Two highly related odorant receptors specifically detect α-bile acid pheromones in sea lamprey (Petromyzon marinus)

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Pheromones play critical roles in habitat identification and reproductive behavior synchronization in the sea lamprey (Petromyzon marinus). The bile acid 3-keto petromyzonol sulfate (3kPZS) is a major component of the sex pheromone mixture from male sea lamprey that induces specific olfactory and behavioral responses in conspecific individuals. Olfactory receptors interact directly with pheromones, which is the first step in their detection, but identifying the cognate receptors of specific pheromones is often challenging. Here, we deorphanized two highly related odorant receptors (ORs), OR320a and OR320b, of P. marinus that respond to 3kPZS. In a heterologous expression system coupled to a cAMP-responsive CRE-luciferase, OR320a and OR320b specifically responded to C24 5α-bile acids, and both receptors were activated by the same set of 3kPZS analogs. OR320a displayed larger responses to all 3kPZS analogs than did OR320b. This difference appeared to be largely determined by a single amino acid residue, Cys-792.56, the C-terminal sixth residue relative to the most conserved residue in the second transmembrane domain (2.56) of OR320a. This region of TM2 residues 2.56–2.60 apparently is critical for the detection of steroid compounds by odorant receptors in lamprey, zebrafish, and humans. Finally, we identified OR320 orthologs in Japanese lamprey (Lethenteron camtschaticum), suggesting that the OR320 family may be widely present in lamprey species and that OR320 may be under purifying selection. Our results provide a system to examine the origin of olfactory steroid detection in vertebrates and to define a highly conserved molecular mechanism for steroid-ligand detection by G protein–coupled receptors.

Bile acids are amphipathic metabolites that are synthesized from cholesterol and function primarily as a surfactant to emulsify dietary fats in the intestine (1). These metabolites also serve as signals in a wide variety of physiological processes, including cholesterol homeostasis and endocrine regulation in vertebrates (2–5). These functions of bile acids are mediated by six known receptors, including both G protein–coupled receptors (GPCRs) (6) and nuclear receptors such as the farnesoid X receptor (7). In fish species, bile acids, once excreted into the water via the urine (8) or feces (9) or from the gills (10), are known to be potent olfactory stimulants and pheromones. However, little is known about the receptors in the olfactory epithelium that respond to these bile acids.

Olfactory detection of bile acids in fishes is highly sensitive and specific (8, 11–13). This allows fish to discriminate diverse fish bile acids, including 5α and 5β forms, bile alcohol sulfates, and conjugated bile acids (1). Olfactory detection of bile acids has been observed throughout fish taxa, including jawless (14) and jawed fishes (15). A progression has been documented from the internal secretion and detection of the 5α-bile acids of basal vertebrates such as lamprey to the 5β-bile acids of ray-finned fish (16). Olfactory detection of waterborne bile acids also shows an evolutionary trend in that basal vertebrates release and detect 5α-bile acids and teleost fish release and detect 5β-bile acids (13). Recently, zebrafish olfactory class A receptors (ORA) that are orthologous to the type 1 mammalian vomeronasal receptors were reported to detect 5β-bile acids (17). This finding is consistent with a previous hypothesis that odorant receptors (ORs) and type 1 vomeronasal receptors detect steroids (18–20).

Sea lamprey (Petromyzon marinus), a jawless vertebrate animal, provides an advantageous model for matching bile acid pheromones to their olfactory receptors. Although bile acids are known to be highly potent olfactory stimuli in several fish species, only sea lamprey bile acids have been shown unequivocally to be bona fide pheromones (10). The latter are rigorously defined to be stimuli secreted by an individual and received by a second conspecific individual inducing innate and specific reactions (21). Many 5α-bile acids and their derivatives, such as petromyzonol sulfate (PZS), petromyzones (PZ), petromyzonamine disulfate, petromyzosterol disulfate, and allocholic acid (ACA) (14, 22–25), as well as sex pheromones, such as 3-ketopetromyzonol sulfate (3kPZS) and 3-ketoalcoholic acid stimulate the olfactory epithelia of sea lamprey (26–28). Critically, 3kPZS is a major contributor to the male sea lamprey sex pheromone mixture that induces specific mating-related behavioral...
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responses in adult female sea lamprey. It is the first-ever vertebrate pheromone biopesticide registered by the United States Environmental Protection Agency. Another advantage of the sea lamprey model is that its genome encodes one of the smallest known odorant receptor repertoires among all vertebrate genomes sequenced (29), providing a less daunting process to match a pheromone to its cognate receptor.

Here we report the identification of two highly related sea lamprey odorant receptors that respond to 3kPZS and numerous 3kPZS analogs that control sea lamprey migration and nest attraction behavior. We further identified a single amino acid residue in the second transmembrane domain (TM2) that is critical to the regulation of the strength of signaling. Our results provide a system to examine the origin as well as molecular mechanisms of olfactory detection and potential novel modulators of steroid pheromone signaling in vertebrates.

Results

Sea lamprey odorant receptor repertoire contains 40 odorant receptor genes

We used the previously reported 27 OR gene sequences identified in the *P. marinus* primary genome assembly version 2.0 as query sequences (29). Using these sequences, we remapped sequences encoding ORs from the improved genome assembly version 7.0 (30) using BLASTN searches. An additional 13 intact single-exon OR genes were further identified, which increased the number of OR genes encoded in the lamprey genome to 40. Neighbor-joining, maximum parsimony, and maximum likelihood (ML) analyses of the predicted amino acid sequences indicated that these 40 genes clustered into one well-supported ingroup that separated well from the outgroup (Fig. S1). Consistent with previous phylogenetic analyses (31), sea lamprey OR genes separated into two distinct clades, type 1 and type 2 ORs. In particular, the four ORs that formed the type 2 clade in our study were identical to the four ORs placed in the type 2 clade in the previous study (31). These four ORs belonged to the η group of type 2 ORs in vertebrates (31). In addition, sea lamprey type 1 ORs segregated into two major classes, class I and class II, with characteristics similar to those of mammalian class I and class II ORs, respectively.

Sea lamprey ORs were targeted to the HEK293T plasma membrane

We were successful in cloning 32 of 40 ORs from sea lamprey genomic DNA. The clones were inserted into the pCMV-N-Rho eukaryotic expression vector (Fig. S2), in which the first 21 amino acid residues of bovine rhodopsin were introduced at the N terminus. The Rho tag was reported to increase the efficiency of OR cell plasma membrane targeting (32).

We selected HEK293T as our heterologous expression system, as it has been widely used for functional expression of ORs from mammalian systems (33). We co-transfected an accessory protein construct (mRTP1s) and EGF with sea lamprey ORs into HEK293T cells. At 48 h post-transfection, the cell-surface expression of ORs was visualized using an antibody against the N-terminal Rho tag (Fig. 1A). Receptors located on the cell surface showed the ring expected for plasma membrane fluorescence and intracellular staining appearing in puncta near the nucleus. Ultimately, 22 of 32 ORs showed a detectable expression on the plasma membrane (Fig. 1B). A subset of both type 1 and type 2 ORs were robustly expressed and localized on the plasma membrane.

Sea lamprey OR320a and OR320b are potently activated by 3kPZS and PZS

We used the cAMP-responsive CRE-luciferase reporter OR activation system (33) to measure responses of lamprey ORs to 3kPZS and PZS. The 22 surface-expressed Rho-tagged ORs were transfected into HEK293T cells along with mRTP1s, Goαal, and the reporter pGL4.29. The 22 OR-transfected cell preparations were then exposed to two concentrations of 3kPZS (10 and 100 μM). Only OR320a and OR320b showed responses in the luciferase assay (Table S1). Both OR320a and OR320b are type 2 ORs. These two receptors were further examined for their responses to PZS, an analog of 3kPZS and potent odorant in sea lamprey (14). PZS also activated both OR320a and OR320b, at a potency similar to that of 3kPZS (Table S1).

Because the CRE-luciferase reporter can be activated by kinases other than protein kinase A, we excluded the possibility that another signaling pathway was involved by directly measuring cAMP production using a time-resolved FRET cAMP assay. Rho-tagged OR320a and OR320b were co-transfected along with mRTP1s and Goαal into HEK293T cells, which were then stimulated with different concentrations of 3kPZS and PZS. Both OR320a and OR320b induced cAMP production upon exposure to 3kPZS (Fig. 2A) and PZS (Fig. 2B) in a concentration-dependent manner. OR320a responded strongly to both 3kPZS (EC₅₀ = 0.86 μM (CI 0.13–5.8 μM); n = 3) and PZS (EC₅₀ = 0.62 μM (CI 0.2–1.9 μM); n = 3) (values are mean (95% CI)). Compared with OR320a, OR320b responded with a markedly lower potency and modestly lower efficacy to both 3kPZS (EC₅₀ > 30 μM; n = 3) and PZS (EC₅₀ = 8.9 μM (CI 2.8–28 μM); n = 3). These data confirmed that OR320a and OR320b are activated by 3kPZS and PZS and regulate the cAMP signaling pathway. The expression patterns of OR320a and OR320b genes were confirmed by absolute real-time PCR analysis. We found that *or320a* and *or320b* were expressed primarily in olfactory epithelium and expressed at low level in brain in adult sea lamprey (Fig. S3); they were not observed in intestine, gills, and kidney.

The response spectra of OR320a and OR320b are largely limited to C₂₄ 5α-bile acids

A library of natural and potential sea lamprey odorants composed of trace amines, amino acids, and steroids, including α- and β-bile acids (10, 14), was screened against OR320a and OR320b using the CRE-luciferase assay to determine the activation spectra of OR320a and OR320b. OR320a and OR320b were not activated by trace amines or amino acids. Among the 44 bile acids tested, OR320a was activated by 3kPZS and nine analogs at 10 μM and by 10 analogs at 100 μM (Table S2). OR320b was activated by 3kPZS and four analogs at 10 μM and by 10 analogs at 100 μM (Table S2). Although spermidine at 100 μM elicited a 10-fold increase in luciferase activity over the
control (Table S2), the increase represented a false positive based on subsequent experiments that examined the concentration-response relationships. The sea lamprey bile acid, ACA, weakly activated OR320a and OR320b. Because the concentration-response curve for ACA on OR320a was not strong enough to determine efficacy, competition between ACA and 3kPZS for OR320a was measured. As expected, ACA inhibited the response of 10 μM 3kPZS with an IC50 of 0.26 μM (Fig. S4).

The signal did not go all the way to baseline, suggesting that ACA is an allosteric inhibitor.

All active ligands for OR320a and OR320b appear to be 3kPZS analogs, with a C24 5α skeleton and a sulfate conjugation at C-24 (Table S2). It was notable that bile acids with a C24 5β skeleton, including TLC, GLC, and LCS, did not activate OR320a and OR320b. The C24 5β skeleton is characterized by an A ring of the steroid nucleus that bends away, forming an L-shaped molecule, which differs from the C24 5α skeleton that forms a somewhat flat molecule. Also, compounds without a C24 skeleton, such as testosterone sulfate, testosterone, and androstenedione, did not activate OR320a and OR320b. The activation spectra of OR320a and OR320b were thus limited to the C24 5α-bile acid profile.

C-12 hydroxyl and C-24 sulfate on C24 5α-bile acids are critical to activating OR320a and OR320b

We measured concentration-response relationships for all eleven 3kPZS analogs using CRE-luciferase assays to determine

![Figure 1. Sea lamprey ORs were expressed heterologously in HEK293T cell line. A, confocal images of HEK293T cells expressing OR320a and OR320b and detected with immunocytochemistry. Cells were transfected with Rho-tagged receptor constructs, pC1-mRTP1s, and pEGFP-N1 (green). Cells were permeated or nonpermeated to evaluate receptor cytoplasm (right) or membrane target (left), respectively, and underwent immunostaining with Rho antibody (red). Blue, 4′,6-diamidino-2-phenylindole. Mock, empty pCMV-N-Rho vector. Scale bar, 50 μm. B, evaluation of sea lamprey OR membrane expression levels in HEK293T cells by immunocytochemistry without cell permeation. % of total cells, the percentage of receptor membrane–expressing cells over total cells. Receptors expressed on at least 1% of the cells were considered to show surface expression. Empty vector (PR) alone and EGFP vector alone serve as negative controls. Data are presented as mean ± S.D. (error bars) of seven experiments.

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Cys-79 in OR320a is critical for its enhanced response compared with OR320b

We next sought to determine the transmembrane domain(s) and amino acid residue(s) that play critical roles in the differential response of OR320a and OR320b to 3kPZS and its analogs. OR320a and OR320b share 92.8% amino acid identity, with only 22 amino acid residues that differ (Fig. 4A). Because OR320a and OR320b have virtually identical specificity but different sensitivity to the same set of analogs, this receptor pair offers an opportunity to decipher the molecular mechanism for receptor sensitivity at the olfactory epithelium. We further reasoned that, because three transmembrane domains, TM3, TM4, and TM6, were identical between OR320a and OR320b, these three regions could not explain the different sensitivity between the two receptors. To narrow down our search, we separated each receptor sequence into three regions. The first region contained the N terminus, TM1–3, and ICL2. The second region included TM4, TM5, and ICL3. The remaining sequence, TM6, TM7, and the C terminus, was assigned as the third region. We replaced each of the three regions of OR320a with the corresponding region of OR320b while maintaining the remaining sequence intact. Conversely, we replaced each of the three regions in OR320b with those of OR320a.

We first assessed the surface expression level of the six chimeras. All constructs showed expression in a similar fraction of cells (Fig. S5; one-way ANOVA, F = 0.5, p = 0.7). Subsequently, we compared responses of all receptor chimeras to 3-keto-1-ene PZS and DKPES using CRE-luciferase assays (Fig. 4, B and C). These two ligands were chosen because their EC\(_{50}\) values differed the most for both OR320a and OR320b. The receptor chimeras in which the first region of OR320a (TM1–3; e.g. ABB) was substituted into the intact OR320b (e.g. BBB) showed lower EC\(_{50}\) and higher E\(_{\text{max}}\) values. We therefore decided to focus on this region thereafter.

There are nine amino acid residues that differ between OR320a and OR320b in the first region. We sequentially replaced each of the nine residues and measured the response of each mutant to 3-keto-1-ene PZS and DKPES. There is no significant difference among the mutated receptors’ membrane expression (Fig. S6; Kruskal–Wallis test, p = 0.17).

Among the nine residues on OR320a, only one mutation (OR320a-C79Y) significantly affected the receptor EC\(_{50}\) to 3-keto-1-ene PZS based on the Kruskal–Wallis ANOVA test (Fig. 5A; EC\(_{50}\), p < 0.05, Dunn’s multiple-comparison test, p < 0.05; E\(_{\text{max}}\), p > 0.05). The OR320a-C79Y responses to 3-keto-1-ene PZS were comparable with that of WT-OR320b (Mann–Whitney test; EC\(_{50}\), p = 0.85; E\(_{\text{max}}\), p > 0.9) but were weaker than those induced by the other eight mutants of OR320a and WT-OR320a. OR320a-C79Y showed the lowest E\(_{\text{max}}\) to DKPES, even weaker than WT-OR320b (Fig. 5B; EC\(_{50}\), unpaired t test, t (3) = 0.84, p = 0.46; E\(_{\text{max}}\), unpaired t test, t (3) = 8.4, p < 0.01).

Likewise, among the nine residues on OR320b, only one mutation (OR320b-Y79C) significantly affected the receptor EC\(_{50}\) to 3-keto-1-ene PZS (Fig. 5C; Kruskal–Wallis ANOVA test, EC\(_{50}\), p < 0.05, Dunn’s multiple-comparison test, p < 0.05; E\(_{\text{max}}\), p > 0.05). The responses of OR320b-Y79C and WT-OR320a to 3-keto-1-ene PZS were no different (unpaired t test,
EC$_{50}$, $t(6) = 1.29, p = 0.25; E_{\text{max}}$, $t(6) = 0.57, p = 0.59$). OR320b-Y79C showed the highest $E_{\text{max}}$ to DKPES, comparable with that of WT-OR320a (Fig. 5D, unpaired $t$ test, EC$_{50}$, $t(4) = 0.59, p = 0.6$; $E_{\text{max}}$, $t(4) = 0.50, p = 0.64$). In addition, OR320b-Y79C induced a stronger response to 3-keto-1-ene PZS and DKPES than the other eight mutants of OR320b and WT-OR320b did. Because the amino acid residue at position 79 in both receptors affects both EC$_{50}$ and $E_{\text{max}}$ of the receptors, this residue likely is essential to the responses associated with the functional differences in OR320a and OR320b and affects their signaling efficiency.

To determine whether the effect of residue 79 on ligand responsiveness is a general property or limited to 3-keto-1-ene PZS and DKPES, we also tested the responses of OR320a-C79Y and OR320b-Y79C to three other ligands. Interestingly, when the residue was switched between OR320a and OR320b, a dramatic reversal was observed in EC$_{50}$ and $E_{\text{max}}$ values in response to 3kPZS, PZS, and 3-keto-4-ene PZS (Fig. 6). OR320b-Y79C showed a 5-fold lower EC$_{50}$ and a 2-fold higher $E_{\text{max}}$ than OR320a-C79Y. Conversely, WT-OR320b had a 5-fold higher EC$_{50}$ and a 2-fold lower $E_{\text{max}}$ than WT-OR320a. Because the switch in amino acid residue 79 induced a reversal in the $E_{\text{max}}$ for multiple ligands that vary in response magnitudes, we propose that this residue is critical for the efficacy of receptor activation rather than to ligand-binding specificity.

**OR320a and OR320b variants with a nonpolar hydrophobic side chain at position 79 exhibit greater response**

The above results indicate that position 79 is critical for the efficacy of ligand response. In other words, Cys-79 in OR320a was likely involved in a receptor activity network. To test our speculation and discover the mechanism behind it, we further performed amino acid residue substitutions at position 79 in both OR320a and OR320b. The substituted amino acid residues were from different physicochemical classes, including nonpolar hydrophobic, polar neutral, and polar hydrophilic classes (Fig. 7). We normalized the activity levels to the percentage of cells expressing receptor to eliminate any effect of receptor expression on the activity of the mutations (Fig. S7; one-way ANOVA, $F = 3.6, p < 0.0005$, Tukey’s multiple-comparisons test, $p < 0.05$). For OR320a and OR320b, the data sets that provided curves that gave interpretable fits are shown in Fig. 7 (see “Experimental procedures” for criteria for undefined curves).

For OR320a, substitutions of cysteine 79 with alanine, leucine, methionine, or phenylalanine resulted in $E_{\text{max}}$ and EC$_{50}$ values for the receptor response to 3-keto-1-ene PZS (Fig. 7A) and DKPES (Fig. 7C) that are similar to those of WT-OR320b (see Table S3 for statistical analysis with one-way ANOVA and Dunnett’s post-test). In contrast, substitutions with tyrosine and asparagine resulted in significant reductions in $E_{\text{max}}$ or increased EC$_{50}$ values (Table S3) that represent reduced function for receptor response to 3-keto-1-ene PZS (Fig. 7B) and DKPES (Fig. 7D). The arginine mutant responses were so small to both ligands that undefined curves resulted.

For OR320b and 3-keto-1-ene PZS, the responses were significantly smaller with the OR320a receptor, making statistical analysis more challenging. There were no significant differences in $E_{\text{max}}$ between the WT-OR320b (Tyr at position 79) and any of the mutants (leucine, methionine, asparagine, asparagine, arginine, and lysine atmides).
**Table 1**

Structure-activity relationship at carbon positions 3, 12, and 24 of 3kPZS analogs

| Name                  | C-3 | C-7 | C-12 | C-24 | ene | A,B | OR320a | OR320b |
|-----------------------|-----|-----|------|------|-----|-----|--------|--------|
| PZS                   | -   | -   | -    | -    | OSO3| -   | 0.26 (0.2, 0.4) | 1.1 (0.9, 1.5) |
| PZ                    | -   | -   | -    | -    | -   | -   | 1.8 (0.8, 4.2) | undefined |
| 3kPZS                 | =O  | -   | -    | -    | OSO3| -   | 1.1 (0.9, 1.5) | 0.92 (0.1, 0.9) |
| 3kPZ                  | =O  | -   | -    | -    | -   | -   | 5.4 (2.3, 13)  | undefined |
| 3-keto-1-ene PZS      | 1   |     |      |      |     |     | 0.83 (0.6, 1.2) | 1.2 (1.0, 1.4) |
| 3-keto-4-ene PZS      | 4   |     |      |      |     |     | 1.0 (0.8, 1.4) | 1.1 (0.9, 1.5) |
| 3kPZS                 | =O  | -   | -    | -    | OSO3| -   | 1.1 (0.9, 1.5) | 0.92 (0.1, 0.9) |
| 3,12-diketo-4-ene PZS | 4   |     |      |      |     |     | 11 (7.9, 16)   | 0.48 (0.3, 0.6) |
| 3,12-diketo-1,4-diene PZS | 1, 4 |     |      |      |     |     | 13 (9.1, 20)   | 0.44 (0.3, 0.6) |
| DKPES                 | =O  | -   | -    | -    | OSO3| -   | 5.6 (4.2, 7.5) | 0.64 (0.4, 0.8) |
| PZS                   | -   | -   | -    | -    | OSO3| -   | 0.26 (0.2, 0.4) | 1.1 (0.9, 1.5) |
| 3kPZS                 | =O  | -   | -    | -    | OSO3| -   | 1.1 (0.9, 1.5) | 0.92 (0.1, 0.9) |
| PZ                    | =O  | -   | -    | -    | -   | -   | 1.8 (0.8, 4.2) | undefined |
| 3kPZ                  | =O  | -   | -    | -    | -   | -   | 5.4 (2.3, 13)  | undefined |
| Androstenedione       | =O  | -   | -    | -    | C20 =O|     | -      |        |
| Testosterone sulfate  | =O  | -   | -    | C20 =O| -    |     | -      |        |
| GCA                   | -   | -   | -    | -    | OHOH|     | -      |        |
| TCL                   | -   | -   | -    | -    | -   | -   | -      |        |

or cysteine; Fig. 7 (E and F) and Table S3). As for OR320a, the response to the Arg mutant was completely abolished preventing statistical analysis due to an S.D. of 0. There were significant reductions in EC_{50} for the Cys and Asn mutants (5 and 4.6 μM compared with 24 μM for the WT Tyr residue), indicating enhanced receptor sensitivity. This is consistent with the opposite effect of the Cys-Tyr mutant in OR320a to reduce receptor signaling. For DKPES signaling through OR320b, responses were smaller still, and no significant differences were observed (Fig. 7 (G and H) and Table S3), though the arginine mutant had no signal detected.

In summary, for 3-keto-1-ene PZS and DKPES, when position 79 was substituted with a nonpolar hydrophobic side chain, both OR320a and OR320b showed the most robust response. When position 79 was replaced with a charged side chain (Arg), both OR320a and OR320b showed the worst response. Nonpolar hydrophobic side chains tend to face the inside of protein cores and are frequently engaged in Van der Waals interactions in α-helices; however, charged polar hydrophilic side chains tend to face toward the outside of protein. Position 79 faces inside the protein and appears to be involved in the receptor activation network rather than belonging to the ligand-binding pocket.

**Cys-79 in OR320a and Tyr-79 in OR320b are conserved in another lamprey species**

Because both OR320a and OR320b responded to the same set of bile acids that are released and specifically detected by sea lamprey, we questioned whether homologs of OR320a and OR320b exist in other lamprey species. We identified five OR320 homologs in the Japanese lamprey (Lethenteron japonicum) genome (34) (Fig. 8). JLOR320a and JLOR320b are orthologs of OR320a and OR320b, respectively, whereas JLOR320-like-1, JLOR320-like-2, and JLOR320-like-3 occurred independently in Japanese lamprey. Amino acid sequence identities among the five JL genes and two sea lamprey (SL) genes were >70%. Interestingly, the amino acid sequences of OR320a in JL and SL were 100% identical. The amino acid residues of OR320b in the two lamprey species were 98.1% identical. Also, Cys-79 is conserved among SLOR320a, JLOR320a, and JL320-like-3, whereas Tyr-79 is conserved in SLOR320b, JLOR320a,
and JL320-like-2. JLOR320-like-1 has a serine at position 79. These levels of sequence conservation may indicate that detection of sulfated bile acids is highly conserved in these two lamprey species, even though the OR gene family has been shown to evolve rapidly (35). The genomic locations of sea lamprey or320a and or320b indicate that they were not derived from a local duplication event. Our phylogenetic analysis suggests that sea lamprey and Japanese lamprey OR320 diverged; 3.8 million years ago (MYA), which is later than the divergence of these two species (30–10 MYA) according to a previous study (36). Furthermore, a selection test based on the ratio of the nonsynonymous/synonymous substitution rate (\( r = d_s/d_{ns} \)) showed that jlor320a, jlor320b, slor320a, and slor320b have been under purifying selection, with \( r = 0.72 \). Therefore, we postulate that the OR320 subfamily potentially represents an advantageous model for studying how evolutionary changes in receptor sequences drive changes in odorant response profiles and pheromone functions.

**Discussion**

In the present study, we matched two sea lamprey odorant receptors, OR320a and OR320b, to their cognate ligands, \( C_{24} \) 5α-bile acids, a class of compounds known to function as sex pheromones. \( C_{24} \) 5α-bile acids are the most ancestral biological bile acids and are produced only by jawless fish such as sea lamprey (1, 4). These two receptors responded strongly to all sulfated \( C_{24} \) 5α-bile acids but did not respond to \( C_{24} \) 5β-bile acids that are found in jawed vertebrates. Another remarkable observation is that amino acid residue 792.56 is critical in activation of these two ancestral receptors. The residues in this region of TM2 (2.56–2.60) also appear to be critical for the detection of steroid compounds by odorant receptors in zebrafish (17) and humans (37). Our results provide a system to examine the origin as well as molecular mechanisms and potential novel modulators of olfactory detection of steroids in vertebrates.

Our analyses of amino acid residue 792.56 in OR320 receptors, when combined with previous studies on other receptors for steroidal odorants, suggest a unique molecular mechanism for olfactory recognition of steroids. TM2 has been implicated in allosteric or conformational changes in GPCRs during the binding of agonist ligands (38–41). Cys-792.56 located in TM2 (Fig. S8) is critical to the different activity levels of OR320a and OR320b. Similarly, in human OR7D4, a natural polymorphism (OR7D4-P79L, Pro2.59 (42)) shows a limited response to androsterone (37), a steroidal pig sex pheromone (43). Moreover, in the zebrafish receptors for lithocholic acid, ORA1–ORA6,
there are two consensus-binding pocket residues on TM2. Mutations R612.57A and P622.60Y in ORA2 and ORA5, respectively, diminished the responses of the receptors to lithocholic acid. Typically, in class A GPCRs, the region from 2.57 to 2.60 of TM2 has an $\alpha$-helix–distorting motif, GG$X$TT (2.56–2.60) (44), T$X$P (2.57–2.59) (45), or Pro2.58–2.60 (46, 47). The $\alpha$-helix–distorting motifs make the shape of TM2 at the extracellular part bend toward TM1 and lean away from TM3. This provides a remarkable ability for conformational change in the ligand-binding pocket (45) related to receptor activation (48). For receptors that lack $\alpha$-helix–distorting motifs, like cannabinoid receptor 2, Ser$^{2.55}$ introduces distortion from normal helicity in TM2 (49). Human OR7D4 has Pro$^{2.60}$, although lacking $\alpha$-helix–distorting motifs, sea lamprey OR320a and OR320b have Ser$^{2.57}$ and zebrafish ORA2 has Ser$^{2.55}$, which serve as a site to introduce distortion to magnitude of receptor conformational change ability. Hence, the region from 2.56 to 2.60 in TM2 of examined steroid receptors (sea lamprey OR320a Cys2.56, zebrafish ORA2 Arg2.57, and ORA5 Pro$^{2.60}$, and human OR7D4 Pro$^{2.59}$) represents a major

Figure 5. Effect of single site switch between OR320a and OR320b. Concentration-dependent responses of the OR320a (A and B) and OR320b (C and D) mutants induced by 3-keto-1-ene PZS and DKPES were measured using the CRE-luciferase assay. Receptor construct DNA concentration was 1 ng/μl. Normalized luciferase value, ratio of OR activation induced by the indicated concentration of the ligand divided by that of the WT OR320a induced by 30 μM PZS. Data are presented as mean ± S.D. (error bars) from three experiments. Statistical comparisons of $EC_{50}$ and $E_{\text{max}}$ values for ligands at each mutant receptor were done by Kruskal–Wallis ANOVA with Dunn's post-test. Detailed $E_{\text{max}}$ and $EC_{50}$ information is provided in Table S8.

Figure 6. Altering OR320a and OR320b response to the ligands by switching the 79th amino acid residue. Sequences of OR320a and OR320b were swapped by exchanging residue Cys-79 of OR320a (OR320a-C79Y) and Tyr-79 of OR320b (OR320b-Y79C). Receptor responses to the indicated ligands were measured using the CRE-luciferase assay. Receptor construct DNA concentration was 1 ng/μl. Normalized luciferase value, ratio of OR activation induced by the indicated concentration of the ligand divided by that of WT OR320a induced by 30 μM PZS. Data are presented as mean ± S.D. (error bars) from three experiments. The mean $EC_{50}$, 95% confidence interval of $EC_{50}$ in μM, and number of experiments are indicated in parentheses. Detailed $E_{\text{max}}$ information is provided in Table S8.
functional hot spot in receptor activation mechanisms and sug-
uggests a conserved interaction pattern of steroidal odorants with
their olfactory receptors throughout vertebrate species.

The specific detection of C_{24} 5α-bile acids by OR320a and
OR320b, in contrast to the specific detection of 5β-bile acids by
zebrafish ORA receptors (17), suggests that jawless and jawed
fish each evolved olfactory receptors for their own bile acids.

Sea lamprey is an extant representative of the agnathan super-
class of jawless fishes, which are known to produce and release
C_{24} 5α-bile acids (1), some of which function as pheromones
(27). Our data indicate that OR320a and OR320b specifically
detect 5α-bile acids, but not 5β-bile acids. In contrast, teleost

Figure 7. Effect of amino acid residue substitution on position 79. CRE-luciferase assay was used to evaluate the activation of OR320a and OR320b mutants by 3-keto-1-ene PZS and DKPES. Receptor construct DNA concentration was 1 ng/μl. Normalized luciferase value, ratio of OR activation induced by the indicated concentration of the ligand divided by the response of WT OR320a induced by 30 μM 3-keto-1-ene PZS (A, B, E, and F) or 30 μM DKPES (C, D, G, and H). Data are presented as the mean ± S.D. (error bars) from three experiments. Statistical comparisons of EC_{50} and E_{max} values for 3-keto-1-ene PZS and DKPES at each mutant receptor were done by one-way ANOVA with Dunnett’s post-test. Detailed E_{max} and EC_{50} information is provided in Table S8.

Figure 8. Molecular phylogenetic analysis indicates that OR320a and OR320b are conserved in another lamprey species. An evolution time tree of type 2 ORs of sea lamprey and Japanese lamprey was constructed using the RelTime method. Divergence times (MYA) for all branching points were calculated using maximum likelihood (bootstrap, 1000) based on the general time-reversible model. Gray bars around each node represent 95% confidence intervals. The mouse ORs were selected as an outgroup. Evolutionary analyses were conducted in MEGA6. SLOR320a and SLOR320b are highlighted with red dots. The divergence time of OR320a and OR320b is ~3.8 MYA.
fishes release and detect mostly 5β-bile salts. In zebrafish, six ORAs, which are orthologous to the mammalian vomeronasal receptors type 1, detect C24 5β-bile salts or acids, but they do not detect any of the four 5α-bile acids examined (17). This striking difference in the specificity of lamprey and zebrafish bile acid receptors in discriminating particular configurations at the C5 position epitomizes a co-evolution of olfactory receptors and the ligands. This co-evolution is likely critical to the species survival, as OR320s have evidently been under purifying selection. The conserved Cys-79 in OR320a and the Tyr-79 in OR320b presumably support their conserved function in detecting C24 5α-bile acids. Notably, both OR320a and OR320b belong to the η group of vertebrate olfactory receptors, the most ancestral ORs of the type II OR family (50), which may have given rise to all ORs that detect water-soluble odorants. Similar profiles in the production and release of C24 5α-bile acid pheromone components have been observed in several Petromyzontidae species, including sea lamprey (genus Petromyzon), sliver lamprey (genus Ichthyomyzon), and pacific lamprey (genus Entosphenus) (13), which allows an opportunity to examine how evolutionary changes in receptor sequences drive changes in odorant response profiles and pheromone functions.

The observed repertoire of OR320a and OR320b responses to sea lamprey C24 5α-bile acids further supports the pheromone function of bile acids and these receptors in sea lamprey. Heterologous expression of receptors in lamprey olfactory epithelium is very challenging, and the presence of endogenous receptors would greatly complicate experiments to show function in that system. However, the HEK cell expression system with Gαolf is widely used for studying mammalian olfactory receptors (33) and is likely to appropriately reflect the function of these receptors in the native tissue. In addition to 3kPZS, DKPES is known to function as a sea lamprey sex pheromone that attracts ovulatory females (51). In our in vitro assays, DKPES activated OR320a and OR320b with roughly half the efficacy of 3kPZS. Likewise, in a previous in vivo assay (electroolfactogram (EOG)), DKPES evoked a response magnitude that is ~50% of the magnitude induced by 3kPZS. Moreover, 3-keto-1-ene PZS, which is slightly more efficacious than 3kPZS in inducing cAMP responses by OR302a and OR320b, is another pheromone that attracts ovulating females (51). In our in vitro assays, DKPES activated OR320a and OR320b with roughly half the efficacy of 3kPZS. Likewise, in a previous in vivo assay (electroolfactogram (EOG)), DKPES evoked a response magnitude that is ~50% of the magnitude induced by 3kPZS. Moreover, 3-keto-1-ene PZS, which is slightly more efficacious than 3kPZS in inducing cAMP responses by OR302a and OR320b, is another pheromone that attracts ovulating females at concentrations as low as 10⁻¹² M, which is comparable with the effective concentrations of 3kPZS (52). As 3kPZS elicits female sea lamprey behavioral response at concentrations as low as 10⁻¹⁴ M (26) and elicits an olfactory epithelium EOG response at a threshold as low as 10⁻¹² M (53), we believe that in native olfactory sensory neuron, 3kPZS could activate olfactory neurons that express O320a or OR320b at much lower concentrations than in a heterologous system. Another potent ligand for OR320a and OR320b, PZS, is an odorant equally potent as 3kPZS. Likewise, in a previous

In conclusion, we have identified a pair of paralogous OR genes that are specifically tuned to C24 5α-bile acids known to function as sex pheromones in the sea lamprey. The difference in the efficacy of these two receptors is largely attributable to the difference in their amino acid residue 79, located in TM2 (2.56), a small region critical to detection of steroid compounds by odorant receptors in vertebrates. This remarkable conservation in the function of a particular position provides an advantageous model to examine how evolution has driven a convergence on olfactory detection of steroids throughout vertebrate animals.

Experimental procedures

Odorants

The odorants used in this study were mainly purchased from Sigma–Aldrich, Santa Cruz Biotechnology, Inc., and Cayman Chemical unless otherwise specified. Lamprey-specific compounds were custom-synthesized by Bridge Organics (Vicksburg, MI, USA) and confirmed by NMR and LC–MS analysis. IUPAC names for abbreviations are listed in Table S4. Purity of all of the compounds was 97% or higher. The odorants were first dissolved in DMSO (Sigma) at a concentration of 20 mM and then diluted to working concentrations in Dulbecco’s modified Eagle’s medium (DMEM, HyClone). All odorant solutions were stored at −20°C.

Phylogenetic analysis

Identification and phylogenetic analysis of sea lamprey ORs—We used the same method as reported previously (29) to identify 13 new putative sea lamprey OR genes. We used the previously reported 27 ORs identified from the P. marinus primary genome assembly version 2.0 as queries (29) to mine sequences encoding ORs from the improved quality genome assembly version 7.0 (30) using BLASTN searches. Predicted amino acid sequences of the 40 identified ORs were aligned using the multiple-sequence alignment program ClustalX version 2.0 (55). Then a phylogenetic tree was constructed using MEGA 6.0 (56) with the sea lamprey TAARs as well as mouse Taar1 (NP_444435.1) and zebrafish Taar1 (NP_001076373.1) as the outgroup. Three algorithms (neighbor-joining, maximum likelihood, and maximum parsimony) were run at 1000 bootstrap replications. The Jones–Taylor–Thornton model was used for the neighbor-joining and maximum likelihood methods, and the default parameters were used for the maximum parsimony algorithm.

Phylogenetic analysis of lamprey OR320—Japanese lamprey type 2 OR amino acid sequences were identified by BLASTP method in the Japanese lamprey genome (34) using OR320a and OR320b as the search queries. A multiple alignment was generated for sea lamprey and Japanese lamprey type 2 OR amino acid sequences using ClustalX version 2.0. Mouse Olfr121 and mouse Olfr446 were used as the outgroup. A phylogenetic time tree was constructed using the RfTime method in MEGA 6.0 (57) to estimate divergence time of the or320 gene. An ML tree was constructed based on the GTR+G model (GTR, general time-reversible; G, a discrete γ distribution) by using the ML method. The calibration constraints were inferred according to a method proposed previously (58). The mouse and zebrafish ORs and TAARs were selected as outgroups, and divergence
times were calibrated (59). For analysis preferences, default settings were used. To explore the evolutionary process of or320, a selection test was performed based on the ratio of the non-synonymous/synonymous substitution rate (ω = dS/dN) (60). The codon selection test was performed via HyPhy in MEGA 6.0 by using the Tamura–Nei model with the maximum likelihood statistical method. Gaps were treated as partial deletions, and the site coverage cutoff was 95%.

**Plasmid constructs**

*Sea lamprey or constructs*—Coding regions of sea lamprey ORs (single-exon) were amplified from sea lamprey genomic DNA and ligated into the pCMV-Tag-2b (Agilent Technologies) vector in which the FLAG tag was replaced with a Rho tag (32). Sea lamprey ORs sequences were confirmed by sequencing (Sangon Biotech, Shanghai, China) using both forward and reverse universal primers of the vector.

**Homology modeling and transmembrane domain recombinant constructs**—To generate TM recombinants (chimeras) of OR320a and OR320b, homology modeling was used to predict the location of TMs of OR320a and OR320b. The SWISS-MODEL Automated Protein Modeling Server was used to search for the structure template based on sequence alignments. Five templates that shared the highest sequence identity or coverage with OR320 were selected to generate models by SWISS-MODEL. Models using the human apelin receptor (Protein Data Bank entry 5VBL.1.B) were rated the best in the webserver scoring program MolProbity. The receptor TM sequences were marked and aligned with ClustalX. Chimeras of OR320a and OR320b were generated by recombinant PCR. First, the three transmembrane domain fragments of each receptor were cloned by PCR using primers listed in Table S5. Then the fragments were annealed into chimeras by PCR and confirmed by sequencing.

**Site-directed mutagenesis**—To generate OR320a and OR320b mutations, forward and reverse mutation primer pairs (Tables S6 and S7) were designed by CE Design version 1.04 (Vazyme, Nanjing, China). WT Rho-tagged OR320 constructs were used as templates in high-fidelity PCR. Mutated receptor coding regions were confirmed by sequencing.

**Cell culture and transfection**

HEK293T cells were maintained in antibiotic-free DMEM containing 10% (v/v) fetal bovine serum (Gibco) at 37 °C and 5% CO2. All of the constructs were transiently transfected into HEK293T cells by using X-treme GENE HP DNA transfection reagent (Roche Applied Science). Transfection was performed in 384-well plates (Greiner Bio-One) by a reverse transfection method. Five ng of the Rho-tagged OR construct and 5 ng of pCI-mRTP1s (gift from Prof. Matsunami, Duke University) were mixed with X-treme GENE HP DNA transfection reagent (1:1, w/v) in 5 μl of DMEM and added into each well. Then HEK293T cells in 25 μl of DMEM contain 0.5% fetal bovine serum were seeded into each well and incubated for 48 h at 37 °C with 5% CO2.

**Odorant receptors that detect bile acid pheromones**

**Immunocytochemistry**

Cells were transfected with 5 ng of the Rho-tagged OR construct, 5 ng of pCI-mRTP1s, and 5 ng of pEGFP-N1 (Clontech) in a 384-well black clear-bottom plate (Greiner Bio-One). Forty-eight h after transfection, cells were fixed with 4% formaldehyde for 15 min at room temperature (RT). After washing three times, cells were incubated in blocking solution for 1 h at RT. Then a primary anti-Rho tag antibody solution (mouse anti-rhodopsin, Millipore, catalog no. MABN15) was applied overnight at 4 °C. The secondary antibody Alexa Fluor 594 (Invitrogen) was used to visualize receptor, and 4’,6-diamidino-2-phenylindole (Life Technologies) was used to visualize cell nuclei. Triton X-100 (0.3%) was added in the stain solution to visualize intracellular receptor expression. Micrographs were taken using a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek). Cells were seeded, transfected, and stained in a 12-well glass-bottom chamber (Ibidi, Munich, Germany) and imaged using a Leica DMI88 confocal laser-scanning microscope (Leica).

**Luciferase assay**

Cells were transfected with 5 ng of the Rho-tagged OR construct, 5 ng of pCI-mRTP1s, 5 ng of pCI-Gαolf (gift from Prof. Matsunami, Duke University), and 20 ng of the CRE-luciferase vector pGL4.29 (cAMP reporter; Promega) in a 384-well white plate (Greiner Bio-One). Forty-eight h after transfection, the cells were incubated in odorant working solution (diluted in DMEM) for 4 h at 37 °C with 5% CO2. For the inhibition test with ACA and 3kPZS, cells expressing OR320a were first incubated with ACA for 1 h and then co-incubated with 1 μM 3kPZS for 4 h. After incubation, luciferase activity was determined using the Steady-Glo Luciferase Assay System (Promega) according to the manufacturer’s instructions.

**cAMP assay**

The LANCE Ultra cAMP detection kit (PerkinElmer Life Sciences) was used by following the manufacturer’s instructions. Briefly, HEK293T cells were seeded in a 100-mm dish (Corning). One μg of Rho-tagged OR320a or OR320b construct, 1 μg of pCI-mRTP1s, and 1 μg of pCI-Gαolf were transfected on the next day. Forty-eight h after transfection, the cells were detached, survival rate was determined using trypsin (Gibco), and cells were resuspended in stimulation buffer (1× Hanks’ balanced salt solution, 5 mM HEPES, pH 7.4, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.1% BSA). A total of 3000 viable cells in 5 μl of stimulation buffer were added in each well of a white 384-well plate (PerkinElmer Life Sciences). Subsequently, 5 μl of prepared odorant stimulation buffer was added in each well, incubated for 30 min at RT. Eu-CAMP tracer and ULightTM-anti-cAMP reagents were then added and incubated in the dark for 1 h at RT. Finally, the time-resolved FRET signal was measured at 615 and 665 nm in a Synergy Neo microplate reader. Concentration–response curves were fitted using the equation in three-parameter logistic GraphPad Prism version 6.0.
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**Absolute real-time quantitative PCR**

RNA was extracted from tissues (olfactory epithelium, brains, intestine, gills, and kidneys) of adult sea lamprey and purified by RNase kits with on-column DNA digestion by DNase I (Qiagen). First-strand cDNA was synthesized by a high-capacity cDNA reverse transcriptase kit (Life Technologies). Real-time quantitative PCR was performed using SYBR Green PCR master mix (Applied Biosystems). Purified pCMV-N-Rho-OR320a and pCMV-N-Rho-OR320b plasmids were used as standards and were run on the same sample plate. β-Actin was used for confirming the RNA sample quantity. All samples were run in three replicates. Cycling conditions were one cycle at 50 °C for 2 min and one cycle at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s. The specificities of primers were validated by sequencing amplicons generated by real-time PCR. The sequences for primers were as follows: L.12164

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GACATGAG (reverse).
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**Data analysis**

The normalized luciferase activity was calculated using the formula,

$$(L_N - L_{\text{Negative control}}) / (L_{\text{Positive control}} - L_{\text{Negative control}})$$

(Eq. 1)

where $L_N$ is luminescence of firefly luciferase of a certain well, $L_{\text{Negative control}}$ is luminescence of firefly luciferase of OR320a response to DMSO on the plate, and $L_{\text{Positive control}}$ is luminescence of firefly luciferase of OR320a response to 3kPZS, PZS, 3-keto-1-ene PZS, or DPKES at the concentration used.

All data were analyzed with GraphPad Prism 6.0 software. Concentration-response curves were fitted using non-linear least squares regression with the three-parameter logistic equation in GraphPad Prism version 6.0, $Y = \text{Bottom} + \{(\text{Top} - \text{Bottom}) / (1 + 10^{(\text{logEC}_{50} - X)})\}$. The convergence criteria were set as medium strict with $R^2$ used to quantify goodness-of-fit. Curves that met one of the following two criteria were considered undefined: 1) log$_{10}$ Δ of EC$_{50}$ (or CI$_{\text{high}}$/CI$_{\text{low}}$ > 10 or 2) percentage coefficient of variation of $E_{\text{max}} >$ than 50.

The normality of the residuals was tested using D’Agostino–Pearson omnibus (K2). For data with two treatment levels, t-tests were used with the option for residuals that follow a normal distribution and show equal variance. For data sets with residuals that violated the normality assumption, we used the Mann–Whitney tests to determine treatment effects. Data sets with more than two treatment levels were analyzed with one-way ANOVA if the residuals followed normal distribution and showed equal variance. If the overall ANOVA had a p value of <0.05, then Dunnett’s multiple-comparison tests were used as a post-test to find significant differences between each mutant and the WT receptor. If the overall ANOVA had a p value of >0.05, a post-test was not used. If the residuals did not follow a normal distribution, Kruskal–Wallis ANOVA test and Dunn’s multiple-comparison test were used.

**Data availability**

The mRNA sequences of slor320a and slor320b are available in GenBank™ and have been assigned accession numbers MK095129 and MK095130, respectively. All other data related to receptor expression and activity are contained in the article. The other data related to lamprey phylogenetic analysis will be shared upon request to Zhe Zhang (zhang677@msu.edu).

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**Author contributions**—Z. Z., R. R. N., and W. L. conceptualization; Z. Z. data curation; Z. Z. and T. S. D. formal analysis; Z. Z. and Q. Z. investigation; Z. Z. visualization; Z. Z., T. S. D., and R. R. N. methodology; Z. Z. writing-original draft; Q. Z. and W. L. supervision; Q. Z. project administration; Q. Z., R. R. N., and W. L. writing-review and editing; J. R. resources; J. R. software; W. L. funding acquisition.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: GPCR, G protein-coupled receptor; $E_{\text{max}}$ maximum effect; 3kPZS, 3-keto-petromyzonolate; PZS, petromyzonolate; ACA, allocholic acid; DPKES, 3,12-diketo-4,6-petromyzonene-24-sulfate; 3-keto-1-ene PZS, 7,12,24-trihydroxy-3-keto-1-cholene-24-sulfate; TM, transmembrane; CRE, cAMP-response element; OR, odorant receptor; EGFP, enhanced green fluorescent protein; Go$_{\alpha}$, olfactory G protein; ORA, olfactory class A receptor; PZ, petromyzones; ML, maximal likelihood; CI, confidence interval; ANOVA, analysis of variance; IL, Japanese lamprey; SL, sea lamprey; MYA, million years ago; EOG, electro-olfactogram; DMEM, Dulbecco’s modified Eagle’s medium; RT, room temperature.

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