Neural activation in olfactory epithelium of East African cichlid in response to odorant exposure

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Keyword

Cichlid, neural activity, olfactory, *c-fos*, V1R, V2R, amino acid, urine collection

Abstract

Fish use olfaction to gain a variety of information. To know what they receive and how they receive is important for understanding the species. However, studies on fish olfactory or pheromone receptors are still few and most of them are based on the neural response from a cultured cell. Here, we established a method to detect a biological-derived neural response from the olfactory epithelium of East African cichlid, the most diversified fish lineage, by *c-fos in situ* hybridization. We tested the response of microvillous neurons, which are expected to be dominated by *V2R*-expressing neurons, against several odorants. We showed microvillous neurons respond to amino acids the most whereas they do not respond to conjugated steroids. We next tested the response of V2R receptors. Although some subfamilies of V2R responded to amino acids, other did not respond which contrasts with the traditional hypothesis. Especially, one V2R subfamily responded to arginine. Not all the copies in this subfamily responded to arginine, which indicates the ligand differentiation in the cichlid-specifically expanded subfamily. Finally, we tested the response of putative pheromone receptor V1R to male urine. For this experiment, we established a new method to collect urine from cichlid. We showed two V1R receptors responded to male urine. Moreover, we showed that V1R2 receptor responded to 4-hydroxyphenyl acetate and lithocholic acid. This study is expected to provide a basis for the study on the olfaction of East African cichlids.

Introduction

Animals use olfaction in many situations such as finding foods, finding and judging mates, recognizing territories, migration, or detecting predators. A large number of olfactory or pheromone receptor genes in their genomes play a key role in these behaviors. Thus, it is important to reveal the receptor function for understanding their specific behavior.

In fish, several kinds of soluble chemicals are known to be detected by olfactory epithelium (OE) as odor. Amino acids drive feeding behavior in many 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 given off 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 (AD), 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). Furthermore, bile acids are also detected by several species, and they drive migration behavior in lamprey, although the functions in other fishes are still 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 4 G protein-coupled receptor (GPCR) 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); 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 many fishes (Sato and Suzuki, 2001; Hansen et al., 2003; Sato and Sorensen, 2018). Moreover, heterologous expression experiments deorphanized zebrafish OR113 receptor as prostaglandin F2α receptor (Yabuki et al., 2016) and several zebrafish TAARs as polyamine receptors (Hussain et al., 2009, 2013). On the other hand, 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). Several 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).

Most of these studies are detecting the neural response from cultured cells not derived from the species of interest. Also, they only had focused on Ostariophysi and Salmoniformes which are relatively old lineage, and there is no research focused on Neoteleostei, which make up nearly 60% of species within fish.

In this study, we focused on cichlid, one of the most diversified lineages in vertebrates. Especially cichlids in the East African Great Lakes are known to be the most striking example of adaptive radiation (Kocher, 2004). Although visual sense was kept focused because of the highly diversified nuptial coloration, 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 sexual imprinting of *Pundamilia* species (Verzijden and Ten Cate, 2007). Other studies show that male tilapia evaluate 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). Furthermore, we previously found that there are several highly diverse alleles in V1R receptor of East African cichlid (Nikaido et al., 2014), and the copy number of V2R increased also 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 (OSN) of East African cichlid *Haplochromis chilotes* by *in situ* hybridization using *c-fos* riboprobe (Figure 1). We first identified the optimum condition for detecting *c-fos* expression in response to odor exposure. Next, we exposed cichlid to food extraction, amino acid mixture, male urine, and conjugated steroid mixture and tested the activation of microvillous neurons. Conjugated steroids are known to induce EOG response in East African cichlid *Astatotilapia burtoni* whereas unconjugated steroids and prostaglandins do not induce EOG response (Cole and Stacey, 2006). Urine collection was conducted by a newly established method (Figure 5A, B). Significantly more microvillous neurons were activated in response to amino acids and food extraction, whereas fewer were activated in response to conjugated steroid mix, which implies that amino acids are detected by V2R receptors and conjugated steroids are detected by OR receptors. To further investigate which V2R receptor detect amino acids, we tested the activation of OSN expressing *V2R* subfamily 4, 8, 14, and 16 as lineage-specifically expanded subfamily and subfamily 2, 7 as a single-copy subfamily. Although OSN expressing V2R subfamily 14 or 16 strongly responded to amino acids, and OSN expressing subfamily 14 especially responded to arginine, the rest of the *V2R*-expressing neurons did not respond to amino acids, which differs from the hypothesis that fish V2R receptors bind amino acids. Within subfamily 14, 14-2 expressing neurons strongly responded to arginine whereas 14-1 or 14-6 expression neurons less responded to arginine which indicates that the lineage-specific expansion of subfamily resulted in ligand differentiation. Finally, we tested the activation of V1R-expressing neurons. We found that OSN expressing *V1R2* or *V1R5* responded to male urine, suggesting the role of V1R receptor as social interaction. We further confirmed that V1R2-expressing neurons responded to 4HPAA and lithocholic acid (LCA) as similar to zebrafish (Behrens et al., 2014; Cong et al., 2019). Although previous research tested the olfactory response in cultured cells, these are not detecting the biological-derived neural response. Furthermore, many receptors especially vomeronasal receptors are known to be difficult to translocate to the surface of the cultured cell. Our method can overcome these problems and be able to examine any kinds of receptors in OE.

Material and Methods

Fish

Cichlid (*Haplochromis chilotes*) were maintained at 27°C on a 12 h light/12 h dark cycle. 6-12 individuals were in a plastic tank (40 cm × 25 cm × 36 cm). Mature adults were used for the experiment.

Odorant solutions

20 proteinogenic amino acids (arginine, histidine, lysine, aspartate, glutamate, serine, threonine, asparagine, glutamine, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, valine), 4-hydroxyphenyl acetate (4HPAA), lithocholic acid (LCA) were purchased from Wako Pure Chemical Industries, Sigma Chemical Co. Each amino acid (except tyrosine) and 4HPAA were dissolved in ultrapure water to 12 mM. Tyrosine and LCA were dissolved in 6 mM NaOH to 12 mM. 3 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., 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 minutes, extraction liquid was centrifuged at 8,000 × g for 5 minutes, 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 non-diluted 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 the method to collect urine from masu salmon used in Yambe et al., 1999, we developed a new method to collect urine directly from cichlid, whose size is around 6-

9 cm, under swimming condition (Figure 5A, B). We used a dental root canal cleaning probe needle with the hole in the side (28G, 490703, B.S.A Sakurai CO.) to construct a sampling catheter. About 0.5-1.2 cm from the tip of this probe needle was gently bent about 90 degrees 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 were anesthetized with ice for 1 minute. 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 to be held in place. Catheterized cichlid was placed in the net chamber made from a pot bottom net to restrict the movement. Cichlid was able to accumulate for 15 minutes. After aspirating for 3-5 hours, about 500-1000 μL of urine was trapped ins the centrifuge tube placed on ice. Urine collected during the first 30 minutes 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).

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, 3L) which is covered with black paper to make inside dark and served with clean dechlorinated water. The fish was kept in this tank for 1.5-3 hour before the exposure. These procedures were done to reduce the background expression of *c-fos* in the olfactory epithelium. The water supply was then stopped, and 15 mL of odorant solution was delivered to the tank by 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:

4mM, male urine: 30-fold dilution, food extraction: 75-fold dilution). Water was exposed in control. After exposing fish for 1 minute, the water was supplied again, and the fish was kept in the tank for 20 minutes for the expression of *c-fos*. The fish was then anesthetized on ice for 5 minutes and killed by decapitation. Olfactory epithelia were dissected in 4% PFA (paraformaldehyde, Wako) / 0.7 × phosphate-buffered saline (PBS). Dissected tissues were fixed in 4% PFA / 0.7 × PBS at 4°C for 7.5 hours. Fixed tissues were treated with 20% sucrose / 0.7 × PBS at 4°C overnight for cryoprotection before embedding. Tissues were embedded in Tissue Tek O.C.T. compound (Sakura) and frozen by liquid nitrogen. Embedded tissues were sliced into 10 µm section in a horizontal direction and loaded on glass slide (MAS-01, Matsunami). Sections were kept at -80°C until using for the following experiments.

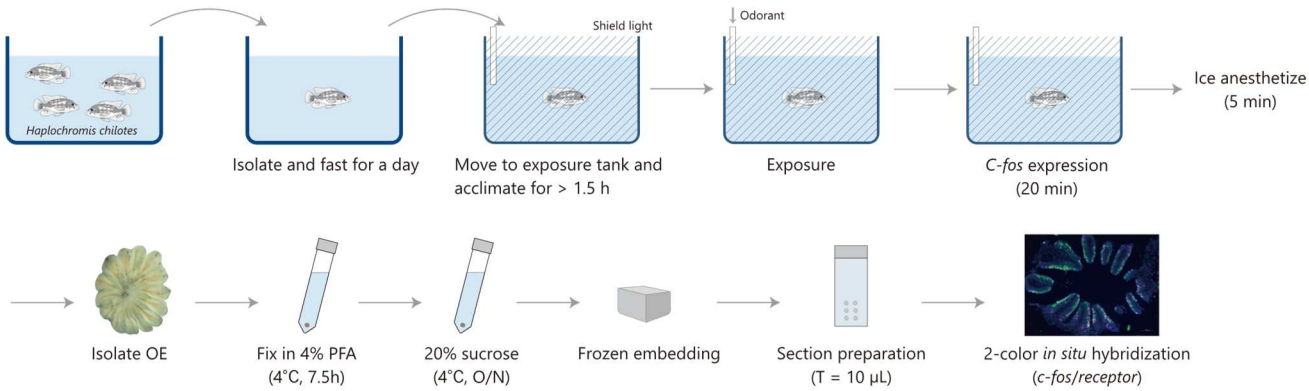

Figure 1. Schematic flow of the experiment.

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 by 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 fluorescein RNA labeling mix (Roche), respectively.

In situ hybridization

Single-color and two-color ISH were performed according to the method which is modified the previous one (Suzuki et al., 2015). Briefly, in single-color 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). In two-color 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 by 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. Signal from FITC-riboprobes were detected with HRP-conjugated anti-FITC antibody (1:500, PerkinElmer). Signals were then 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). All images were digitally captured on a fluorescence microscope (Carl Zeiss).

Quantification of neuron number

The image was first integrated into a single section by Adobe Photoshop CC 2018. The tone of the image was correlated by Curves in Photoshop. Expressing neurons were determined by the threshold of brightness (85) and the shape, and marked by Count Tool in Photoshop. Co-localization was also tested on the marked neuron by Photoshop. The area was measured by fluorescent from DAPI using Photoshop.

Results

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

In initial experiments, we exposed cichlid to food extraction and assessed the up-regulation of 5 immediate-early genes (IEGs) *c-fos*, *egr1*, *c-jun*, *fra1*, *junb* by *in situ* hybridization in the olfactory epithelium. By comparing the expression of non-exposed and exposed olfactory epithelium, we concluded that *c-fos* is the most suitable neural activity marker for cichlid olfactory epithelium (Figure 2A).

To quantitatively confirm the up-regulation of *c-fos* in cichlid olfactory epithelium, we exposed cichlid to food extraction and tested whether the number of *c-fos*-expressing neurons would increase with time (control/10/20/30 minutes) since exposure (Figure 2B, C). We compared the number of *c-fos*-expressing neurons in 5 sections which were evenly selected and found that the number in 20 minutes and 30 minutes were 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 minutes. RNA

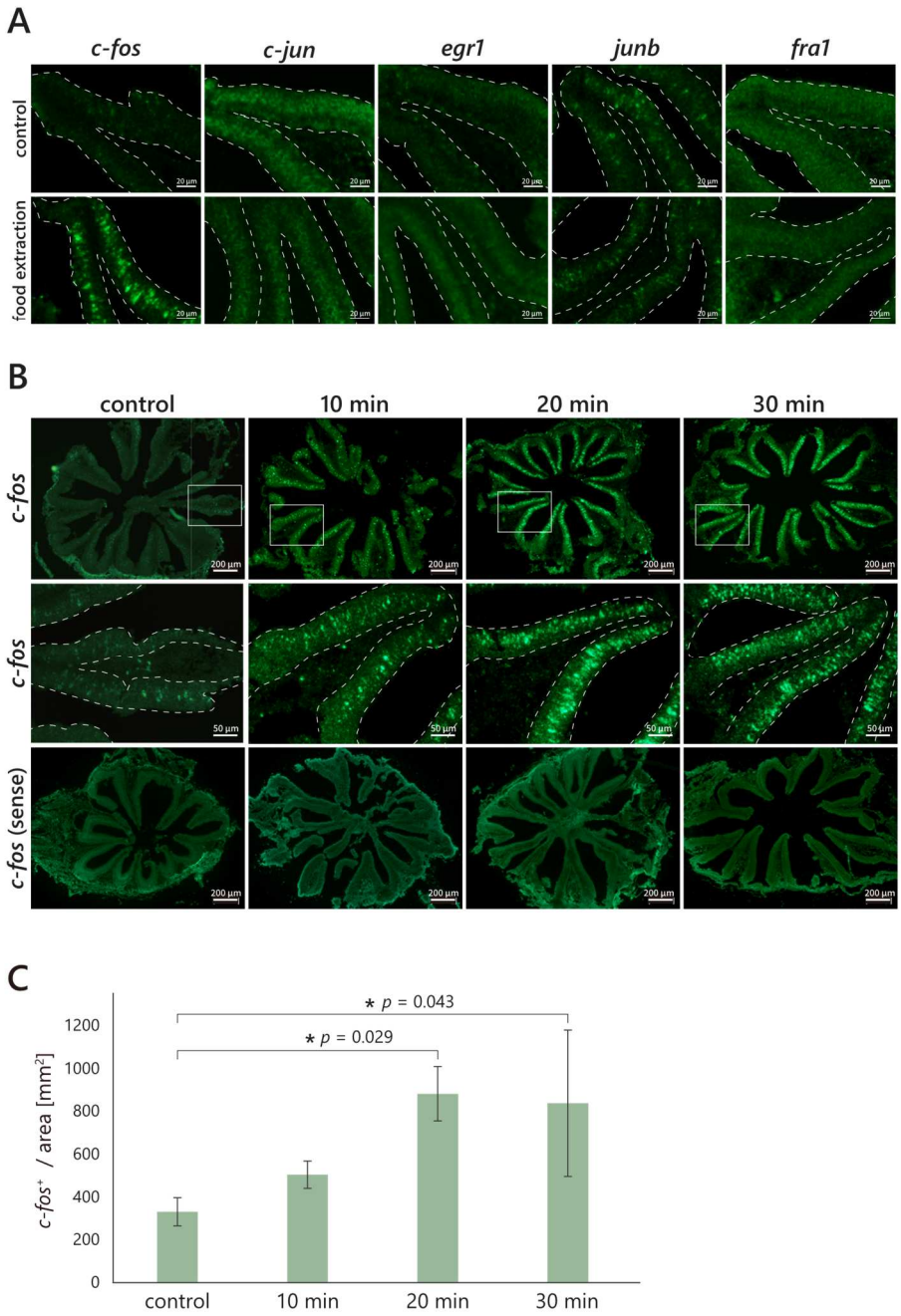

Figure 2. *C-fos* expression robustly induced in OE by exposure of food extraction. Cichlid was exposed to water (control) or food extraction (final conc.: 9.5 mg/L). **(A)** *In situ* hybridization with cRNA probes for *c-fos*, *c-jun*, *egr1*, *junb*, or *fra1*. The dotted line represents the outline 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*$^+$ (3 individuals each, 5 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 (Hansen et al., 2004; Sato et al., 2005), against several odorants: the mixture of 20 proteinogenic amino acids (final conc. at 2 μM each); food extractions; male urine; and the mixture of 3 conjugated steroids (final conc. at 33 nM each). Exposure of each 4 odorants significantly increased the number of *c-fos*-expressing neurons compared to control with stronger S/N ratio (n = 4; $p = 0.047$, $p = 0.035$, $p = 0.024$, $p = 0.043$; Welch's *t*-test, Figure 3A, B). We also calculate the rate of microvillous neurons among *c-fos*-expressing neurons. It became 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). Food extraction also made it high similar to amino acids (Figure 3C). On the other hand, it became 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).

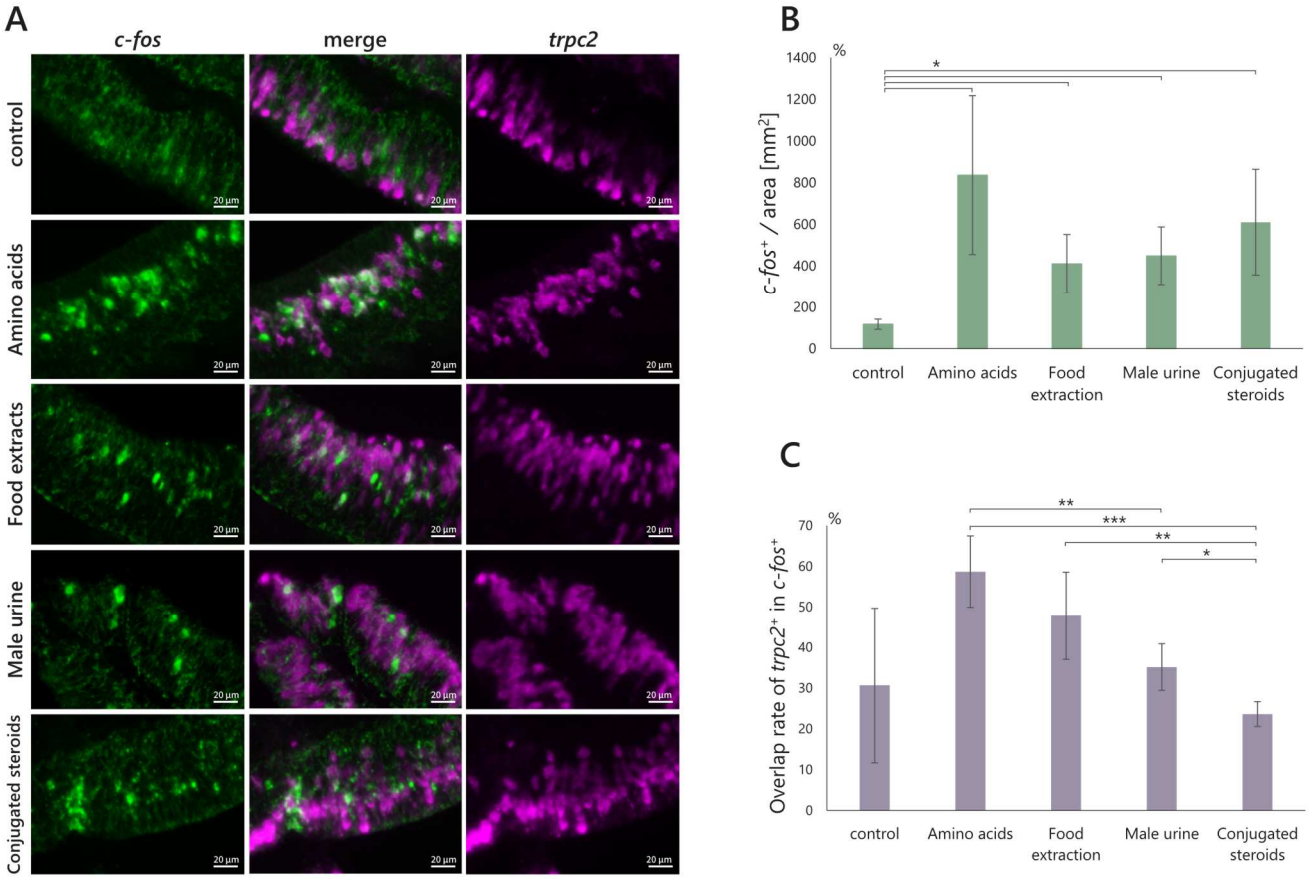

Figure 3. Response of microvillous neurons. (A-C) Double *in situ* hybridization with cRNA probes for *c-fos* (green) and *TrpC2* (magenta) of OE sections of cichlid exposed to water (control), amino acids (final conc.: 2 µM each), food extraction (final conc.: 9.5 mg/L), male urine (final conc.: 6000-fold dilution), or conjugated steroids (final conc.: 33 nM). **(B)** Bar plot of density of *c-fos*$^+$ (4 individuals each, 5 section per individual; Welch's *t*-test). **(C)** Bar plot of overlap rate of *TrpC2*$^+$ in *c-fos*$^+$ (4 individuals each, 5 section 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 traditional 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 possess 61 intact *V2R* genes which is one of the largest number among teleost (Nikaido et al., 2013). We first exposed cichlid to a mixture of 20 amino acids (final conc. at 2 μM each) and tested the response of V2R subfamily 4, 8, 14, and 16 as an expanded subfamily, and V2R subfamily 2-1, 7-1 as a single-copy family (Figure 4A, B). The responding rate was calculated from the co-locate rate of *c-fos*-expressing neurons among *V2R*-expressing neurons. Conversely to the hypothesis that fish receive amino acid via V2R receptors, only a small number of subfamily 4/8-expressing neurons responded to amino acids (3.6/0.9%; Figure 4A, B). On the other hand, 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). To find out which amino acid does V2R subfamily 14 and 16 receive, we exposed cichlid to 4 groups of amino acids: A including non-polar or neutral amino acids (Gly, Ala, Ser, Pro, Thr); B including aromatic or carbamic amino acid (Phe, Tyr, Trp, His, Asn, Gln); C including branched or sulfur-containing amino acids (Val, Ile, Leu, Met, Cys); and D including charged amino acids (Arg, Lys, Asp, Glu) (final conc. 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 high an S/N ratio to amino acids D including charged amino acids (Figure 4C, D). The other amino acids groups also made a response in subfamily 14-expressing neurons, although the responding rate was much smaller. 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 conc. at 10 μM) makes response (46%; Figure 4C, D). We further tested the response to arginine of 3 single copies in subfamily 14 which are 14-1, 14-2, 14-6. Respectively, they

responded 0%, 28%, and 9.1% (Figure 4E, F). On the other hand, subfamily 16-expressing neurons most responded to amino acids group C, although the rate was not high as subfamily 14-expressing neurons which exposed to group D (9.2%; Figure 4D, Supplementary Figure 1A). We also tested the response against amino acids group C of 3 single copies in subfamily 16 which are 16-1, 16-3, and 16-6, but no response was observed in any copy (Supplementary Figure 1B).

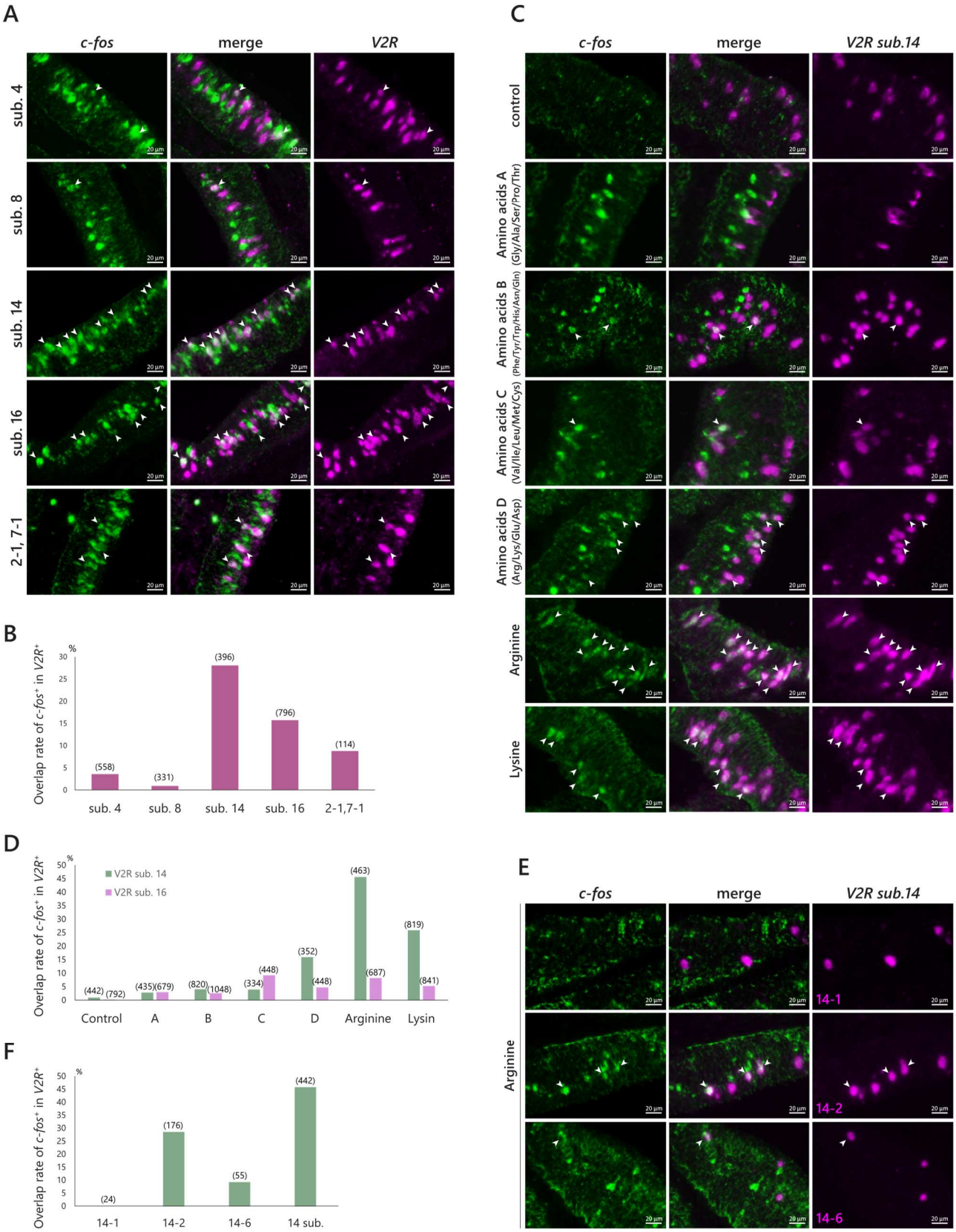
A
c-fos
merge
V2R
sub. 4
sub. 8
sub. 14
sub. 16
2-1, 7-1
20 μm
B
Overlap rate of c-fos+ in V2R+
%
(558)
(331)
(396)
(796)
(114)
sub. 4
sub. 8
sub. 14
sub. 16
2-1,7-1
C
c-fos
merge
V2R sub.14
control
Amino acids A (Gly/Ala/Ser/Pro/Thr)
Amino acids B (Phe/Tyr/Trp/His/Asn/Gln)
Amino acids C (Val/Ile/Leu/Met/Cys)
Amino acids D (Arg/Lys/Glu/Asp)
Arginine
Lysine
D
V2R sub. 14
V2R sub. 16
Overlap rate of c-fos+ in V2R+
%
(442)
(792)
(435)
(679)
(820)
(1048)
(334)
(448)
(352)
(448)
(463)
(687)
(819)
(841)
Control
A
B
C
D
Arginine
Lysin
E
c-fos
merge
V2R sub.14
Arginine
14-1
14-2
14-6
F
Overlap rate of c-fos+ in V2R+
%
(24)
(176)
(55)
(442)
14-1
14-2
14-6
14 sub.

Figure 4. Response of V2R receptor to amino acids. Double in situ hybridization with cRNA probes for *c-fos* (green) and *V2R* (magenta) of OE section exposed to amino acids. **(A, C, E)** Representative image. Arrowheads represent the co-localization of *c-fos* and *V2R*. **(B, D, F)** Bar plot of overlap rate of *c-fos*$^+$ in *V2R*$^+$ (1 individual each). The numbers in the bracket represent the number of *V2R*$^+$ counted in a single section. **(A-B)** OE section of cichlid exposed to amino acids (final conc.: 2 μM each). Each probe is identical over 80% to all copies in each subfamily. **(C-D)** OE section of cichlid exposed to 4 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 conc.: 2 μM each), arginine (final conc. at 10 μM) or lysine (final conc. 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, 14-6 belong to 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 3 conjugated steroids using probes for the mixture of 6 *V1R* (Figure 5D). *V1R*s-expressing neurons responded to male urine the most (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 4-hydroxyphenyl acetate (4HPAA) and lithocholic acid (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).

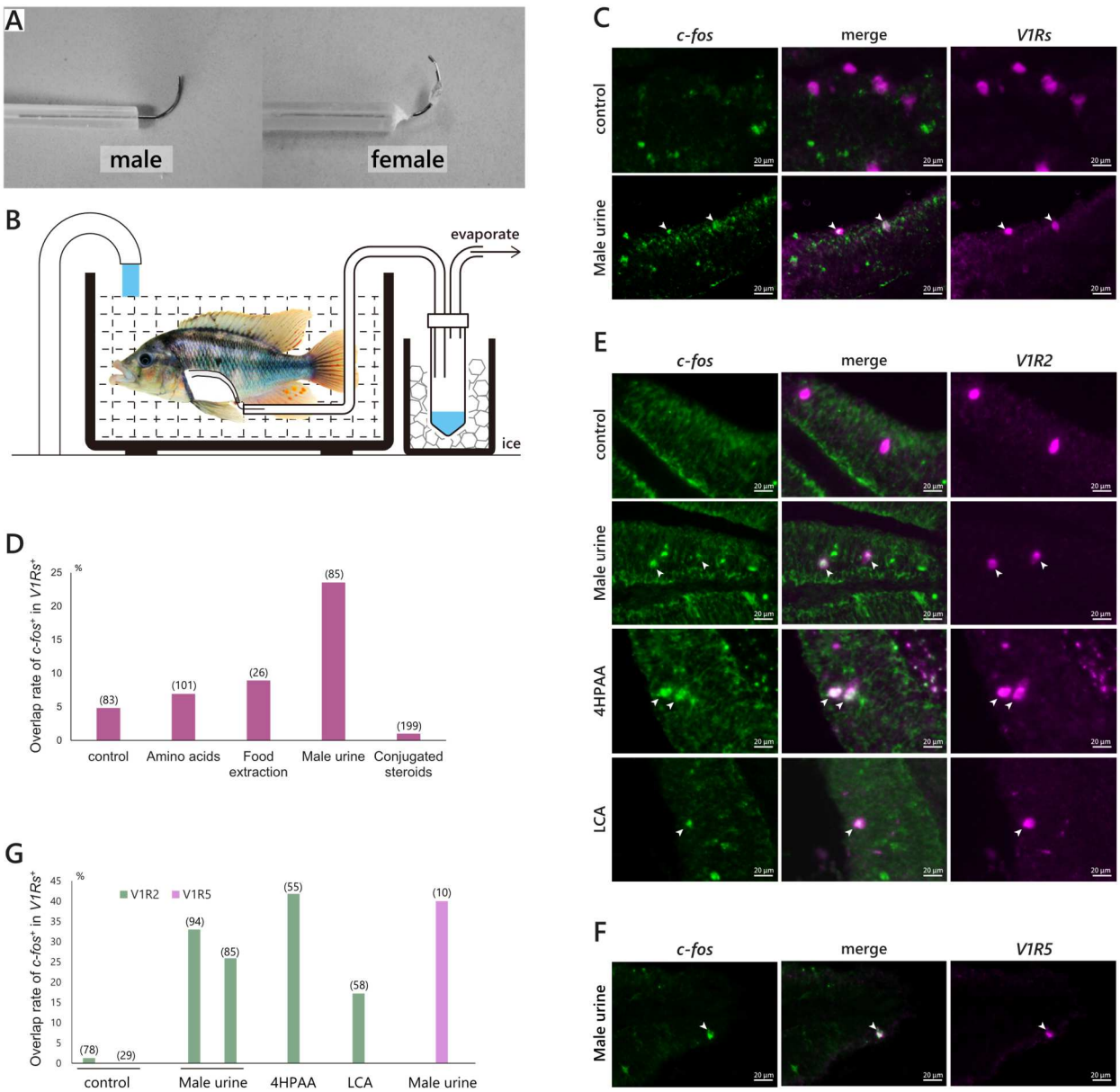

Figure 5. Response of V1R receptor. (A) The catheter used for urine collection. These catheters have a hole in the side. The catheter for female has a silicone plug attached to it. **(B)** Schematic drawing of urine collection. **(C-F)** Double *in situ* hybridization with cRNA probes for *c-fos* (green) and *V1R* (magenta) of OE section. **(C, E, F)** Representative image. Arrowheads represent the co-localization of *c-fos* and *V1R*. **(D, G)** Bar plot of overlap rate of f *c-fos*$^+$ in *V1R*$^+$ (1 individual each). The numbers in the bracket represent the number of *V1R*$^+$ counted in a single section. **(C-D)** Response of V1Rs receptor to amino acids (final conc.: 2 μM each), food extraction (final conc.: 9.5 mg/L), male urine (final conc.: 6000-fold dilution), or conjugated steroids (final conc.: 33 nM). **(E)** Response of V1R2 receptor to male urine (final conc.: 6000-fold dilution), 4-hydroxyphenyl acetate (4HPAA) (final conc.: 10 μM), or lithocholic acid (LCA) (final conc.: 10 μM). **(F)** Response of V1R5 receptor to male urine (final conc.: 6000-fold dilution).

Discussion

Here, we established a method to detect biological-derived neural responses from OE of East African cichlid by *c-fos in situ* hybridization and showed different neural response to each odorant stimuli. We first identified the most suitable neural activity marker gene as *c-fos*. *Egr1*, which is another major marker gene for neural activity (Isogai et al., 2011), was reported to be useful for an active marker of the cichlid brain (Burmeister and Fernald, 2005), but did not show up-regulation in OE. *C-fos*-expressing neurons were significantly increased 20 minutes after exposure of food extraction. Also, an S/N ratio was increasing after 20 minutes. Thus, we concluded that 20 minutes is the best time length between the 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 was also high when exposed to food extract. Because microvillous neurons are dominated by *V2R*-expressing neurons, this result suggests cichlid receive amino acids in food via V2R receptors. This is consistent with the traditional hypothesis that fish receive amino acids via V2R receptors (Koide et al., 2009; DeMaria et al., 2013). On the other hand, some electrophysical research (Sato and Suzuki, 2001; Hansen et al., 2003; Sato and Sorensen, 2018) showed that ciliated neurons also respond to amino acids. 45% *c-fos*-expressing neurons still did not co-label with *TrpC2* when exposed to amino acids. The function of amino acid reception of ciliated neurons 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 V2R receptors to amino acids. African cichlids have 61 copies of *V2R* which is one the largest number among teleost. Although fish V2R receptors are thought 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 amino acids. This is contrary to the traditional hypothesis that V2R receptors receive amino acids indicating that they receive other chemicals. One candidate for this is a peptide. Mouse V2R receptors receive peptides (Kimoto et al., 2005), and stickleback and zebrafish receive 9-mer MHC peptides in the olfactory epithelium (Milinski et al., 2005; Hinz et al., 2013). Compared to single amino acids, peptides can make many different types by the combination of amino acids, so peptide is more suitable for species-specific odors. Within teleost *V2R*s, subfamily 4 and 16 are diversified in many lineages (Nikaido et al., 2013), and these subfamilies may act as species-specific odor by receiving peptides (Zhou et al., 2021). In fact, subfamily 4 did not respond to amino acids, and although subfamily 16 did respond to amino acids group C, 3 copies consisting of subfamily 16 did not respond to amino acids group C. Perhaps these receptors receive peptide.

In addition, the *V2R* gene cluster is adjacent to *neprilysin*, which is encoding metallopeptidase, suggesting that neprilysin may produce degraded peptides which are received by V2R receptors (Hashiguchi and Nishida, 2006; Johnstone et al., 2009; Nikaido et al., 2013). We confirmed that *neprilysin* is expressed in cichlid OE (Supplementary Figure 2). Most *neprilysin* were expressed in cells in the basal region of OE. Perhaps these cells excrete the free enzymatically active domain of neprilysin. Furthermore, some *neprilysin* co-located with *TrpC2* which suggest that V2R receptor receive proximally-cleaved peptide.

We also showed that among the three copies in subfamily 14 which is expanded specifically in a cichlid, one copy was receptive to arginine, one was slightly receptive, and one was not receptive (Figure 4E, F). Moreover, we showed that subfamily 16 which is also expanded responded to amino acids C, while the three copies in subfamily 16 did not respond to amino acids C (Supplementary Figure 1B). These results suggest that copy expansion of cichlid *V2R* led to increasing receivable odorants by ligand differentiation. This hypothesis is also supported by our previous study that the residues putatively related to ligand binding in cichlid-specifically expanded subfamilies were much diverse than that of whole teleost (Nikaido et al., 2013).

V1R2 might be 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. On the other hand, *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), they might be received via OR receptor.

We further tested every 6 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 might be used to find food since 9% of expressing neurons responded to food extract (Figure 5D).

We finally showed *V1R2*-expressing neurons responded to 4HPAA (4-hydroxyphenyl acetate) and LCA (lithocholic acid) (Figure 5E). Previous research was also reporting 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), and our preliminary experiments confirmed that 4HPAA exist in the cichlid urine, V1R2 receptor might be a fundamental pheromone (= 4HPAA) receptor which is common across teleost. Besides, 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. By further investigation on the function of the other derivative allele, it might help to understand the impact of the V1R2 receptor on adaptive radiation in cichlid via assortative mating.

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Declaration of Competing Interests

The authors have no conflicts of interest to declare.

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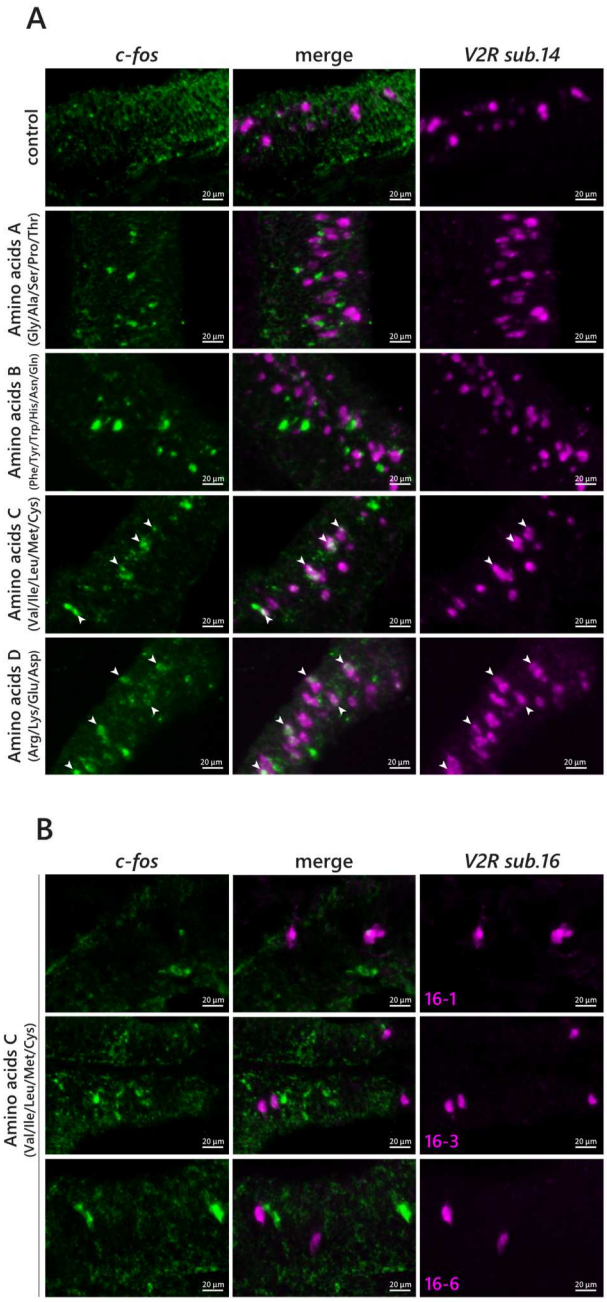
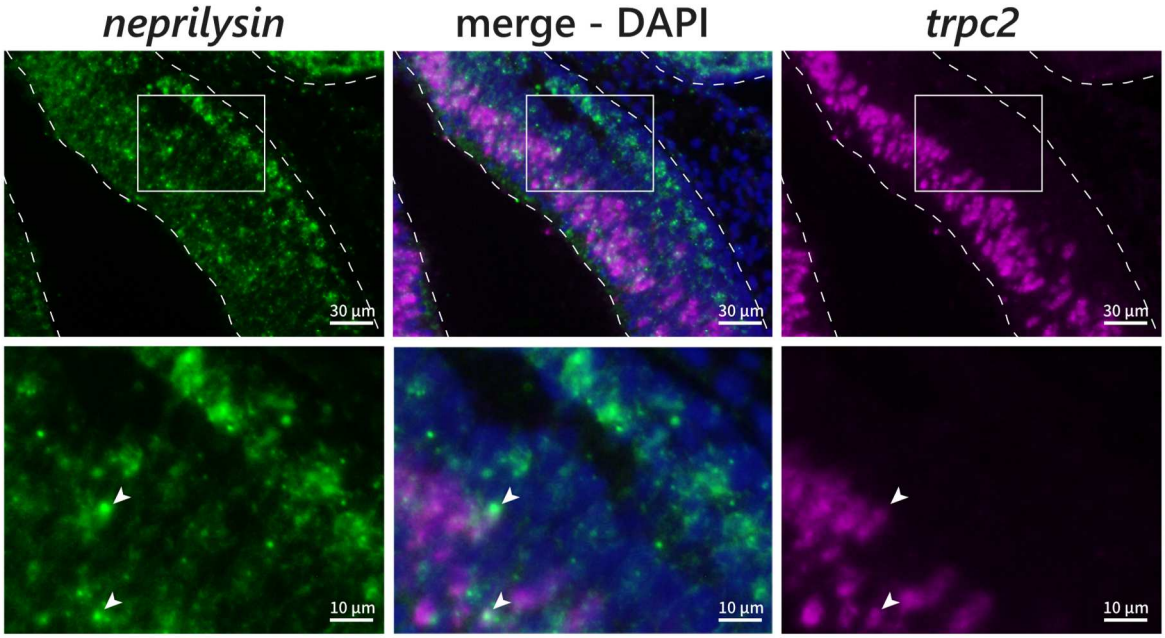
Supplementary Data



Supplementary Figure 1. Response of V2R sub. 16 receptors to amino acids. (A) Double in situ hybridization with cRNA probes for *c-fos* (green) and *V2R sub. 16* (magenta) of OE section exposed to o 4 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 conc.: 2 μM each), arginine (final conc. at 10 μM) or lysine (final conc. 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 conc. at 10 μM). Arrowheads represent the co-localization.



Supplementary Figure 2. Expression of *neprilysin* in OE. Double *in situ* hybridization with cRNA probes for *neprilysin* (green) and *TrpC2* (magenta) of 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 mostly expressed in the basal region of OE except little in middle region. The lower panels represent the magnified image of the box in the upper panel. Arrowheads represent the co-localization of *neprilysin* and *TrpC2*.

Supplementary Table 1. Primer list used in this study.

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