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RESEARCH ARTICLE

c-fos expression in the olfactory epithelium of the East African cichlid (*Haplochromis chilotes*) in response to odorant exposure

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ABSTRACT

Fishes use olfaction to gain varied information vital for survival and communication. To understand biodiversity in fishes, it is important to identify what receptors individual fish use to detect specific chemical compounds. However, studies of fish olfactory receptors and their ligands are still limited to a few model organisms represented primarily by zebrafish. Here, we tested the *c-fos* expression of olfactory sensory neurons (OSNs) in an East African cichlid, the most diversified teleost lineage, by *in situ* hybridization with a *c-fos* riboprobe. We confirmed that microvillous neurons contributed the most to the detection of amino acids, as in other fishes. Conversely, we found that ciliated neurons contributed the most to the detection of conjugated steroids, known as pheromone candidates. We also found that V2Rs, the major receptor type in microvillous neurons, exhibited differential responsiveness to amino acids, and further suggest that the cichlid-specific duplication of *V2R* led to ligand differentiation by demonstrating a differential response to arginine. Finally, we established a non-lethal method to collect cichlid urine and showed how various OSNs, including *V1R*$^{+}$ neurons, respond to male urine. This study provides an experimental basis for understanding how cichlids encode natural odours, which ultimately provides insight into how olfaction has contributed to the diversification of cichlids.

KEY WORDS: Olfactory sensory neuron, V1R, V2R, Amino acid, Urine collection

INTRODUCTION

Animals use olfaction to guide many complex behaviours, such as foraging for food, choosing mates, recognizing territories, migrating and avoiding predators. This olfaction is initiated by the reception of odours by olfactory receptors expressed on the peripheral olfactory neurons. Hence, it is important to identify both the specific receptors and the corresponding odorants to understand diverse behaviours in animals.

In fishes, several soluble chemical compounds are detected by the olfactory epithelium (OE) as odour. A number of odorants are known to drive feeding behaviour in fishes; for example, amino acids in zebrafish and salmonid species (Hara, 2006; Koide et al., 2009; Valentinčič et al., 1999), polyamines in goldfish (Rolen et al., 2003), and nucleotides in zebrafish (Wakisaka et al., 2017). These odorants sometimes work differently in other species, e.g. amino acids work as home stream substances for homing or sex pheromones in salmonid species (Shoji et al., 2003; Yamamoto et al., 2010, 2013; Yambe et al., 2006) and as signals of social status in tilapia (Kutsyna et al., 2016), and polyamines drive aversive behaviour in zebrafish (Hussain et al., 2013). Sex steroids and prostaglandins have been shown to act as pheromones in goldfish (Dulka et al., 1987; Sorensen et al., 1988; Stacey et al., 1989) and prostaglandins in zebrafish (Yabuki et al., 2016). Bile acids are detected by several species, and they drive migration behaviour in lamprey, although their functions in other fishes remain controversial (Huertas et al., 2010; Li et al., 1995; Michel and Lubomudrov, 1995; Zhang et al., 2001).

Fishes detect odorants by olfactory 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 known as OlfC; Herrada and Dulac, 1997). The single adenosine receptor A2c is also found in the zebrafish OE (Wakisaka et al., 2017). These receptors are expressed in several types of olfactory sensory neurons (OSNs). For example, ORs are expressed in ciliated neurons (Sato et al., 2005; Hansen et al., 2003), which detect a broad range of odorants such as amino acids, sex steroids, prostaglandins and bile acids (Sato and Suzuki, 2001; Hansen et al., 2003; Yabuki et al., 2016; Sato and Sorensen, 2018). TAARs are also expressed in ciliated neurons and detect polyamines (Hussain et al., 2013; Li et al., 2015). V2Rs are expressed in microvillous neurons; both microvillous neurons (Hansen et al., 2003; Koide et al., 2009; Sato and Sorensen, 2018; Sato and Suzuki, 2001) and V2Rs (DeMaria et al., 2013; Luu et al., 2004) were shown to respond to amino acids. V1Rs are also expressed in microvillous neurons and detect 4-hydroxyphenyl acetate (4HPAA) and bile acids in zebrafish (Behrens et al., 2014; Cong et al., 2019). A single V1R (V1R4/ora4) is specifically expressed in crypt neurons (Oka et al., 2012), of which the ligand has not been identified. A2C is expressed in pear-shaped neurons and detects adenosine (Wakisaka et al., 2017). Another minor neuron, kappe neuron, is also found in the zebrafish OE (Ahuja et al., 2015); however, the receptor and ligand have not yet been identified.

In general, relatively few olfactory receptors have been studied and these studies are limited to organisms such as zebrafish, salmon and goldfish. However, the clade Neoteleostei, which includes approximately 60% of fish taxonomic diversity, has not been the focus for studies of olfaction. Hence, investigating this large diverse clade of non-model species remains crucial for understanding fish evolution.

In this study, we focused on cichlids, one of the most diversified lineages of vertebrates. Cichlids in the East African Great Lakes represent one of the most striking examples of vertebrate adaptive radiation (Kocher, 2004). Because of their highly diversified nuptial coloration, the visual ecology of cichlids has been an important area of research demonstrating the significance of vision in driving cichlid speciation (Seehausen et al., 2008; Terai et al., 2006).

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Although olfaction has drawn less attention from cichlid biologists compared with vision, cichlids also utilize olfaction in many different ecological contexts (Keller-Costa et al., 2015). For example, olfaction contributes to conspecific recognition of *Pseudotropheus emmiltos* (Plenderleith et al., 2005) and sexual imprinting of *Pundamilia* species (Verzijden and Ten Cate, 2007). Other studies show that male tilapia evaluate the sexual status of potential mates from female urine (Miranda et al., 2005), and that a glucuronidated steroid in male tilapia urine is detected by at least two distinct receptors and works as a priming pheromone (Keller-Costa et al., 2014a,b). Moreover, we previously found several highly diverse polymorphic alleles in the V1Rs of East African cichlids (Nikaido et al., 2014), and the copy number of V2Rs has increased in East African cichlid genomes (Nikaido et al., 2013), which suggests the functional importance of olfaction potentially driving cichlid adaptive radiation. However, as for other fishes, little is understood about the ligand specificity of individual OSNs in cichlids.

Here, we performed *in situ* hybridization with a riboprobe of the neural activity marker gene *c-fos* to investigate OE in the East African cichlid *Haplochromis chilotes*. *Haplochromis chilotes* is relatively fast growing and easy to breed, a high-resolution genome is available (Nakamura et al., 2021) and olfactory receptor genes have been identified (Nikaido et al., 2013, 2014). We tested the response of three types of OSNs, i.e. microvillous neurons, $V2Rs^{+}$ neurons and $V1Rs^{+}$ neurons, and reported the ligand specificity of several stimulants. We also tested the specific detection of several V2Rs for amino acids and V1Rs for cichlid male urine. This study offers important initial experimental insights into how cichlids encode natural odours, which may allow us to elucidate the mechanism of olfaction-based species recognition in future studies.

MATERIALS AND METHODS

Fish

Cichlids, *Haplochromis chilotes* (Boulenger, 1911), were collected in Mwanza gulf of Tanzania during an exploration that was led by the Nikaido Laboratory in 2018. Fish were maintained and bred 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) and fed with feed pellets twice a day. Only mature males were used in order to ensure that the experimental conditions were comparable.

Preparation of stimulants

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 and lithocholic acid (LCA) were purchased from Wako Pure Chemical Industries (Chuo-ku, Osaka, Japan) and Sigma Chemical Co. (St Louis, MO, USA). Each amino acid (except tyrosine) and 4HPAA was dissolved in ultrapure water to 12 mmol l^{-1} to create a stock solution. Tyrosine and LCA were dissolved in 6 mmol l^{-1} NaOH aqueous solution to 12 mmol l^{-1} as the stock solution. 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 (Chuo-ku, Tokyo, Japan), Cayman Chemical Co. (Ann Arbor, MI, USA) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively, and dissolved in DMSO to 10 mmol l^{-1} to create a stock solution. Food extract was prepared by the following procedure. First, 2 g of crushed feed pellets (Otohime EP1, Marubeni Nisshin Feed Co., Chuo-ku, Tokyo, Japan) was added to ultrapure water up to 14 ml and vortexed. After incubating at room temperature for 5 min, the extraction liquid was centrifuged at 8000 ***g*** for 5 min, and the supernatant was collected as the food extract stock solution, and stored at 4°C until the exposure experiment. Stock solutions were diluted with ultrapure water prior to the exposure, and a 15 ml diluted solution was applied experimentally. Each solution was diluted as follows: a mixture of 20 amino acids/amino acids group A–D was diluted to 400 µmol l^{-1} (final concentration of 2 µmol l^{-1} in the exposure tank); a mixture of conjugated steroids was diluted to 6.6 µmol l^{-1} each (final concentration of 33 nmol l^{-1} in the exposure tank); arginine/lysine/glutamate/aspartate/4HPAA was diluted to 2 mmol l^{-1} (final concentration of 10 µmol l^{-1} in the exposure tank); LCA was diluted to 4 mmol l^{-1} (final concentration of 20 µmol l^{-1} in the exposure tank); food extract was diluted 75-fold (final concentration of 15,000-fold dilution in the exposure tank).

Urine collection

Urine was collected from mature male cichlids. Although several studies have collected non-diluted urine from fish, they were limited to larger species such as Masu salmon, rainbow trout, Mozambique tilapia and Senegalese sole (Fatsini et al., 2017; Keller-Costa et al., 2014a; Sato and Suzuki, 2001; Yambe et al., 1999). By adapting methods to collect urine from Masu salmon employed in Yambem et al. (1999), we developed a non-lethal method to collect urine directly from cichlids, whose size is approximately 6–9 cm (Fig. 1A,B). We used a dental root canal cleaning probe needle (28G, 490703, BSA Sakurai Co., Nagoya, Aichi, Japan) to construct a sampling catheter (Fig. 1A). This needle has a hole in the side, which is less prone to clogging than a needle with a hole in the tip. Approximately 0.5–1.2 cm from the tip, the needle was gently bent inward in the direction that the hole faces at and angle of approximately 90 deg so that the hollow tube structure remained open (Fig. 1A). The bent needle was connected to a 15 ml centrifuge tube using silicon tubing (o.d. 10 mm, i.d. 0.5 mm) fixed with adhesive (Aron Alpha EXTRA Fast-Acting Versatile, Konishi, Osaka-shi, Osaka, Japan) to trap the urine. The centrifuge tube was then further connected to an aspirator (DAS-01, As one, Osaka-shi, Osaka, Japan) to aspirate the urine.

Cichlids were anesthetized with ice water for 1 min. The catheter was inserted via the urogenital papilla into the urinary bladder. The silicon tubing connecting the catheter was fixed to the anal fin by a wire and then clipped with a bulldog clip to hold it in place. Catheterized cichlids were placed in a polyethylene net chamber to restrict their movement and placed underwater. Urine was aspirated through the catheter for 3–5 h. Approximately 500–1000 µl of urine was collected in the centrifuge tube and 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, Blacksburg, VA, USA). Urine was diluted 30-fold with ultrapure water prior to exposure treatment, and 15 ml diluted urine was applied experimentally (final concentration at 6000-fold dilution in the exposure tank).

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

Exposure and tissue preparation

Adult cichlids were isolated in a glass tank (40 cm×25 cm×36 cm) the day prior to exposure and were not fed (Fig. 1C). The following day,

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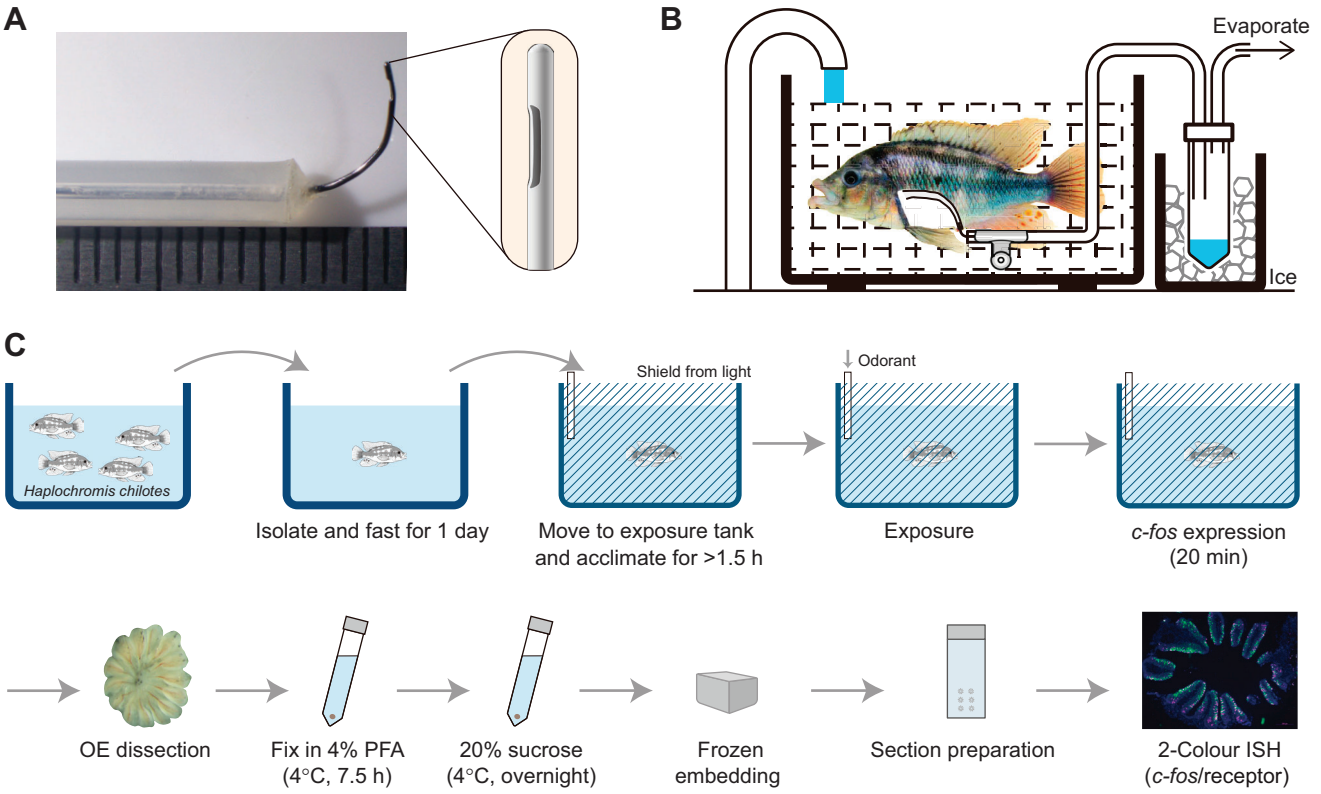

Fig. 1. Schematic drawing of methods. (A) Catheter for urine collection. The catheter has a hole in the side near the tip of the needle to prevent clogging. (B) Schematic drawing of the non-lethal urine collection protocol. (C) Schematic workflow for the detection of odorant-induced *c-fos* expression. Individual cichlids were exposed to a stimulant (odorant); olfactory epithelia (OE) were then isolated, fixed (in paraformaldehyde, PFA), cryoprotected in 20% sucrose, embedded and frozen. Thin sections (10 μm) were prepared and used for *in situ* hybridization (ISH).

fish were transferred to the exposure tank (30 cm×11 cm×9 cm, 3 l) which was covered with black paper to create a dark environment. Clean dechlorinated water flowed into the tank at one end and out from the opposite end. The fish were kept in this tank for 1.5–3 h before exposure to odorant in order to minimize *c-fos* expression in the OE. Immediately prior to exposure, water inflow was temporarily stopped, and 15 ml of stimulant (odorant) was delivered to the same end of the tank as the water inflow using a peristaltic pump (SJ-1211II-H, Atto, Taito-ku, Tokyo, Japan). Water alone was applied as a negative control. The stimulant was delivered into the tank over a period of approximately 1 min, and the water inflow was then resumed. Fish were kept in the tank for 20 min after exposure to allow for expression of *c-fos*. The fish were then quickly decapitated and the OE were dissected out in 4% paraformaldehyde (PFA, Wako)/phosphate-buffered saline (PBS). Dissected tissues were fixed in 4% PFA/PBS at 4°C for 7.5 h, then treated with 20% sucrose/PBS at 4°C overnight for cryoprotection before embedding in Tissue Tek O.C.T. compound (Sakura Finetek, Chuo-ku, Tokyo, Japan) and were frozen using liquid nitrogen. Embedded tissues were sliced into 10 μm horizontal sections and placed on a glass slide (MAS-01, Matsunami, Bunkyo-ku, Tokyo, Japan). Sections were kept at −80°C until use for downstream experiments.

Preparation of riboprobes

Riboprobes for *in situ* hybridization (ISH) were designed to the coding region or untranslated region. Each sequence was amplified from cDNA of the OE by Ex-Taq (Takara Bio, Kusatsu, Shiga, Japan) with the primers shown in Table S1. PCR products were ligated to pGEM-T (Promega, Madison, WI, USA) or pBluescript SKII (−) plasmid and sequenced. Plasmids were extracted with the QIAfilter Plasmid Midi Kit (Qiagen, Venlo, Netherland) and then linearized using an appropriate restriction enzyme (Takara). Digoxigenin (DIG)-labelled or fluorescein (FITC)-labelled riboprobes were synthesized with T7, T3 or SP6 RNA polymerase (Roche) from the linearized plasmids with DIG or FITC RNA labelling mix (Roche, Basel, Switzerland), respectively.

ISH

Single-colour and two-colour ISH was performed according to the method of Suzuki et al. (2015) with several modifications. Briefly, for single-colour ISH, sections were treated with 5 μg ml^{-1} proteinase K for 8 min at 37°C and hybridized with DIG-labelled riboprobes (5 ng μg^{-1}) at 60°C overnight. The sections were washed, treated with 2 μg ml^{-1} RNase A in TNE (Tris–NaCl–EDTA) for 30 min at 37°C, then with streptavidin/biotin blocking kit (Vector Laboratories, Newark, CA, USA), and then 1% blocking reagent (PerkinElmer, Waltham, MA, USA) in Tris-buffered saline (TBS) for 1 h. Signals were detected with peroxidase-conjugated anti-DIG antibody (1:100, 11207733910, Roche), amplified by Tyramide Signal Amplification (TSA) Plus Biotin kit (PerkinElmer), and visualized with Alexa Fluor 488-conjugated streptavidin (1:200, Thermo Fisher Scientific, Waltham, MA, USA). Sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). In the case of two-colour ISH, sections were hybridized with DIG- and FITC-labelled riboprobes (2.5 ng μg^{-1} at each) at 60°C overnight. Signals from DIG-riboprobes were detected with peroxidase-conjugated

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anti-DIG antibody (1:100, 11207733910, Roche), amplified using TSA Plus DIG kit (PerkinElmer) and visualized with DyLight 594-conjugated anti-DIG antibody (1:150, DI-7594-.5, Vector Laboratories). Sections were treated with 15% H_2O_2 in TBS for 30 min to inactivate peroxidase activity. Then, signals from FITC-labelled riboprobes were detected using anti-Fluorescein-POD Fab fragments (1:80, 11426346910, Roche), amplified with TSA Plus Biotin kit (PerkinElmer) and visualized with Alexa Fluor 488-conjugated streptavidin (1:200, Thermo Fisher Scientific). Sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). All images were digitally captured using a Zeiss Axioplan SP fluorescence microscope with a Zeiss Axiocam 503 color CCD camera (Carl Zeiss, Oberkochen, Germany). The images were level corrected, contrast adjusted, pseudo-coloured (magenta for DIG signal, green for FITC signal) and merged using Adobe Photoshop CC 2018.

Analysis of ISH images

For determination of the marker gene for neural activity, cichlids were exposed to food extract, and single-colour ISH (30 min between stimulant exposure and ice anaesthesia) was performed to find the early marker gene with the greatest increase of positive neurons and the highest signal intensity. For quantification of neurons, typical shapes of OSNs (ciliated/microvillous/crypt/kappe/pear-shaped neuron; Ahuja et al., 2015; Hamdani and Døving, 2007; Wakisaka et al., 2017) were used as reference criteria. In addition, for quantification of *c-fos*-positive (*c-fos*$^+$) neurons, signals that have a luminance value greater than 85 were considered positive. For both single-colour and two-colour ISH, the number of sections per individual and the number of individuals is given in the figure legends. The area used to normalize the number of neurons was measured from the DAPI image of the OE section. Double-positivity was scored when both signals were positive, overlapped and determined to have the same cell shape. These procedures were all performed using Adobe Photoshop CC 2018. Statistical analysis was done using Tukey–Kramer multiple comparison, Welch's *t*-test, Student's *t*-test and *F*-test of equality of variances, as indicated in the figure legends. Significance was determined at $P \leq 0.05$.

RESULTS

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

We initially exposed cichlids to food extract and assessed the upregulation of five immediate-early genes, *c-fos*, *egr1*, *c-jun*, *fra1* and *junb* by ISH in the OE. The results of these initial experiments were used to decide on the most suitable gene for neural activity marker detection. *c-fos* was the gene with the greatest increase in signal intensity and number of positive neurons, so it was selected as the most suitable neural activity marker for cichlid OE (Fig. 2A).

To quantitatively confirm the upregulation of *c-fos* in the cichlid OE, we exposed cichlids to food extract and tested whether the number of *c-fos*$^+$ neurons would increase with time after exposure (i.e. from exposure until ice anaesthesia; control and 10/20/30 min; Fig. 2B,C; Table S2). We compared the number of *c-fos*$^+$ neurons in five sections, evenly selected from top to bottom of the vertical

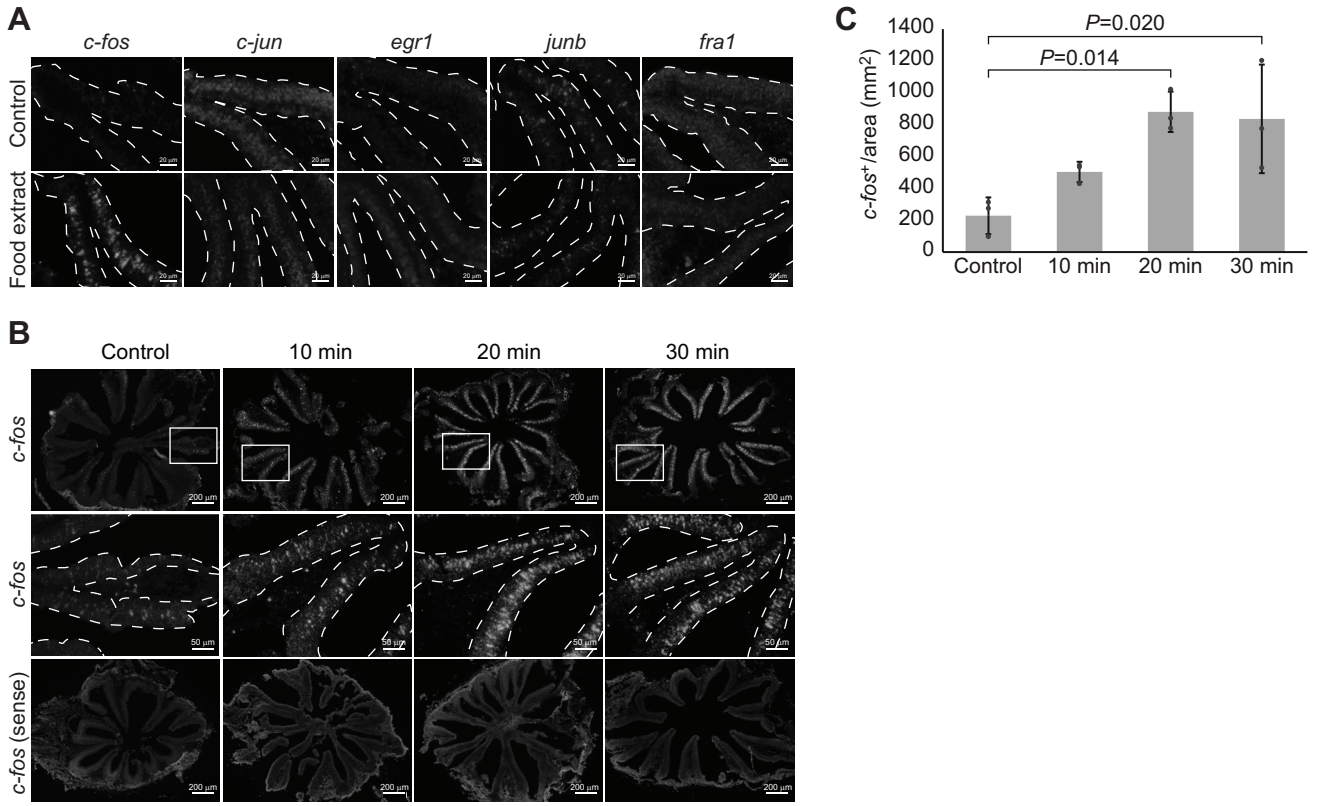

Fig. 2. *c-fos* expression is induced robustly in the OE by exposure to food extract. Cichlids were exposed to water (control) or food extract (final concentration: 15,000-fold dilution). (A) ISH with riboprobes for the immediate-early genes *c-fos*, *c-jun*, *egr1*, *junb* and *fra1*. The dashed line indicates the outline of the OE. (B) ISH with riboprobes for *c-fos*. The columns represent the length of time between stimulant exposure and ice anaesthesia. The middle row shows a magnified image of the boxed region in the top row. The bottom row is the control (sense probe). (C) Bar graph (means±s.e.m.) of the number of *c-fos*-positive (*c-fos*$^+$) neurons in 1 mm^2. The total number of *c-fos*$^+$ neurons in five sections was counted for each individual (three individuals each; Tukey–Kramer test; Table S3).

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z-axis from each individual and found that the number at 20 and 30 min intervals was significantly greater than in the control (3 individuals; P=0.014, P=0.020; Tukey–Kramer test; Fig. 2B; Table S3). Thus, we set the time between stimulant exposure and ice anaesthesia as 20 min.

c-fos expression of microvillous neurons

Next, we tested the neural response of microvillous neurons. In several fishes, microvillous neurons are known to detect amino acids (Hansen et al., 2003; Sato and Sorensen, 2018; Sato and Suzuki, 2001), and the majority of microvillous neurons are expected to express *V2R* (Fig. S2A). V2R receptors are also suggested to detect amino acids (DeMaria et al., 2013; Koide et al., 2009). Here, we tested the *c-fos* expression of microvillous neurons in response to a mixture of 20 proteinogenic amino acids (final concentration: 2 µmol l^{-1} each) and amino acid-rich food extract to examine whether our method is useful for testing the specificity of OSNs. In addition, we also tested the *c-fos* expression in response to cichlid male urine (final concentration: 6000-fold dilution) and a mixture of three conjugated steroids (DHEA-s, E2-17g and E2-3,17s, final concentration: 33 nmol l^{-1} each). Urine is considered to be the main source of pheromones in cichlids (Keller-Costa et al., 2014a; Maruska and Fernald, 2012). Conjugated steroids are candidates for cichlid pheromone (Keller-Costa et al., 2014a, 2016; Miranda et al., 2005); the three conjugated steroids we tested are known to be detected by independent receptors in African cichlid *Astatotilapia burtoni* (Cole and Stacey, 2006). Exposure to the four stimulants significantly increased the number of *c-fos*$^+$ neurons with strong intensity compared with the control (4 individuals; P=0.047, P=0.035, P=0.024, P=0.043, respectively; Welch's *t*-test, Fig. 3A,B; Tables S2 and S3). We calculated the percentage of *Trpc2*$^+$ neurons, which indicates microvillous neurons (Sato et al., 2005), among *c-fos*$^+$ neurons to test the contribution of microvillous neurons to the detection of each stimulant (4 individuals each). The percentage of *Trpc2*$^+$ neurons among *c-fos*$^+$ neurons was highest following exposure to amino acids (55±8.8%) and was significantly higher than that following exposure to male urine (35±5.7%) or conjugated steroids (24±3.0%) (4 individuals; P=0.0080, P=6.1×10^{-4}, respectively; Student's *t*-test; Fig. 3C; Tables S2 and S3). It also became high following exposure to food extract (48±11%; Fig. 3C; Table S2). However, it was lowest following exposure to conjugated steroids, being significantly lower than when exposed to amino acids, food extract or male urine (P=6.1×10^{-4}, P=0.0095, P=0.022, respectively; Fig. 3C; Table S3). We further tested the *c-fos* expression (one individual each) of another major type of OSN, ciliated neurons, which is identified by *Golf2* expression (Jones and Reed, 1989; Koide et al., 2009). In contrast to microvillous neurons,

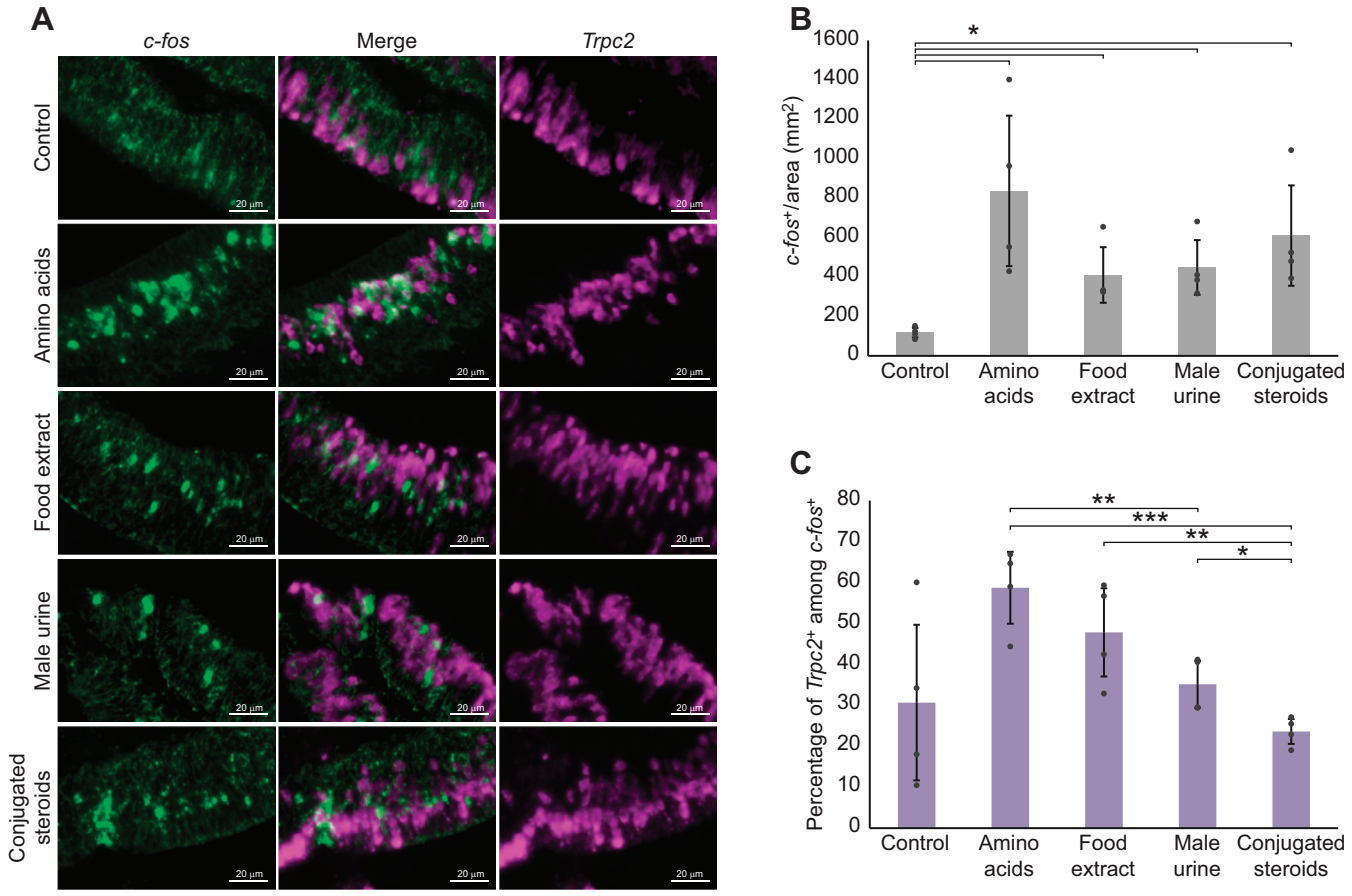

Fig. 3. Specificity of microvillous neurons. (A) Two-colour ISH with riboprobes for *c-fos* (green) and *Trpc2* (magenta) of OE sections of cichlids exposed to water (control), a mixture of 20 proteinogenic amino acids (final concentration: 2 µmol l^{-1} each), food extract (final concentration: 15,000-fold dilution), male urine (final concentration: 6000-fold dilution), or a mixture of three conjugated steroids (final concentration: 33 nmol l^{-1}). (B) Bar graph (means±s.e.m.) of the number of *c-fos*$^+$ neurons in 1 mm^2 (one section per individual, four individuals each; Welch's *t*-test, and *F*-test of equality of variances; Table S3). (C) Bar graph (means±s.e.m.) of the percentage of *Trpc2*$^+$ neurons among *c-fos*$^+$ neurons (one section per individual, four individuals each; Welch's *t*-test, Student's *t*-test, and *F*-test of equality of variances; Table S3). *P<0.05, **P<0.01, ***P<0.001.

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the percentage of *Golf2*$^{+}$ neurons among *c-fos*$^{+}$ neurons was highest (67%) following exposure to conjugated steroids, and it became lower with amino acids (42%) and food extract (41%) versus other treatments (58% in male urine), except in control which was 29% (Fig. S1A,B, Table S2).

c-fos expression of *V2R*-expressing neurons in response to amino acids

East African cichlids experienced a lineage-specific expansion in the *V2R* multigene family and possess 61 intact *V2R* genes, one of the largest repertoires among teleosts (Nikaido et al., 2013). It can

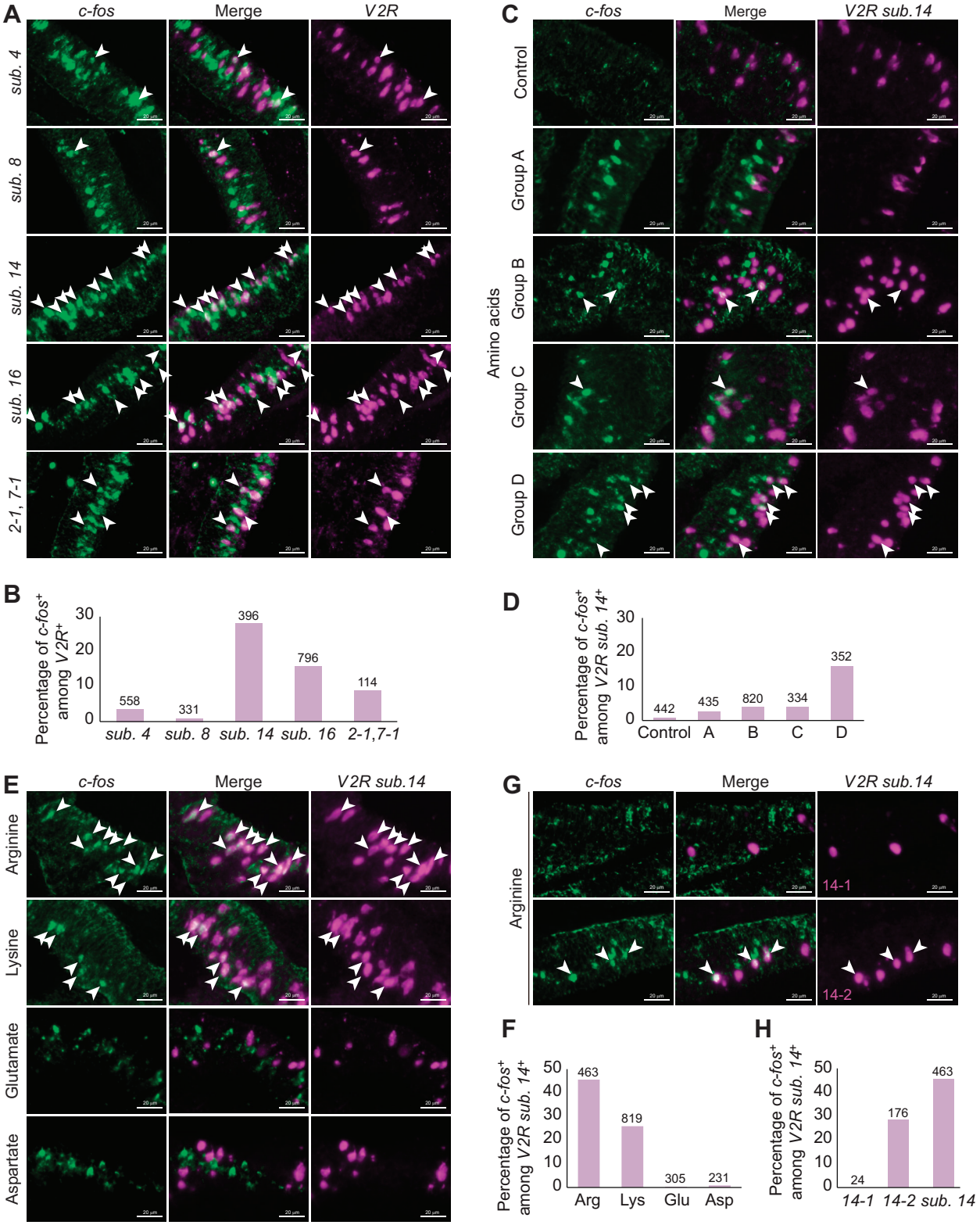

Fig. 4. See next page for legend.

Fig. 4. Specificity of *V2R*$^+$ neurons to amino acids. (A,B) Two-colour ISH with riboprobes for *c-fos* (green) and *V2R subfamily 4/8/14/16/2-1,7-1* (magenta) of OE sections exposed to a mixture of 20 proteinogenic amino acids (final concentration: 2 µmol l^{-1} each) (A) and bar graph of the percentage of *c-fos*$^+$ neurons among *V2R*$^+$ neurons (one individual each) (B). (C,D) Two-colour ISH with riboprobes for *c-fos* (green) and *V2R subfamily 14* (magenta) of OE sections 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 µmol l^{-1} each) (C) and bar graph of the percentage of *c-fos*$^+$ neurons among *V2R subfamily 14*$^+$ neurons (one individual each) (D). (E,F) Two-colour ISH with riboprobes for *c-fos* (green) and *V2R subfamily 14* (magenta) of OE sections exposed to arginine, lysine, glutamate or aspartate (final concentration: 2 µmol l^{-1}) (E) and bar graph of the percentage of *c-fos*$^+$ neurons among *V2R subfamily 14*$^+$ neurons (one individual each) (F). (G,H) Two-colour ISH with riboprobes for *c-fos* (green) and *V2R 14-1/14-2* (magenta) of OE sections exposed to arginine (final concentration: 2 µmol l^{-1}) (G) and bar graph of the percentage of *c-fos*$^+$ neurons among *V2R subfamily 14*$^+$ neurons (one individual each) (H). (A,C,E,G) Representative images. Arrowheads represent the colocalization of *c-fos* and *V2R*. (B,D,F,H) The number of *V2R*$^+$ neurons counted in a single section is indicated above each bar.

be hypothesized that this expanded number of *V2R* genes led to the expansion of detectable odours. Cichlids possess 13 of the 16 V2R subfamilies previously identified in the teleosts (Hashiguchi and Nishida, 2006) (Fig. S3, Supplementary Materials and Methods). Within these 13 V2R subfamilies, four subfamilies in particular (4, 8, 14 and 16) have expanded the number of genes by tandem duplication. We therefore tested the *c-fos* expression of these four expanded subfamilies (4, 8, 14 and 16) plus 2-1,7-1 as a single-copy subfamily, with a mixture of 20 proteinogenic amino acids (final concentration: 2 µmol l^{-1} each; Fig. 4A,B; Table S2). We designed the riboprobes for each subfamily to have >80% homology with every gene in each subfamily. We made sure in advance that these four subfamilies would not colocalize each other by two-colour ISH (Fig. S2C). The response rate of *V2R*$^+$ neurons was calculated from the percentage of *c-fos*$^+$ neurons among *V2R*$^+$ neurons. Consistent with the hypothesis that teleosts detect amino acids via V2R receptors, a large fraction of *V2R*$^+$ neurons responded to amino acids (Fig. 4A,B; Table S2). Relatively larger fractions of neurons responded in V2R subfamilies 14 and 16 (28%/16%), and an intermediate fraction of neurons responded in V2R subfamily 2-1,7-1 (8.8%). However, only a small fraction of neurons responded to amino acids in V2R subfamilies 4 and 8 (3.6%/0.9%).

To determine which amino acids are detected by V2R subfamilies 14 and 16, we exposed cichlids to four groups of proteinogenic amino acids: A, including non-polar or neutral amino acids (Gly, Ala, Ser, Pro and Thr); B, including aromatic or carbamic amino acids (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: 2 µmol l^{-1} each). This grouping is based on electrical properties and a cluster analysis of zebrafish odorant-induced activity patterns (Friedrich and Korsching, 1997). The largest fraction (16%) of *V2R subfamily 14*$^+$ neurons responded with stronger intensity to D group amino acids, including charged amino acids (Fig. 4C,D; Table S2). Although *V2R subfamily 14*$^+$ neurons also responded to other amino acid groups, the response rate was much lower (control: 0.9%, A: 2.3%, B: 4.0%, C: 3.9%; Fig. 4C,D). In contrast, the largest fraction of *V2R subfamily 16*$^+$ neurons responded to amino acids in group C including branched or sulfur-containing amino acids, and a small fraction responded to other amino acid groups (control: 0.12%, A: 2.9%, B: 2.8%, C: 9.2%, D: 4.7%; Fig. S4A,B, Table S2).

To further narrow down the amino acids that induce a response in *V2R subfamily 14*$^+$ neurons, we exposed cichlids to the four individual amino acids in group D (final concentration: 10 µmol l^{-1}) and found that basic amino acids, especially arginine, produced a strong response in *V2R subfamily 14*$^+$ neurons (arginine: 46%, lysine: 26%, glutamate: 0.33%, aspartate: 0.87%; Fig. 4E,F). We further tested the response to arginine of two individual genes in V2R subfamily 14 (*14-1*, *14-2*) which are expressed in a mutually exclusive manner (Fig. S2C). The response rates to arginine between these two genes were substantially different at 0% and 28%, respectively (Fig. 4G,H).

We also tested the *c-fos* expression of *V2R subfamily 16*$^+$ neurons in response to group C amino acids of three individual genes in *V2R subfamily 16* (*16-1*, *16-3*, *16-6*). However, no colocalization with *c-fos* was observed in any copy (Fig. S4C, Table S2). This suggests that group C amino acids are detected by OSNs expressing V2Rs other than 16-1/3/6 in the V2R subfamily 16.

We also tested the *c-fos* expression of four subfamilies of *V2R*$^+$ neurons (one individual each) in response to male urine to examine the possibility that V2R subfamilies that do not respond well to amino acids may be involved in social behaviour. However, only a small fraction of *V2R*$^+$ neurons responded to male urine (subfamily 4: 1.5%, subfamily 8: 1.7%, subfamily 14: 1.6%, subfamily 16: 1.0%; Fig. S4D,E).

c-fos expression of *V1R*-expressing neurons

Finally, we tested the response of *V1R*$^+$ neurons to stimulation. Although zebrafish V1R/ORA has been shown to detect 4HPAA and bile acids (Behrens et al., 2014; Cong et al., 2019), its function remains unclear. We first tested the response of *V1R*$^+$ neurons to four stimulants – a mixture of proteinogenic amino acids, food extract, male urine and the mix of three conjugated steroids – using a series of probes for six *V1R*s (Fig. 5A–C; Table S2). *V1R*$^+$ neurons responded to male urine with the highest response rate among olfactory stimuli tested (16±5.7%; Fig. 5A,B), which was significantly higher than that of the control (P=0.038; Tukey–Kramer test; Table S3). In contrast, only a small fraction of *V1R*$^+$ neurons responded to amino acids (5.5±1.3%), food extract (5.5±2.8%) and conjugated steroids (4.3±3.4%), with no significant difference from the control. Moreover, although the percentage of *V1R*$^+$ neurons among *c-fos*$^+$ neurons when exposed to male urine was not significantly higher than that of the control (P=0.067), it was significantly higher than that following exposure to amino acids, food extract and conjugated steroids (P=0.026, P=0.030, P=0.028, respectively; Tukey–Kramer test; Table S3).

We next tested the response to male urine for each of the six *V1R*s and found only *V1R2*/*ORA1*$^+$ neurons and *V1R5*/*ORA5*$^+$ neurons were responsive (33%, 40%, respectively; Fig. 5D–G; Table S2). Additionally, we tested the response of *V1R2*/*ORA1*$^+$ neurons to 4HPAA (final concentration: 10 µmol l^{-1}) and LCA (final concentration: 20 µmol l^{-1}), which are candidates for the ligand of zebrafish V1R2/ORA1 (Behrens et al., 2014; Cong et al., 2019). Many *V1R2/ORA1*$^+$ neurons responded to 4HPAA (42%), and a smaller fraction responded to LCA (17%) (Fig. 5D,E; Table S2).

DISCUSSION

Odorant-induced neural responses of cichlid OSNs can be tested by ISH with *c-fos* riboprobe

We performed ISH with a riboprobe of *c-fos* to test the odorant-induced neural responses of cichlid OSNs. Immediate-early genes such as *c-fos* are useful neural activity markers that have been utilized in teleosts (Hussain et al., 2013; Kress and Wullimann,

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2012; Okuyama et al., 2011; Yabuki et al., 2016). In cichlids, although *c-fos* (Butler et al., 2016; Field and Maruska, 2017) and *egr1* (Burmeister and Fernald, 2005; Burmeister et al., 2005) were both shown to be useful for mapping neural activity in brain, only *c-fos* showed obvious upregulation in the OE. *c-fos*$^+$ neurons were significantly increased 20 min after exposure to food extract and the intensity of *c-fos* signals was stronger after 20 min. Furthermore, a large fraction of *Trpc2*$^+$ neurons and *V2R*$^+$ neurons responded to amino acids, which supports previous studies that demonstrate teleosts can detect amino acids via microvillous neurons and V2Rs (DeMaria et al., 2013; Hansen et al., 2003; Koide et al., 2009; Sato and Sorensen, 2018; Sato and Suzuki, 2001). In contrast, 41% of *c-fos*$^+$ neurons were not *Trpc2*$^+$ (Fig. 3C), and 42% of *c-fos*$^+$ neurons were *Golf2*$^+$ (Fig. S1B) when exposed to amino acids, which suggests that ciliated neurons also respond to amino acids. This is consistent with previous electrophysical research on rainbow

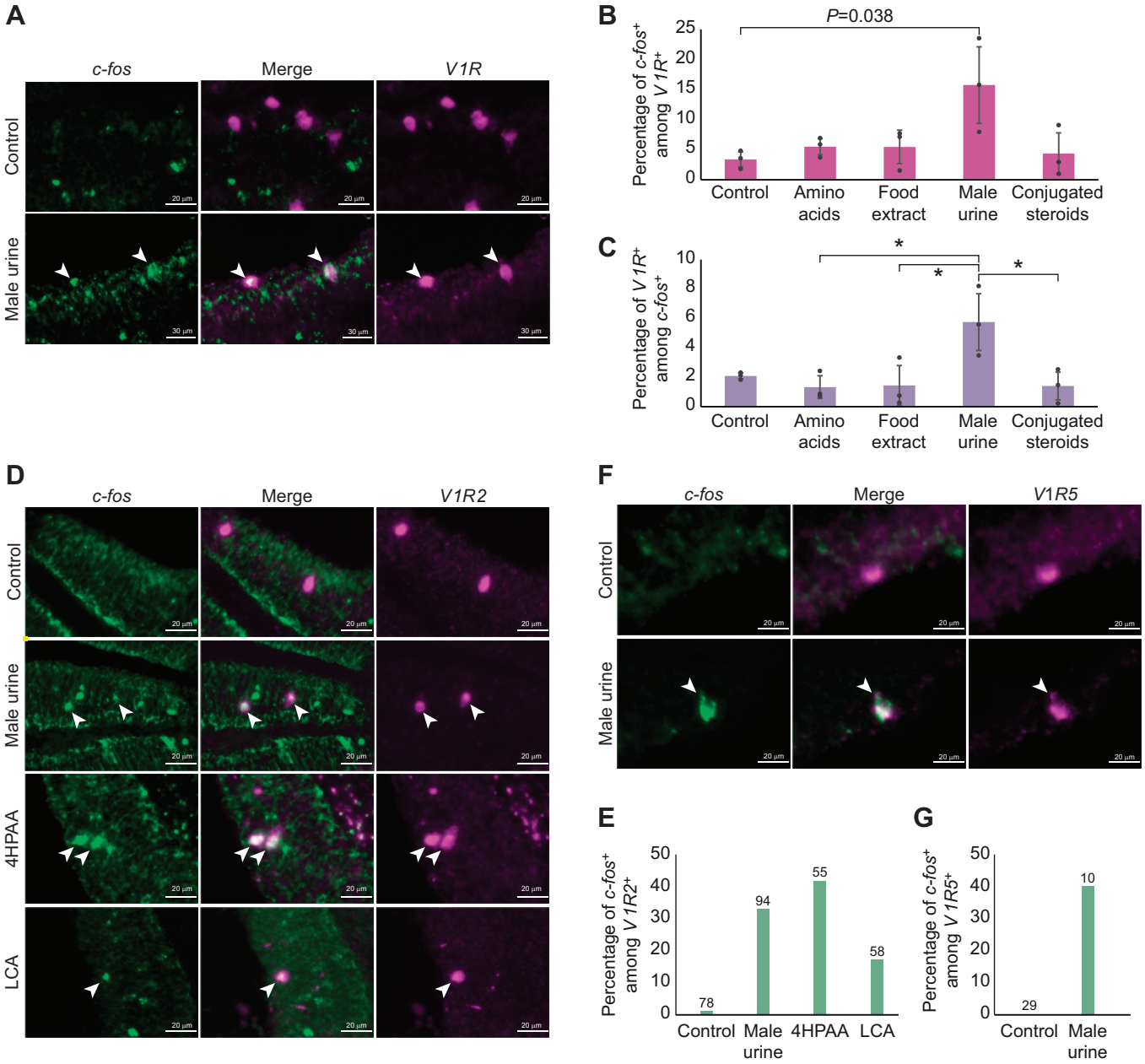

Fig. 5. Specificity of V1R receptor. (A) Two-colour ISH with riboprobes for *c-fos* (green) and *V1R* (magenta) of OE sections exposed to male urine (final concentration: 6000-fold dilution). (B,C) Bar graph (means±s.e.m.) of the percentage of *c-fos*$^+$ neurons among *V1R*$^+$ neurons (B) and the percentage of *V1R*$^+$ neurons among *c-fos*$^+$ neurons (C) for OE sections exposed to water (control), a mixture of 20 proteinogenic amino acids (final concentration: 2 µmol l^{-1} each), food extract (final concentration: 9.5 mg l^{-1}), male urine (final concentration: 6000-fold dilution), or a mixture of three conjugated steroids (final concentration: 33 nmol l^{-1}) (one section, three individuals). (D,E) Two-colour ISH with riboprobes for *c-fos* (green) and *V1R2* (magenta) of OE sections exposed to male urine (final concentration: 6000-fold dilution), 4HPAA (final concentration: 10 µmol l^{-1}) or LCA (final concentration: 10 µmol l^{-1}) (D) and bar graph of the percentage of *c-fos*$^+$ neurons among *V1R2*$^+$ neurons exposed to male urine, 4HPAA and LCA (one individual each) (E). (F,G) Two-colour ISH with riboprobes for *c-fos* (green) and *V1R5* (magenta) of OE sections exposed to male urine (final concentration: 6000-fold dilution) (F) and bar graph of the percentage of *c-fos*$^+$ neurons among *V1R5*$^+$ neurons exposed to male urine (one individual each) (G). (A,D,F) Representative images. Arrowheads represent *c-fos* and *V1R* colocalization. (B,C,E,G) The number of *V1R*$^+$ neurons counted in a single section is indicated above each bar. *P<0.05.

trout, channel catfish and goldfish (Hansen et al., 2003; Sato and Sorensen, 2018; Sato and Suzuki, 2001). These results also suggest that the upregulation of *c-fos* induced by stimulant exposure is properly detected.

Large fractions of *V2R subfamily 14*$^+$ and *subfamily 16*$^+$ neurons responded to amino acids, which supports previous studies (DeMaria et al., 2013; Koide et al., 2009). However, we showed that *V2R subfamily 4*$^+$ and *8*$^+$ neurons only marginally responded to proteinogenic amino acids. This indicates that the majority of V2R receptors in subfamilies 4 and 8 receive other chemical compounds, such as non-proteinogenic amino acids. In other teleosts, the non-proteinogenic amino acid kynurenine is detected by Masu salmon as a sex pheromone (Yambe et al., 2006). Within teleost *V2R* subfamilies, 4 and 16 are independently diversified in several lineages (Nikaido et al., 2013), suggesting that these subfamilies could possibly receive species-specific odours. V2R subfamily 4 only marginally responded to proteinogenic amino acids and at least three genes of V2R subfamily 16 did not respond to proteinogenic amino acids (Fig. 4B; Fig. S4C).

Duplicated *V2R* genes may help cichlids to detect new odorant

We also showed that among the two V2Rs in subfamily 14, only one was receptive to arginine (Fig. 4G,H). Moreover, some V2Rs in subfamily 16 responded to amino acids in group C, whereas the three V2Rs in subfamily 16 that we tested did not (Fig. S4C). The different ligand selectivity in the expanded cichlid-specific V2R subfamily suggests that the specific expansion of V2R led to an expansion of detectable odours. Previous studies also supported this hypothesis from the finding that the residues predicted to be related to ligand selectivity (Alioto and Ngai, 2006; Luu et al., 2004) were much more diverse in V2R subfamilies specifically expanded in cichlids than in those of other teleosts (Nikaido et al., 2013). To further examine this hypothesis, additional combinations of amino acids and V2Rs need to be experimentally investigated.

Detection of urine in cichlid OE

Until now, olfactory responses to urine have been shown by electrophysiological studies in teleosts (e.g. Fatsini et al., 2017; Frade et al., 2002; Keller-Costa et al., 2016; Sato and Suzuki, 2001). In this study, we showed responses of OSNs to urine using *c-fos* expression as neural activity marker. We found that 35% of *c-fos*$^+$ neurons, which were induced by the exposure of male urine, were microvillous neurons (*Trpc2*$^+$), 58% were ciliated neurons (*Golf2*$^+$) and 0% were crypt neurons (*V1R4/ORA4*$^+$). Ciliated neurons contributed the most to the detection of urine, which is consistent with an electrophysiological study showing the response of ciliated neurons to urine in rainbow trout (Sato and Suzuki, 2001). Given that ciliated neurons detect conjugated steroids in goldfish (Sato and Sorensen, 2018), it is possible that the activation of ciliated neurons by male urine in cichlids was actually due to the conjugated steroids contained in urine.

Furthermore, 15.8% of *V1R*$^+$ neurons responded to male urine. As at least *V1R2* colocalizes with *Trpc2* (Fig. S2B), a subset of urine-responding microvillous neurons may contain *V1R*$^+$ neurons. However, as the population of *V1R*$^+$ neurons is much smaller than that of *V2R*$^+$ neurons, and the percentage of *V1R*$^+$ neurons among *c-fos*$^+$ neurons is 5.8% when exposed to male urine (Fig. 5C), *V2R*$^+$ neurons may be also responding to male urine. Although four *V2R subfamily*$^+$ neurons did not respond to male urine (Fig. S4D,E), this does not preclude the possibility that other V2Rs responded to male urine. In fact, rainbow trout and tilapia urine contain amino acid concentrations of the order of millimolar (Kutsyna et al., 2016; Sato and Suzuki, 2001).

Among six V1R receptors, *V1R2/ORA1*$^+$ and *V1R5/ORA5*$^+$ neurons responded to male urine (Fig. 5D–G). Although V1R receptors other than V1R2/ORA1 and V1R5/ORA5 did not respond to urine, they might be responsible for responses to other odour sources such as female urine and faeces. Another possibility is that they are used to find food as 9% of *V1R*$^+$ neurons responded to food extract (Fig. 5B).

V1R2 has a higher number of positive neurons in the OE compared with other V1Rs, suggesting that it is particularly important for urine detection. We demonstrated that *V1R2/ORA1*$^+$ neurons responded to 4HPAA and LCA (Fig. 5D), and it has been shown previously that cultured cells expressing zebrafish V1R2/ORA1 responded to 4HPAA and bile acids (Behrens et al., 2014; Cong et al., 2019). Previous research showed that exposure to 4HPAA induces spawning of zebrafish (Behrens et al., 2014). As the V1R2/ORA1 receptor is well conserved across teleosts (Saraiva and Korsching, 2007), it may also relate to reproduction in cichlids. Notably, two distinct types of *V1R2/ORA1* alleles (Nikaido et al., 2014) occur in East African cichlids, and the individuals used in this study had the ancestral allele. Further investigation of the function of the alternative alleles should help illuminate the potential impact of V1R2/ORA1 receptors on adaptive radiation in cichlid fishes via assortative mating.

Conclusion

In summary, we demonstrated that ISH with a *c-fos* riboprobe is useful for testing the odorant-induced neural responses of cichlid OSNs by showing that: (1) the number of *c-fos*$^+$ neurons increased with stimulant exposure; and (2) microvillous neurons responded to amino acids and food extract, which is consistent with previous research on zebrafish. We also showed that (3) each V2R subfamily has different responsiveness to amino acids; and (4) there is a difference in response to arginine between two copies in the V2R subfamily 14, suggesting that duplication of V2R may have led to the expansion of detectable odorants in cichlids. Furthermore, we (5) established a new method to collect urine non-lethally from cichlids, and (6) showed various OSNs, including *V1R*$^+$ neurons (especially *V1R2* and *V1R5*), responded to male urine. Taken together, the results of our study verify the ligand specificity of OSNs to odorants in cichlids, which we anticipate will continue to be revealed experimentally as fundamentally important to adaptive radiation in this extraordinarily biodiverse group of teleost fishes.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.K., M.N.; Methodology: R.K., M.N.; Validation: R.K.; Formal analysis: R.K.; Investigation: R.K.; Resources: R.K., M.N.; Writing - original draft: R.K.; Writing - review & editing: R.K., M.N.; Visualization: R.K.; Supervision: M.N.; Project administration: M.N.; Funding acquisition: R.K., M.N.

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