± SEM.

Neural response of Vomeronasal type-2 receptor-expressing neurons to amino acid

To test the hypothesis that fish receive amino acid via V2R receptors (Koide *et al*., 2009; DeMaria *et al*., 2013), we next exposed cichlid to amino acids and examined the response of *V2R*-expressing neurons. East African cichlids experienced a lineage-specific expansion in the *V2R* multigene family and possessed 61 intact *V2R* genes, one of the largest among teleost (Nikaido *et al*., 2013). Sixty-one genes comprise 13 subfamilies (Supplementary Figure 2). Within these 13 subfamilies, four subfamilies (4, 8, 14, and 16) have expanded the number of genes by tandem duplication. We first exposed cichlid to a mixture of 20 amino acids (final concentration at 2 μM each) and tested the response of these four subfamilies (4, 8, 14, and 16) plus subfamily 2-1, 7-1 as a single-copy subfamily (Figure 4A, B, Supplementary Table 2). The responding rate was calculated from the colocalization rate of *c-fos*-expressing neurons among *V2R*-expressing neurons. In support of the hypothesis that fish receive amino acid via V2R receptors, relatively more fraction responded in subfamily 14, 16 (28%/16%; Figure 4A, B), and intermediate value of fraction responded in subfamily 2/7 (8.8%; Figure 4A, B). Alternatively, only a small number of subfamily 4/8-expressing neurons responded to amino acids (3.6%/0.9%; Figure 4A, B). To determine which amino acid does V2R subfamily 14 and 16 receive, we exposed cichlid to four groups of amino acids: A, including nonpolar or neutral amino acids (Gly, Ala, Ser, Pro, and Thr); B, including aromatic or carbamic amino acid (Phe, Tyr, Trp, His, Asn, and Gln); C, including branched or sulfur-containing amino acids (Val, Ile, Leu, Met, and Cys); and D, including charged amino acids (Arg, Lys, Asp, and Glu) (final concentration at 2 μM each). This grouping is based on electrical properties and the cluster analysis of goldfish odorant-induced activity patterns (Friedrich and Korsching, 1997). High rate (16%) of V2R subfamily 14-expressing neurons responded with stronger intensity to amino acids D, including charged amino acids (Figure 4C, D, Supplementary Table 2). The other amino acid groups also responded in subfamily 14-expressing neurons, although the responding rate was much lower. To further narrow down the amino acids, which made a response in subfamily 14-expressing neurons,

we exposed cichlid to individual amino acids and found that basic amino acid, especially arginine (final concentration at 10 μM), makes response (46%; Figure 4C, D). We further tested the response to arginine of three single copies in subfamily 14, which are 14-1, 14-2, and 14-6. Respectively, they responded 0%, 28%, and 9.1% (Figure 4E, F, Supplementary Table 2). Alternatively, subfamily 16-expressing neurons most responded to amino acids group C, although the rate was not high as subfamily 14-expressing neurons, which were exposed to group D (9.2%; Figure 4D, Supplementary Figure 3A). We also tested the response to amino acids group C of three single copies in subfamily 16, which are 16-1, 16-3, and 16-6. However, no colocalization with *c-fos* was observed in any copy (Supplementary Figure 3B).

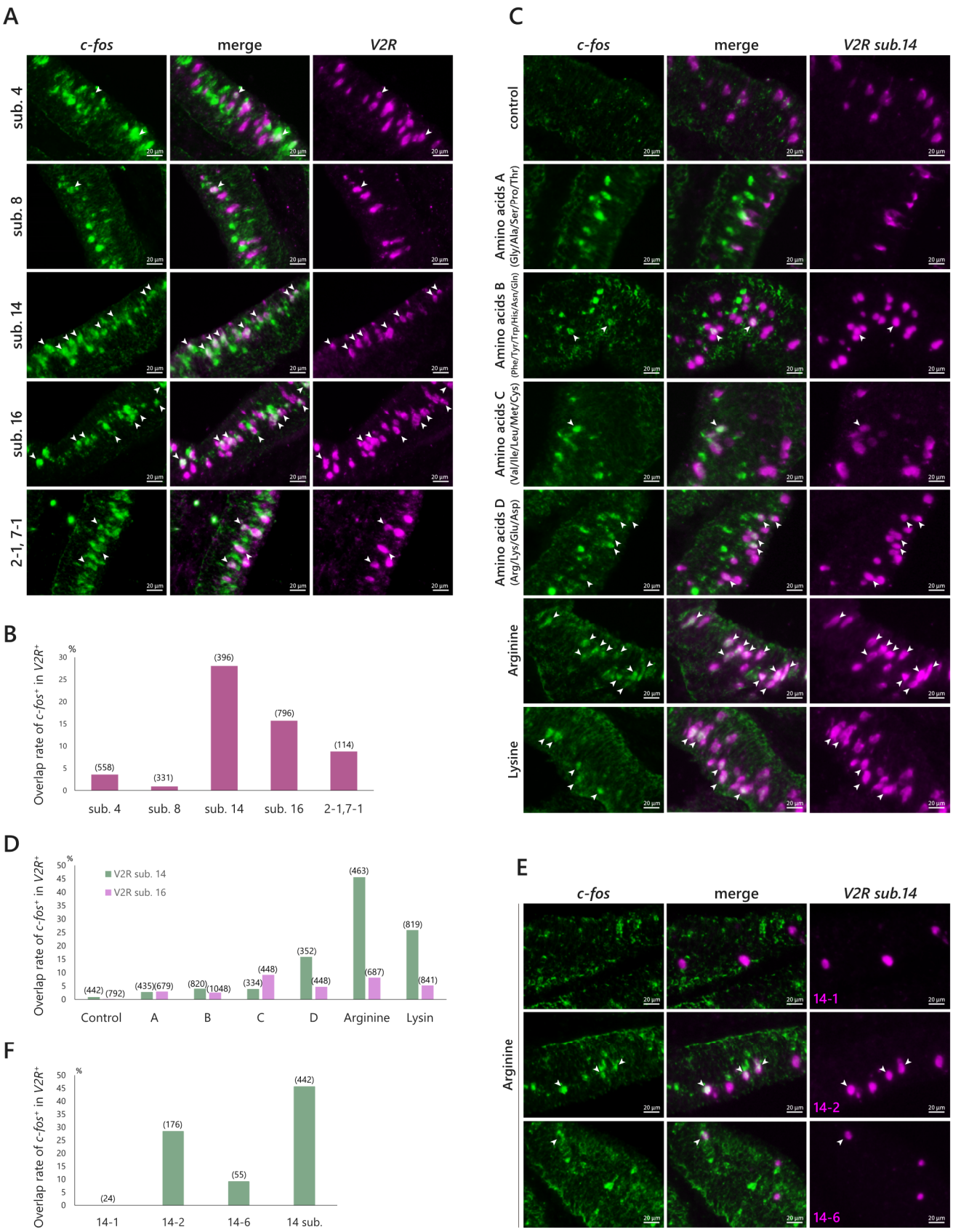

Figure 4. Response of V2R receptor to amino acids. Double *in situ* hybridization with cRNA probes for *c-fos* (green) and *V2R* (magenta) of olfactory epithelium (OE) section exposed to amino acids. **(A, C, E)** Representative image. Arrowheads represent *c-fos* and *V2R* colocalization. **(B, D, F)** Bar plot of overlap rate of *c-fos*$^+$ in *V2R*$^+$ (one individual each). The numbers in the brackets represent the number of *V2R*$^+$ counted in a single section. **(A, B)** OE section of cichlid exposed to amino acids (final concentration: 2 μM each). Each probe is identical above 80% to all copies in each subfamily. **(C, D)** OE section of cichlid exposed to four groups of amino acids (A: Gly, Ala, Ser, Pro, Thr; B: Phe, Tyr, Trp, His, Asn, Gln; C: Val, Ile, Leu, Met, Cys; D: Arg, Lys, Asp, Glu; final concentration: 2 μM each), arginine (final concentration at 10 μM) or lysine (final concentration at 10 μM). **(E, F)** OE section of cichlid exposed to arginine. **(E)** The upper image for 14-1, the middle image for 14-2, the bottom image for 14-6. 14-1, 14-2, and 14-6 belong to the V2R subfamily 14.

Neural response of V1R-expressing neurons to odorants

Finally, we tested the response of *V1R*-expressing neurons. We first test the response to the mixture of proteinogenic amino acids, food extraction, male urine, and the mix of three conjugated steroids using probes for the mixture of 6 *V1R* (Figure 5D, Supplementary Table 2). *V1R*s-expressing neurons responded the most to male urine (24%; Figure 5C, D). We next tested the response to male urine of each 6 *V1R*s and found *V1R2* and *V1R5*-expressing neurons were responding (29%, 40%; Figure 5E, F, G). Additionally, we tested the response of *V1R2*-expressing neurons to 4HPAA and LCA, which are the candidate of the ligand of zebrafish V1R2 (Behrens *et al.*, 2014; Cong *et al.*, 2019). Many *V1R2*-expressing neurons responded to 10 μM 4HPAA (42%), and a relatively large number also responded to 20 μM LCA (17%) (Figure 5E, G, Supplementary Table 2).

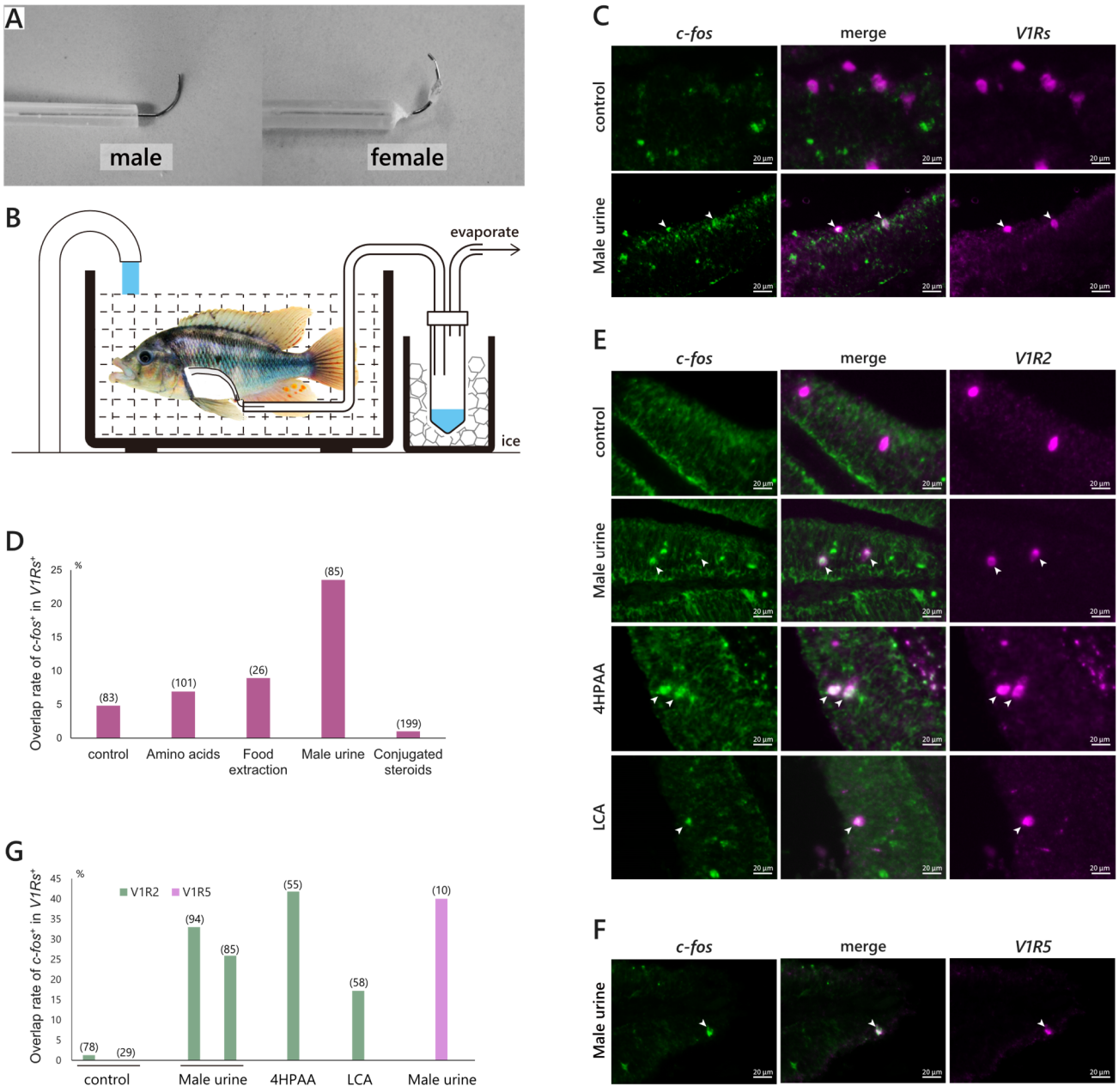

Figure 5. Response of V1R receptor. (A) The catheter was used for urine collection. These catheters have a hole in the side near the tip of the needle to prevent clogging. The catheter for females has a silicone plug attached to it. **(B)** Schematic drawing of urine collection. **(C–F)** Double *in situ* hybridization with cRNA probes for *c-fos* (green) and *V1R* (magenta) of olfactory epithelium section. **(C, E, F)** Representative image. Arrowheads represent *c-fos* and *V1R* colocalization. **(D, G)** Bar plot of overlap rate of f *c-fos*$^+$ in *V1R*$^+$ (1 individual each). The numbers in the brackets represent the number of *V1R*$^+$ counted in a single section. **(C, D)** Response of V1Rs (V1R1-6) receptor to amino acids (final concentration: 2 μM each), food extraction (final concentration: 9.5 mg/L), male urine (final concentration: 6000-fold dilution), or conjugated steroids (final concentration: 33 nM). **(E)** Response of V1R2 receptor to male urine (final concentration: 6000-fold dilution), 4HPAA (final

concentration: 10 μM), or LCA (final concentration: 10 μM). **(F)** Response of V1R5 receptor to male urine (final concentration: 6000-fold dilution).

Discussion

Here, we established a method to detect biological-derived neural responses from OE of East African cichlid via *in situ* hybridizations of *c-fos* and showed different neural responses to each odorant stimuli. We first identified the most suitable neural activity marker gene as *c-fos*. *Egr1*, another major marker gene for neural activity (Isogai *et al*., 2011), was reported to be helpful for an active marker of the cichlid brain (Burmeister and Fernald, 2005) but did not show upregulation in OE. *C-fos*-expressing neurons were significantly increased 20 min after exposure to food extraction. Also, the intensity was stronger after 20 min. Hence, we concluded that 20 min is the best time between exposure and ice anesthesia for detecting olfactory response.

V2R-expressing neuron responded to amino acids

The rate of microvillous neurons (*TrpC2*-expressing neurons) among the *c-fos*-expressing neurons was highest when exposed to amino acids and high when exposed to food extract. Because microvillous neurons are expected to be dominated by *V2R*-expressing neurons since *V1R*s are sparsely expressed (Ota *et al*., 2012; Supplementary Figure 1A), this result suggests that cichlids receive amino acids in food via V2R receptors. This is consistent with the hypothesis that fish receive amino acids via V2R receptors (Koide *et al*., 2009; DeMaria *et al*., 2013). Alternatively, similar to some electrophysical research (Sato and Suzuki, 2001; Hansen *et al*., 2003; Sato and Sorensen, 2018) that showed that ciliated neurons also respond to amino acids, 45% *c-fos*-expressing neurons were not colabeled with *TrpC2* when exposed to amino acids indicating that the rest *c-fos*-expressions were in ciliated neurons. The function of amino acid reception of ciliated neurons is unknown, but at least, V2R receptors are expected to trigger feeding behavior by receiving amino acids (Koide *et al*., 2009).

Next, we tested the response of individual subfamilies of V2R receptors to amino acids. East African cichlids have 61 copies of *V2R*, which is one of the largest numbers among teleosts.

Although fish V2R receptors are shown to receive amino acids (Koide *et al*., 2009; DeMaria *et al*., 2013), it is interesting that cichlid has *V2R* copies far more than the number of proteinogenic amino acids. We showed that subfamilies 4- and 8-expressing neurons did not respond to proteinogenic amino acids. This indicates that these V2R receptors receive other chemicals, such as nonproteinogenic amino acids and peptides. Kynurenine is one example of nonproteinogenic amino acid that masu salmon receive as a sex pheromone (Yambe et al., 2006). Mouse V2R receptors recognize peptides (Kimoto *et al*., 2005), and stickleback and zebrafish receive 9-mer MHC peptides via OE (Milinski *et al*., 2005; Hinz *et al*., 2013). These chemicals are possibly playing a role other than foraging. V2R subfamily 9 are suggested to relate to fright reaction in Ostariophysan fishes (Yang et al., 2019). Furthermore, peptides are more suitable for species-specific odor since peptides can be more variable than single amino acids by combining several amino acids. Within teleost *V2R*s, subfamilies 4 and 16 are independently diversified in several lineages (Nikaido *et al*., 2013), so that these subfamilies possibly receive species-specific odor. V2R subfamily 4 and at least three copies of V2R subfamily 16 only marginally responded to proteinogenic amino acids (Figure 4B; Supplementary Figure 3B). These V2R receptors possibly receive nonproteinogenic amino acids or peptides.

Also, the *V2R* gene cluster is adjacent to *neprilysin*, which is encoding metallopeptidase, suggesting that neprilysin possibly produce degraded peptides received by *V2R*-expressing neurons (Hashiguchi and Nishida, 2006; Johnstone *et al*., 2009; Nikaido *et al*., 2013). We confirmed that *neprilysin* is expressed in the cichlid OE (Supplementary Figure 4). Most *neprilysin* was expressed in cells in the basal region of OE. These cells likely excrete the free enzymatically active domain of neprilysin. Furthermore, some *neprilysin* colocated with *TrpC2*, which suggests that the V2R receptor receives proximally degraded peptides.

We also showed that among the three copies in subfamily 14, explicitly expanded in a cichlid, one copy was receptive to arginine. One was slightly receptive, and one was unreceptive (Figure 4E, F). Moreover, we showed that subfamily 16 also expanded and responded to amino acids C, whereas the

three copies in subfamily 16 did not respond to amino acids C (Supplementary Figure 3B). These results suggest that copy expansion of cichlid *V2R* led to increasing variation of receivable odorants via ligand differentiation. Our previous study also supported this hypothesis that the residues putatively related to ligand selectivity in cichlid-specifically expanded subfamilies were much diverse than that of whole teleost (Nikaido *et al*., 2013).

Possible role of V1R2 as a fundamental pheromone receptor that is common across teleost

Finally, we tested the response of putative pheromone receptor V1R (Behrens *et al*., 2014). *V1R*-expressing neurons most responded to male urine (24%). This suggests that V1R receptors relate to social interaction. Conversely, *V1R*-expressing neurons did not respond to conjugated steroids, putative pheromones of cichlid (Cole and Stacey, 2006). Since conjugated steroids made less response of microvillous neurons (Figure 3C), which indicate that ciliated neurons receive them, they might be received via OR receptor or TAAR receptor.

We further tested every six V1R receptors and found that *V1R2*- and *V1R5*-expressing neurons responded to male urine (Figure 5E, F). Although V1R receptors other than V1R2 and V1R5 did not respond to urine, they might be responsible for other things such as female urine and feces. Another possibility is that it is used to find food since 9% of expressing neurons responded to food extract (Figure 5D).

We finally showed that *V1R2*-expressing neurons responded to 4HPAA and LCA (Figure 5E). Previous research also reported that cultured cells expressing zebrafish V1R2 responded to 4HPAA and bile acids (Behrens *et al*., 2014; Cong *et al*., 2019). Because previous research showed that exposure to 4HPAA induces spawning of zebrafish (Behrens *et al*., 2014), our preliminary experiments confirmed that 4HPAA exists in the cichlid urine and V1R2 receptor might be a fundamental pheromone (= 4HPAA) receptor, which is common across teleost. Additionally, of the two distinct types of V1R2 alleles (Nikaido *et al*., 2014) in East African cichlids, the individuals used

in this study had the ancestral allele. Further investigation of the function of the other derivative allele might help to understand the impact of the V1R2 receptor on adaptive radiation in cichlid via assortative mating.

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Conflict of interest

The authors do not have any conflicts of interest.

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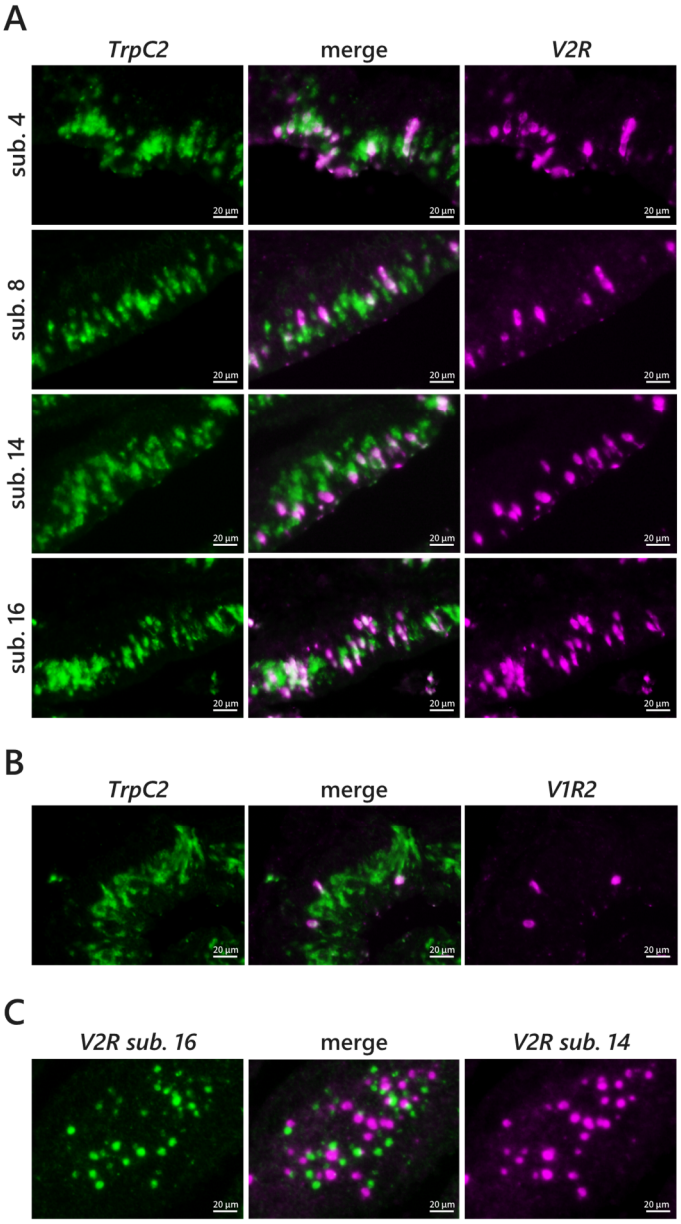
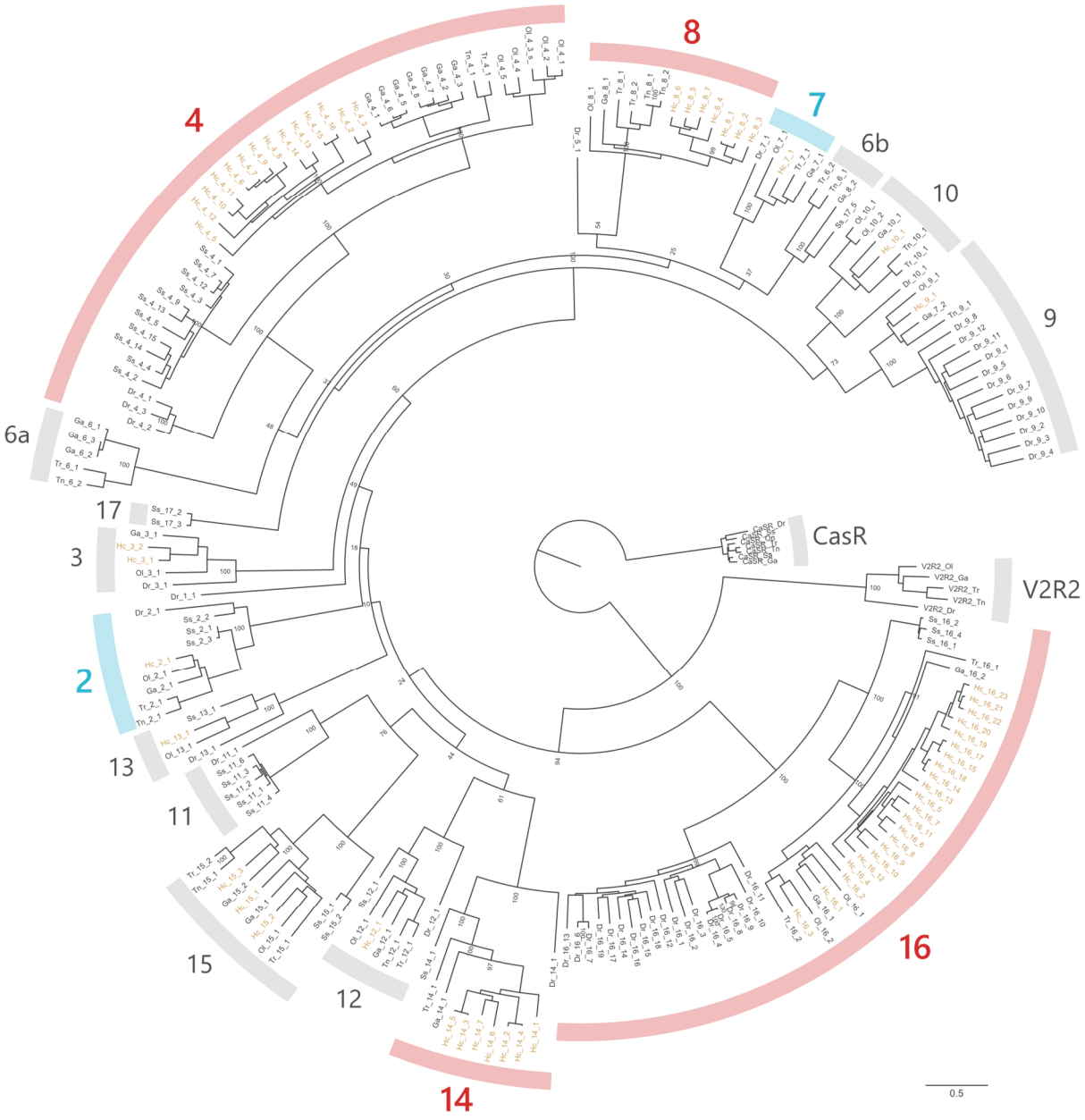
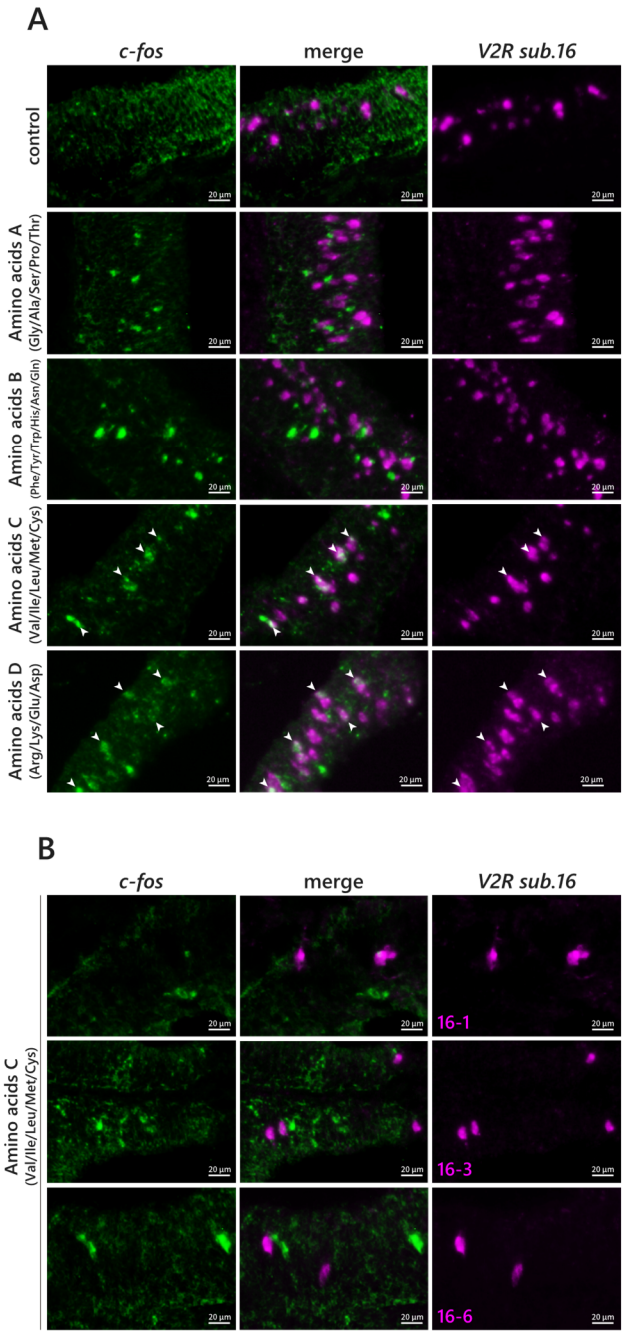
Supplementary Data



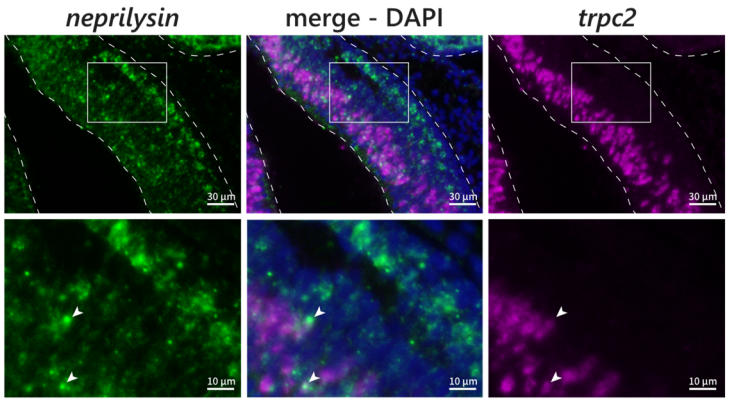
Supplementary Figure 1. Coexpression of *TrpC2* and *V2R* or *V1R*. (A-B) Double *in situ* hybridization with cRNA probes for *TrpC2* (green) and *V2Rs* (A; magenta) or V1R2 (B; magenta) of olfactory epithelium (OE) section. All *V2R*s and *V1R2* colocalized with *TrpC2*. **(C)** Double *in situ* hybridization with cRNA probes for *V2R sub. 16* (green) and *V2R sub. 14* (magenta) of OE section. They are exclusively located with each other.



Supplementary Figure 2. Maximum likelihood (ML) tree for *V2R* genes of seven teleost fishes. All the amino acid sequences were collected from Nikaido *et al*. (2013). RAxML-NG (Kozlov *et al*., 2019) was used to construct an ML tree with 100 bootstrap replicates. Numbers close to the nodes are ML bootstrap percentages. Arcs represent each subfamily. Subfamilies used in this study were coloured red (expanded in cichlid) or blue (not expanded in cichlid). Sequences of cichlid are coloured orange. Hc: East African cichlid (*Haplochromis chilotes*); Dr: zebrafish (*Danio rerio*); Ss: Atlantic salmon (*Salmo salar*); Ga: three-spined stickleback (*Gasterosteus aculeatus*); Tr: fugu (*Takifugu rubripes*), Tn: green-spotted pufferfish (*Tetraodon nigroviridis*); Ol: medaka (*Oryzias latipes*).



Supplementary Figure 3. Response of V2R sub. Sixteen receptors to amino acids. (A) Double *in situ* hybridization with cRNA probes for *c-fos* (green) and *V2R sub. 16* (magenta) of olfactory epithelium (OE) section exposed to four groups of amino acids (A: Gly, Ala, Ser, Pro, Thr; B: Phe, Tyr, Trp, His, Asn, Gln; C: Val, Ile, Leu, Met, Cys; D: Arg, Lys, Asp, Glu; final concentration: 2 µM each), arginine (final concentration at 10 µM) or lysine (final concentration at 10 µM). **(B)** Double *in situ* hybridization with cRNA probes for *c-fos* (green) and *V2R 16-1*/*16-3*/*16-3* (magenta) of OE section exposed to amino acids group C (Val, Ile, Leu, Met, Cys; final concentration at 10 µM). Arrowheads represent colocalization.



Supplementary Figure 4. Expression of *neprilysin* in OE. Double *in situ* hybridization with cRNA probes for *neprilysin* (green) and *TrpC2* (magenta) of olfactory epithelium (OE) sections of cichlid. The middle panels represent the merged image of *neprilysin* and *TrpC2* with DAPI (blue). The dotted line represents the outline of the OE. *Neprilysin* is primarily expressed in the basal region of OE except little in the middle region. The lower panels represent the magnified image of the box in the upper panel. Arrowheads represent the colocalization of *neprilysin* and *TrpC2*.

Supplementary Table 1. Primer list used in this study.

Supplementary Table 2. Summary of quantification data analyzed in this study.