Evolutionary Conservation of 3-Iodothyronamine as an Agonist at the Trace Amine-Associated Receptor 1

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Key Words

Trace amine-associated receptor 1 · 3-Iodothyronamine · β -Phenylethylamine · *p*-Tyramine · Vertebrates

Abstract

Objectives: The trace amine-associated receptor 1 (Taar1) is a G_s protein-coupled receptor activated by trace amines, such as β -phenylethylamine (β -PEA) and 3-iodothyronamine (T₁AM). T₁AM is an endogenous biogenic amine and thyroid hormone derivative that exerts several biological functions. However, the physiological relevance of T₁AM acting via Taar1 is still under discussion. Therefore, we studied the structural and functional evolution of Taar1 in vertebrates to provide evidence for a conserved Taar1-mediated T₁AM function. **Study Design:** We searched public sequence databases to retrieve Taar1 sequence information from vertebrates. We cloned and functionally characterized Taar1 from selected vertebrate species using cAMP assays to determine the evolutionary conservation of T₁AM action at Taar1. **Results:** We found intact open reading frames of Taar1 in more than 100 vertebrate species, including mammals, sauropsids and amphibians. Evolutionary conservation analyses of Taar1 protein sequences revealed a high variation in amino acid residues proposed to be involved in agonist binding,

especially in rodent Taar1 orthologs. Functional characterization showed that T₁AM, β -PEA and *p*-tyramine (*p*-Tyr) act as agonists at all tested orthologs, but EC₅₀ values of T₁AM at rat Taar1 differed significantly when compared to all other tested vertebrate Taar1. **Conclusions:** The high structural conservation of Taar1 throughout vertebrate evolution highlights the physiological relevance of Taar1, but species-specific differences in T₁AM potency at Taar1 orthologs suggest a specialization of rat Taar1 for T₁AM recognition. In contrast, β -PEA and *p*-Tyr potencies were rather conserved throughout all tested Taar1 orthologs. We provide evidence that the observed differences in potency are related to differences in constraint during Taar1 evolution.

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Introduction

In 2004, the natural occurrence of the biogenic amine 3-iodothyronamine (T₁AM), a derivative of thyroxine, was reported to be present in the brain [1]. Further studies revealed the presence of T₁AM in numerous other tissues, including the heart, liver and muscle, as well as in blood [2–4]. Administration of T₁AM to laboratory animals

causes various metabolic responses, such as increases in plasma glucose levels, gluconeogenesis, lipid oxidation and ketogenesis, but also neurological effects including a decreased pain threshold and the modulation of sleep and feeding behavior [5]. Along with the discovery of T₁AM, its action as a high-affinity agonist at trace amine-associated receptor 1 (Taar1) was demonstrated [1]. Taar1 belongs to the family of trace amine-associated receptors, and was first deorphanized as a receptor activated by trace amines, such as β -phenylethylamine (β -PEA), *p*-tyramine (*p*-Tyr) and tryptamine, and couples to the G_s protein/adenylyl cyclase pathway [6, 7]. Taar1 expression was found in leukocytes, brain and peripheral tissues including, among others, heart, kidney, liver, pancreas and spleen [6, 8, 9]. Substantial interspecies differences in functional and pharmacological properties of Taar1 have been reported [10–12]. However, the interplay of an agonist and its receptor usually remains constant during evolution. Exceptions are receptors for exogenous ligands such as receptors of the immune and odorant systems which coevolve with their ligands. The latter scenario is very unlikely for Taar1 since it is rather ubiquitously expressed and not specifically found at environment-body barriers. This raises the question of whether evolution constrained the T₁AM/Taar1 interplay for a distinct physiological function.

In the present study we analyzed the structural and functional evolution of Taar1 in vertebrates. Previous studies revealed the presence of two zebrafish and five fugu genes that were orthologous to the mammalian Taar1 [13]. Due to the expansion of the Taar repertoire in teleost fish [13], our study focused on Taar1 of species belonging to the vertebrate classes Amphibia, Sauropsida and Mammalia. To determine whether T₁AM is the endogenous agonist at Taar1, characterized by the evolutionary as well as functional conservation of the agonist-receptor interaction, we cloned and functionally tested Taar1 of three distantly related mammalian species as well as of humans, and one representative each of birds, Crocodylia and amphibians. We found that all tested Taar1 orthologs were activated by T₁AM, suggesting that T₁AM is a physiologically relevant agonist at Taar1. However, EC₅₀ values of T₁AM were in the high nanomolar to micromolar range (0.75–2.4 μ M) at all Taar1 orthologs, except that of rat Taar1 (<0.1 μ M). To test whether the observed differences in T₁AM potency were due to positive selection in rodents we gathered and analyzed Taar1 sequence information of a total of 111 vertebrate species, which revealed a significantly different evolutionary rate for the rodent Taar1 branch.

Materials and Methods

Materials

If not stated otherwise all substances were purchased from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany) and C. Roth GmbH (Karlsruhe, Germany). Cell culture material was obtained from Sarstedt (Nümbrecht, Germany) and primers were purchased from Life Technologies (Darmstadt, Germany). For the expression of Taar1 in mammalian cell lines the pcDps vector was used [14]. Restriction enzymes were purchased from New England Biolabs (Frankfurt, Germany). 3-Iodothyronamine hydrochloride (CAS 788824-64-6) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Experimental Procedures

Taar1 Ortholog Identification, Alignments and Evolutionary Analyses

For the searching of NCBI trace archives, Taar1 sequences of various species were obtained using the rat ortholog nucleotide sequence as the query sequence in discontinuous megablast and blasting of all available trace archives, as well as nucleotide collection and nonredundant protein sequences. Trace files of sequences producing significant alignments were downloaded followed by assembly, analysis (using SeqManPro of DNASTar Lasergene Software Suite for Sequence Analysis 7.1.) and manual adjustment. Trace identifier numbers or NCBI accession numbers for each Taar1 ortholog achieved are listed in online supplementary table S1 (see www.karger.com/doi/10.1159/000430839 for all online suppl. material). All species with Taar1 pseudogenes and all Taar1-like teleost fish orthologs were excluded from alignment and analyses. Nucleotide alignments were generated with the ClustalW algorithm (Bioedit Sequence Alignment Editor 7.0.9; <http://www.mbio.ncsu.edu/BioEdit/bioedit.html> [15]) followed by manual trimming. The phylogenetic evolutionary history was inferred by the maximum likelihood method based on the Kimura 2-parameter model [16]. Evolutionary analyses were conducted using MEGA6 and PAML [17, 18]. Branch support was estimated with 1,000 bootstrap replicates. The resulting tree (online suppl. fig. S1) is not fully resolved, as is indicated by bootstrap values below 95%, but does not contradict the generally accepted vertebrate phylogeny. Consequently, we used the inferred tree for PAML analyses. Tests of selection ($\omega = d_N/d_S$) were accomplished by maximum likelihood using a codon-based substitution model implemented in PAML version 4.2 [18]. Branch models [19] that allow ω to vary among branches in the phylogeny were applied to determine ω ratios on particular lineages. Likelihood ratio tests (LRT) were performed to test nested competing hypotheses (online suppl. table S5).

Cloning of Taar1 Orthologs

Genomic DNA samples were prepared from tissue of selected species. Tissue samples were digested in lysis buffer (50 mM Tris/HCl, pH 7.5; 100 mM EDTA; 100 mM NaCl; 1% SDS; 0.5 mg/ml proteinase K) and incubated at 55°C for 18 h. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Primer pairs (online suppl. table S2) were used to amplify rat Taar1 (NCBI reference sequence: NM_134328.1), human TAAR1 (NCBI reference sequence: NM_138327.1), elephant Taar1 (NCBI reference sequence: XM_003404009.1), kangaroo Taar1 (as inferred from NCBI trace archive: ti:1649014544, ti:1643994001,

ti:1616200502, ti:156029489, ti:1275352077), chicken Taar1 (NCBI reference sequence: XM_004935740.1), crocodile Taar1 (NCBI reference sequence: XM_006270537.1) and frog Taar1 (NCBI reference sequence: XM_002935811.2). PCR reactions were performed under variable annealing and elongation conditions. A standard PCR reaction (50 µl) contained genomic DNA (200 ng) with primers (400 nM each), Phusion reaction buffer (1×), dNTP (125 µM each) and Phusion high-fidelity polymerase (0.6 U each; Thermo Fisher Scientific, Waltham, Mass., USA). The reactions were initiated with a denaturation at 98°C for 30 s, followed by 25 cycles of denaturation at 98°C for 20 s, annealing at 58°C for 1 min and elongation at 72°C for 2 min. A final extension step was performed at 72°C for 10 min. Specific PCR products were directly sequenced and/or subcloned into the pCR2.1-TOPO vector (Life Technologies) for sequencing. Sequencing reactions were performed with a dye-terminator cycle sequencing kit and applied on an ABI 3700 automated sequencer (both from Life Technologies). In online supplementary figure S2 we provide an alignment of all cloned protein Taar1 sequences versus database protein Taar1 sequences.

The full-length Taar1 from rat, human, elephant, kangaroo, chicken, crocodile and frog were inserted into the mammalian expression vector pcDps and epitope-tagged with an N-terminal hemagglutinin (HA) epitope (YPYDVPDYA) and a C-terminal FLAG-tag (DYKDDDDK) by a PCR-based overlapping fragment approach to allow immunological detection. Point mutations were introduced in rat and crocodile Taar1 using the primer pairs depicted in online supplementary table S2 and the fast cloning technique as described in Li et al. [20]. The identity of all constructs and correctness of all PCR-derived sequences were confirmed by sequencing.

Cell Culture and Functional Assays

CHO-K1 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Cells were maintained at 37°C in a humidified 5% CO₂ incubator. For transient transfection, LipofectamineTM 2000 (Life Technologies) was used.

Enzyme-Linked Immunosorbent Assay

An indirect cellular ELISA was used to estimate cell surface expression of N-terminal HA-tagged receptor constructs. Briefly, CHO-K1 cells were seeded into 48-well plates (4 × 10⁴ cells per well) and transfected the following day with 0.2 µg DNA and 0.5 µl LipofectamineTM 2000 per well according to the manufacturers' protocol. Two days after transfection, the cells were fixed with formaldehyde, without disrupting the cell membrane, and incubated in blocking solution (DMEM with 10% FBS) for 1 h at 37°C. The cells were then incubated with anti-HA-peroxidase-labeled high-affinity rat monoclonal antibody (3F10, Roche Molecular Biochemicals, Mannheim, Germany). After the removal of excess unbound antibody by extensive washing, H₂O₂ and o-phenylenediamine (2.5 mM each in 0.1 M phosphate-citrate buffer, pH 5.0) were added to serve as substrate and chromogen, respectively. After 15 min the enzyme reaction was stopped by adding 1 M H₂SO₄ containing 0.05 M Na₂SO₄, and color development was measured bichromatically at 492 and 620 nm using an ELISA reader (Sunrise Plate Reader, Tecan, Crailsheim, Germany). To assess the total receptor expression of full-length HA/FLAG double-

tagged Taar1 constructs a 'sandwich ELISA' was used as described in Schöneberg et al. [21]. In brief, transfected cells were harvested from 6-cm dishes and membrane preparations were solubilized in lysis buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% deoxycholate, 1% Nonidet P-40, 0.2 mM PMSF, 10 µg/ml aprotinin) overnight. Microtiter plates (MaxiSorp, Nunc Immunoplates) were coated with a monoclonal antibody directed against the C-terminal FLAG tag (10 mg/ml in 0.05 M borate buffer, M2 antibody; Sigma-Aldrich). After incubation with the membrane solubilises, bound full-length Taar1 proteins were detected with a peroxidase-labeled anti-HA monoclonal antibody (3F10, Roche Molecular Biochemicals).

ALPHAScreenTM cAMP Assay

ALPHAScreenTM cAMP assays were performed in 48-well plates (4 × 10⁴ CHO-K1 cells per well), and cells were transfected with 0.15 µg DNA and 0.5 µl LipofectamineTM 2000 per well. Stimulation with various agonist concentrations was performed in triplicate 48 h after transfection. Reactions were stopped by aspiration of the media and cells were lysed in 50 µl of lysis buffer containing 1 mM 3-isobutyl-1-methylxanthine. From each well 5 µl of lysate were transferred to a 384-well plate. According to the manufacturers' protocol, the cAMP content of cell extracts was determined by a nonradioactive cAMP accumulation assay based on the ALPHAScreenTM technology (Perkin Elmer LAS, Rodgau-Jügesheim, Germany).

Data were analyzed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, Calif., USA; www.graphpad.com). All cAMP data are presented as the fold over basal cAMP level of nonstimulated, empty pcDps vector-transfected CHO-K1 cells.

Results

Evolutionary Taar1 Protein Conservation in Comparison to Other G Protein-Coupled Receptors

First, we analyzed the evolutionary conservation of Taar1 using only representatives of mammals, sauropsids and amphibians. We chose two distantly related placental mammals, namely rat (Boreoeutheria) and elephant (Afrotheria), and one marsupial (kangaroo belonging to Metatheria) as representatives for mammals. Furthermore, we included one representative each of Aves (chicken), Crocodylia (crocodile) and Amphibia (frog). This comparison revealed 48.1% amino acid identity positions and a 70.5 ± 5.6% similarity between those Taar1 orthologs (fig. 1a; online suppl. table S3). To place this degree of conservation in a more general, comprehensive frame of G protein-coupled receptors (GPCR) conservation, we analyzed both the percentage identity and average conservation of some other aminergic receptors [α_{2A}-adrenergic receptor (ADRA2A), β₂-adrenergic receptor (ADBR2), dopamine receptor D4 (DRD4), histamine receptor H2 (HRH2), and 5-hydroxytrypta-

Fig. 1. Evolutionary conservation of Taar1 protein sequences in selected vertebrate species and Taar1 expression levels in transiently transfected CHO-K1 cells. **a** Alignment of Taar1 protein sequences of 6 vertebrate species, which were selected as representatives for mammals, sauropsids and amphibians. Identical/conserved residues (as compared to rat Taar1) are shown as dots and similar residues in gray. **b, c** Total cellular and cell surface expression levels of vertebrate and mutant Taar1 orthologs were measured by a sandwich and cell surface ELISA, respectively. Specific

optical density (OD) readings are given as a percentage of HA-/FLAG-tagged rat Taar1. For sandwich ELISA, the nonspecific OD value (empty vector) was 0.019 ± 0.001 , and the OD value of the HA-tagged rat Taar1 was 0.218 ± 0.042 (set 100%). For cell surface ELISA, the nonspecific OD value (empty vector) was 0.008 ± 0.002 (set 0%) and the OD value of the HA-tagged rat Taar1 was 0.037 ± 0.004 (set 100%). Data are presented as the mean $\pm$ SEM of 3 independent experiments carried out in triplicates.

mine (serotonin) receptor 1F (HTR1F)] and two known to be very well conserved GPCR (rhodopsin and melanocortin receptor 4, MC4R) for which sequence information was available for six representative species of the different vertebrate classes (online suppl. table S3; accession numbers are specified in online suppl. table S1). Rhodopsin, MC4R and HTR1F are highly conserved with 87.3, 82.7 and 82.9% similarity, respectively (online suppl. ta-

ble S3). Nevertheless, Taar1 conservation across the chosen species is comparable to other aminergic receptors, such as ADRA2A (76.2%) and ADRB2 (72.6%), and indeed higher than DRD4 (65.3%) and HRH2 (63.1%) conservation (online suppl. table S3). These analyses highlight that the amino acid conservation of Taar1 is not significantly different to other aminergic GPCR.

Table 1. Functional characterization of vertebrate Taar1 orthologs and rat Taar1 mutants

Taar1	Basal (fold over negative control)	T ₁ AM		β-PEA		p-Tyr	
		E _{max} (fold over neg- ative control)	EC ₅₀ , μM	E _{max} (fold over neg- ative control)	EC ₅₀ , μM	E _{max} (fold over neg- ative control)	EC ₅₀ , μM
<i>Orthologs</i>							
Rat	7.78±0.78 (20)	13.95±1.58	0.090±0.017 (9)	22.90±0.77	0.078±0.014 (5)	19.14±2.08	0.122±0.066 (6)
Human	1.60±0.15 (9)***	4.10±0.77	1.69±0.42 (3)***	7.27±1.28	0.66±0.12 (3)**	5.76±0.56	1.29±0.38 (3)*
Elephant	2.41±0.23 (13)***	9.56±1.47	0.75±0.19 (7)**	11.52±0.73	0.20±0.090 (3) n.s.	12.00±0.84	0.066±0.014 (3) n.s.
Kangaroo	1.57±0.09 (13)***	4.43±0.39	1.38±0.37 (7)**	4.64±0.29	0.11±0.053 (3) n.s.	5.97±0.75	0.105±0.030 (3) n.s.
Chicken	1.98±0.26 (13)***	5.27±1.16	2.41±0.41 (7)***	19.65±3.71	0.65±0.22 (3) [#]	26.85±4.85	0.657±0.093 (3)**
Crocodile	4.27±0.31 (20)***	10.76±1.35	2.34±0.41 (9)***	16.73±1.18	0.18±0.057 (5) n.s.	17.58±0.78	0.080±0.025 (6) n.s.
Frog	2.99±0.30 (13)***	5.51±0.80	1.48±0.40 (7)**	8.97±0.30	0.085±0.040 (3) n.s.	7.41±1.00	0.014±0.009 (3) n.s.
<i>Rat</i>							
Rat M268T	6.28±0.83 (9) n.s.	12.43±0.30	0.123±0.066 (3) n.s.	12.70±0.20	0.22±0.063 (3)*	18.39±2.31	0.162±0.020 (3) n.s.
Rat N287I	6.13±0.81 (9) n.s.	12.21±1.87	0.159±0.040 (3) [#]	20.06±2.97	0.15±0.091 (3) n.s.	20.54±1.48	0.195±0.143 (3) n.s.
Rat N287V	3.60±0.56 (9)***	5.21±0.91	0.250±0.129 (3)*	16.87±1.75	0.31±0.070 (3)*	13.03±1.83	0.363±0.289 (3) n.s.
Rat M268T/N287I	2.20±0.29 (9)***	6.32±0.21	0.241±0.120 (3)*	9.25±2.29	1.03±0.365 (3)*	6.87±0.46	0.240±0.075 (3) n.s.
Rat M268T/N287V	2.87±0.47 (6)***	10.49±2.35	0.123±0.023 (2) n.s.	10.85±3.17	0.30±0.042 (2)**	16.30±5.38 n.s.	0.078±0.051 (2) n.s.
<i>Crocodile</i>							
Crocodile T268M	4.45±0.45 (6) n.s.	12.47±1.53	1.02±0.84 (2) n.s.	14.96±2.31	0.28±0.12 (2) n.s.	12.89±0.84	<0.001 (2) n.s.
Crocodile V287N	3.52±0.48 (9) n.s.	7.89±0.94	1.55±0.55 (3) n.s.	9.31±1.01	0.24±0.078 (3) n.s.	20.54±1.49	0.015±0.006 (3) n.s.

CHO-K1 cells were transfected with receptor constructs, and agonist-induced cAMP accumulation was determined with the ALPHAScreen™ technology (see Experimental Procedures). E_{max} and EC₅₀ values were determined from concentration-response curves of T₁AM, β-PEA or p-Tyr using GraphPad Prism (fig. 2). Data are given as the mean ± SEM of the indicated number of independent experiments (in parentheses), each performed in

triplicate. Using a two-tailed unpaired t test, p values were determined comparing EC₅₀ values for each trace amine at Taar1 orthologs or rat Taar1 mutants compared to rat Taar1, respectively. Crocodile Taar1 mutants were compared to crocodile Taar1 wild type. [#] p ≤ 0.1; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001. n.s. = Not significant.

Functional Analyses of Vertebrate Taar1 Orthologs

To test whether the observed structural conservation is reflected in Taar1 function upon agonist stimulation, we cloned Taar1 from rat, human, elephant, kangaroo, chicken, crocodile and frog. All Taar1 orthologs were N-terminally tagged with an HA epitope and C-terminally FLAG-tagged to enable both the determination of total cellular and cell surface expression levels. CHO-K1 cells were transiently transfected and total cellular and cell surface expression were monitored using ELISA. Thereby, we found that all tested Taar1 orthologs had considerably reduced total and cell surface expression levels when compared to rat Taar1 (fig. 1b).

The main aim of our study was to analyze whether T₁AM could be the endogenous agonist of Taar1 which, if true, should display an evolutionary conserved function in vertebrates. Thus, we performed cAMP accumulation assays to determine from concentration-response curves basal, E_{max} and EC₅₀ values for T₁AM action at the various Taar1 orthologs (table 1; fig. 2a).

First, we found that the basal activity of all vertebrate Taar1 orthologs was significantly reduced when compared to rat Taar1 (table 1). Rat Taar1 was activated by

T₁AM with an EC₅₀ value of 90 nM in transfected CHO-K1 cells, which is in the range of the previously reported EC₅₀ value [1, 11, 22, 23]. In great contrast, the EC₅₀ values of T₁AM at all other orthologs tested were in the micromolar range (table 1; fig. 2a) and significantly different when compared to the EC₅₀ value determined for rat Taar1. Wainscott et al. [11] had previously shown that T₁AM activates rat Taar1 with an EC₅₀ value of 22 nM and human TAAR1 with an EC₅₀ value of 1.5 μM, which are both in the range of our obtained functional data. Interestingly, E_{max} values of T₁AM at elephant and crocodile Taar1 were in a similar range compared to rat Taar1, whereas the E_{max} values of all other orthologs were about 2- to 3-fold lower (table 1; fig. 2a).

Next, we analyzed all Taar1 orthologs in cAMP accumulation assays stimulating transiently transfected CHO-K1 cells with increasing concentrations of β-PEA and p-Tyr, both trace amines and known agonists at Taar1. Remarkably, only Taar1 of human and chicken displayed EC₅₀ values for both trace amines that were significantly differing when compared to rat Taar1 (table 1; fig. 2b, c). For Taar1 of human and rat this confirms the results obtained from Wainscott et al. [11] presenting EC₅₀ values

Fig. 2. Functional characterization of 7 vertebrate Taar1 orthologs. CHO-K1 cells were transfected with receptor constructs, and agonist-induced cAMP accumulation was determined with the ALPHAScreen™ technology (see Experimental Procedures). Concentration-response curves are shown of T₁AM (**a**), β-PEA (**b**) and *p*-Tyr (**c**), from which all the values depicted in table 1 were derived. Data are presented as the fold over basal cAMP level of nonstimulated mock-transfected CHO-K1 cells. Data are given as the mean ± SEM of the number of independent experiments indicated in table 1, each performed in triplicate.

of 106 and 209 nM of β -PEA and 425 and 68 nM of *p*-Tyr at human and rat Taar1, respectively. The EC₅₀ values of β -PEA at both human and chicken Taar1 were significantly lower, whereas the EC₅₀ value of *p*-Tyr was significantly higher when compared to rat Taar1 (table 1). All other tested Taar1 orthologs were activated by β -PEA and *p*-Tyr with EC₅₀ values comparable to the one obtained for rat Taar1 (table 1). Moreover, we observed significantly higher E_{max} values for Taar1 of rat, chicken, crocodile and frog when stimulated with β -PEA and *p*-Tyr compared to T₁AM (table 1).

Taar1 Protein Is Highly Conserved during Vertebrate Evolution

One potential explanation for the observed differences in T₁AM potency could be that Taar1 of rat and potentially other rodents evolved under positive selection, thus resulting in a ligand-binding pocket specifically recognizing T₁AM. To test this hypothesis, we gathered the Taar1 ortholog sequence information of 1 amphibian, 29 sauropsids (including 22 birds) and 81 mammals by mining publicly available databases (online suppl. fig. S1; online suppl. table S1). Due to the large expansion of the Taar repertoire in teleost fish (zebra fish, fugu and Atlantic salmon) and the lack of data reporting the physiological relevance and pharmacological characterization of Taar1 in teleost fish, we did not include teleost fish Taar1 orthologs in our analyses [13, 24]. Furthermore, no pseudogenes, namely the previously reported Taar1 pseudogenes reported for species of the Caninae subfamily, were included in the analyses [25].

The evolutionary relation of all Taar1 orthologs was inferred by the maximum likelihood method based on the Kimura 2-parameter model implemented in MEGA6 (online suppl. fig. S1) and subsequently used for both PAML analyses and determination of the relative evolutionary rate per amino acid site (fig. 3; online suppl. table S4) [16–18]. For the latter approach, no sequence divergence times are required because all individual relative rates are scaled such that the average relative rate over all positions is equal to 1. Thus, a position with a relative rate less than 1 is inferred to be more highly conserved than the average conservation of sites in the alignment, whereas a position with a rate greater than 1 is evolving faster than average [26]. Furthermore, the lower the relative evolutionary rate of a site, the higher conserved is this position during evolution. Altogether 77 amino acids are 100% conserved throughout amphibian, sauropsids and mammalian Taar1 orthologs, corresponding to 22.9% identity positions (fig. 3). Evolutionary analyses using

PAML revealed that rodent Taar1 evolves with a significantly different evolutionary rate compared to all other vertebrate Taar1 (online suppl. table S5). Moreover, branch-site models were applied to detect positive selection affecting only a few sites on the prespecified foreground branch, including all species belonging to the superfamily of Muroidea within the rodents (test 2: modified model A vs. modified model A with $\omega_2 = 1$ fixed). These analyses were conducted with both a complete vertebrate tree and a tree including only rodents (online suppl. table S5). Using branch-site model A with the Muroidea branch (see online suppl. fig. S1) as the foreground lineage in the branch-site test of positive selection (null model $\omega_2 = 1$ fixed), LRT provided support ($p = 0.095$) for $\omega_{\text{Muroidea}} = 4.863$, compared to $\omega_0 = 0.108$ for all other rodents, and for Phe176, Arg237 and Asn287 as positively selected sites with a Bayes empirical Bayes (BEB) probability above 0.75 in Muroidea (online suppl. table S5) [27, 28]. Using the complete vertebrate tree, 6 positively selected sites with a BEB probability above 0.75 could be found in Muroidea, but LRT provided no evidence that Muroidea evolve with a rate significantly greater than 1 (online suppl. table S5). Therefore, there is some evidence that positive selection might be responsible for the different agonist profiles found for Taar1.

Next, we inspected the aligned ortholog sequences for obvious alterations and changes. Interestingly, we found that in the rock dove (*Columba livia*) and naked mole-rat (*Heterocephalus glaber*) Taar1 orthologs exhibit a Cys and His, respectively, instead of the Arg in the highly conserved DRY motif. Many GPCR with mutant DRY have been characterized to unveil the role of this highly conserved motif [29]. Exchange of Arg to His mostly causes the inability of the GPCR to couple to its G protein whereas agonist affinity remains unaffected [29]. In the context of odorant receptors, occurrence of a DCY motif has been reported for human OR912-93, which cannot be activated by the ketone that has agonistic activity at other primate OR912-93 with an intact DRY motif [30]. The authors suggest that the human receptor is a pseudogene that has so far not accumulated any other nonsense mutations [30].

Recently, Reese et al. [31] suggested a putative ligand-binding pocket for rat and mouse Taar1 which they derived from a Taar1-ADBR2 homology model. Since all other Taar1 orthologs tested, except rat Taar1, can only be activated by T₁AM in the micromolar range, we investigated the conservation of the amino acid positions specified by Reese et al. [31] to be involved in ligand binding (table 2). Interestingly, of the 18 specified resi-

Table 2. Putative ligand-binding pocket residues as suggested previously [31, 32], modified to reflect the positional evolutionary pattern at each position

Rat Taar1 amino acid position	Amino acid in rat Taar1 (number of vertebrate orthologs with this amino acid)	Amino acids occurring in other vertebrate Taar1 orthologs (species with specified amino acids)
75	Val (87)	Ala (2: prairie vole, guinea pig) Ile (22)
99	Thr (104)	Met (1: rock dove) Ser (6: sperm whale, Atlantic canary, white-throated sparrow, blind mole rat, lesser Egyptian jerboa, medium ground finch)
102	Asp (111)	
103	Ile (111)	
106	Ser (111)	
185	Phe (109)	Leu (2: wild Bactrian camel, alpaca)
193	Ala (99)	Ser (2: rock dove, blind mole rat) Thr (5: human, chimpanzee, bonobo, chicken, wild turkey) Val (5: long-tailed chinchilla, Chinese hamster, golden hamster, prairie vole, degu)
194	Phe (80)	Ser (30: sauropsids, frog) Cys (1: cape rock hyrax)
197	Ser (108)	Cys (1: Carolina anole) Phe (1: golden hamster) Val (1: Chinese hamster)
198	Phe (111)	
257	Phe (111)	
261	Trp (111)	
264	Phe (111)	
265	Phe (111)	
268	Met (25)	Thr (83) Ile (3: common marmoset, star-nosed mole, blind mole rat)
283	Asn (70)	Ile (30: sauropsids, frog) Ser (4: guinea pig, star-nosed mole, nine-banded armadillo, Ord's kangaroo rat) Thr (6: Hoffmann's two-toed sloth, chicken, lesser Egyptian jerboa, golden hamster, white-cheeked gibbon, cape rock hyrax) Arg (1: platypus)
284	Asp (103)	Glu (8: guinea pig, long-tailed chinchilla, cape golden mole, lesser hedgehog tenrec, big brown bat, naked mole rat, degu, platypus)
287	Asn (1)	Ile (67) Val (34: sauropsids, frog) Ala (1: wild Bactrian camel) Met (2: green sea turtle, star-nosed mole) Ser (4: Chinese hamster, golden hamster, prairie vole, blind mole rat) Thr (1: prairie deer mouse) Tyr (1: mouse)
291	Tyr (111)	

Taar1 sequence information of 111 vertebrate species was aligned and analyzed for amino acid conservation at positions proposed to be involved in ligand binding. The numbers in parentheses indicate the number of vertebrate Taar1 orthologs with the shown amino acid at this position.

Met and Val287 to Asn in crocodile Taar1. We tested the total and cell surface expression of all Taar1 mutants, revealing a reduced expression for all mutant Taar1 (fig. 1c). Subsequently, we analyzed all mutant Taar1 in cAMP accumulation assays and determined concentration-

response curves for T₁AM, β -PEA and *p*-Tyr (table 1). We found that the potencies of T₁AM for rat Taar1 N287I, N287V and M268T/N287I were significantly higher when compared to wild-type rat Taar1, supporting a relevance for these residues in T₁AM recognition. However,

the obtained EC₅₀ values for T₁AM were still in the nanomolar range. For the respective vice versa mutations in crocodile Taar1 we observed only trends towards lower EC₅₀ values for T₁AM with 1 µM for crocodile Taar1 T268M and 1.6 µM for V287N, compared to 2.3 µM in wild-type crocodile Taar1 (table 1). EC₅₀ values for β-PEA were significantly higher for all rat Taar1 mutants except N287I, which is in line with the previously published data from Reese et al. [31]. For crocodile Taar1 mutants EC₅₀ values for β-PEA did not significantly differ when compared to wild-type crocodile Taar1 (table 1). No significant differences in EC₅₀ values for *p*-Tyr acting at mutant rat and crocodile Taar1 compared to the respective wild type were observed.

Discussion

Since the deorphanization of Taar1 in 2001, extensive studies have increased our understanding of Taar1 pharmacology [6, 7]. Moreover, it has been demonstrated that, besides being an agonist at Taar1, T₁AM also interacts with ADRA2A receptors, apolipoprotein B100 [33], mitochondrial ATP synthase [34], and membrane monoamine transporters [35, 36], and in Taar1 knockout mice thermoregulatory responses were not altered upon T₁AM administration [37]. Thus, it is still under debate which physiological effects of T₁AM are mediated exclusively through Taar1.

Here, we analyzed the structural and functional evolution of Taar1 in vertebrates. Initial analyses revealed that Taar1 protein conservation is comparable to other aminergic receptors, such as ADRA2A, ADRB2, DRD4 and HRH2, when analyzing the protein sequences of representative species for mammals, sauropsids and amphibians (online suppl. table S3). Expression analyses revealed that rat Taar1 protein expression is considerably higher when compared to all other tested orthologs in our transient expression system using CHO-K1 cells (fig. 1b). Furthermore, functional analyses of Taar1 of those representative species also revealed that rat Taar1 is the only Taar1 ortholog tested that is activated by T₁AM in a nanomolar range, whereas no such differences in potency were observed for the other two trace amines tested (table 1; fig. 3).

We found T₁AM to activate rat Taar1 with an EC₅₀ value of 64 nM, which is in the range of the previously reported EC₅₀ values [1, 20, 21]. However, the EC₅₀ values of T₁AM at all other orthologs tested were in the micromolar range (table 1; fig. 2). Previous studies have reported an approximate 10-fold lower potency of mouse Taar1

(100–300 nM) when compared to rat Taar1 and an EC₅₀ value of 1.5 µM for human TAAR1 [1, 11, 12, 22, 23].

T₁AM was first detected in the brain [1], heart [3] and blood [2] by liquid chromatography mass spectrometry. Saba et al. [4] were able to show the presence of T₁AM in rat tissues and quantify tissue concentrations of 1–90 pmol/g. Serum concentrations of T₁AM were found in the range of 0.2–0.3 nM in humans and rats [4, 38]. Given these low concentrations detected in vivo, one might argue that EC₅₀ values in the micromolar range do not reflect the real agonistic activity of T₁AM at all vertebrate Taar1, except in rats and potentially in mice. There are several options to address this issue. First, local concentrations of T₁AM might be much higher than the concentrations detectable in whole tissue and blood, possibly due to short-lived signals. Second, overexpression of vertebrate Taar1 in CHO-K1 cells disregards the physiological context of each individual receptor in its organism and/or cell populations in vivo. Thus, receptor function is monitored outside its biological networks, which might cause the determination of EC₅₀ values which do not reflect the in vivo reality [39]. Third, due to the high potency of T₁AM at rat Taar1, it is possible that evolutionary selective pressures promoted the Taar1-T₁AM interaction in rats, resulting in a nanomolar potency of T₁AM at rat Taar1.

To test whether positive selection acting on rodent Taar1 might be the reason for the high potency of T₁AM at rat Taar1 when compared to other vertebrate Taar1 orthologs, we acquired Taar1 sequence data of more than 100 vertebrate species and conducted evolutionary analyses using PAML. These analyses showed that rodent Taar1 evolve with a significantly different evolutionary rate compared to all other vertebrate Taar1 (online suppl. table S5), and species belonging to the superfamily of Muroidea within Rodentia especially evolved with an ω_{Muroidea} of 4.863, which is a strong indication for positive selection acting on those Taar1 orthologs.

Evolutionary analyses revealed position 287, which has been demonstrated to be crucial for ligand recognition in rat Taar1 [31], as a positively selected site characterized by high variation across Taar1 of all vertebrate species (online suppl. table S5). A methionine at position 268 was demonstrated to increase the potency of β-PEA at mouse Taar1 [31]. To test the relevance of both Met268 and Asn287, we generated rat Taar1 in which Met was exchanged to Thr and Asn was replaced by Ile or Val. Functional analyses revealed that rat Taar1 with these mutations is activated by T₁AM with about a 2-fold lower potency, whereas the opposite was observed for the vice versa mutations in crocodile Taar1 (table 1). Thus, Met268

and Asn287 play a role in T₁AM recognition, but are not solely responsible for the high T₁AM potency at rat Taar1.

Here, we have provided evolutionary evidence for both the observed species-specific functional differences of the Taar1-T₁AM interaction and its conservation over a long period of evolution, rendering surrogate effects as the only explanation very unlikely.

Conclusion

In sum, we conclude that Taar1 is highly conserved throughout vertebrate evolution. All functionally tested Taar1 orthologs are expressed at the cell surface and can be activated by T₁AM, β -PEA and *p*-Tyr. Thus, the ligand-binding pocket for these biogenic amines is highly preserved in vertebrates. However, we observed species-specific differences in agonist preferences, as previously also reported when comparing the potencies of many different agonists at human, mouse and rat Taar1 [11, 12]. Consequently, it is likely that Taar1 is highly conserved as a promiscuous receptor that detects various species-

specific biogenic amines, with β -PEA being the common denominator structure characterized by the lowest variation in potency at vertebrate Taar1 orthologs. The repertoire of Taar differs considerably even between closely related species, while at the same time the ligand-binding pocket is highly conserved even across Taar paralogs [40]. This suggests overlapping functions for the other Taar paralogs potentially involved in the mediation of species-specific biogenic amine signals, one of which is T₁AM.

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Disclosure Statement

The authors declare that they have no competing financial interests.

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