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Identification of endogenous agonists of extra-oral bitter taste receptors

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Publications

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Abbreviations

7TM	seven transmembrane domain
ASBT	apical sodium-dependent bile acid transporter
ASM	airway smooth muscle
ATP	adenosine triphosphate
BSH	bile salt hydrolase
CA	cholic acid
Ca^{2+}	calcium ion
CALHM	calcium homeostasis modulator
cAMP	cyclic adenosine monophosphate
CaSR	extracellular-calcium-sensing receptor
CD36	cluster of differentiation 36
CDCA	chenodeoxycholic acid
CRD	cysteine rich domain
CXCR	CXC chemokine receptor
CYP	cytochrome P450
ECL	extracellular loop
ENaC	epithelial sodium channel
ER	endoplasmic reticulum
FGF19	fetal growth factor 19

FXR	farnesoid X receptor
GLUT	glucose transporter
GMP	guanosine monophosphate
GPCR/GPR	G-protein coupled receptor
hBAT	human bile acid – CoA:amino acid N – acyltransferase
HEK	human embryonic kidney
IMP	inosine monophosphate
IL	interleukin
IP_3	inositol triphosphate
IP_3R3	inositol triphosphate receptor type 3
LCA	lithocholic acid
MCA	muricholic acid
mGluR	metabotropic glutamate receptor
OST	organic solute transporter
OTOP1	otopetrin-1
P2X2/3	P2X ionotropic purinergic receptors 2 and 3
PKA	protein kinase A
PLC β2	phospholipase C β2
PTC	phenylthiocarbamide
RTP	receptor transporter protein

SGLT1	sodium/glucose-cotransporter 1
SNP	single nucleotide polymorphism
SST3	somatostatin receptor subtype 3
TAS1R	taste receptor subfamily 1
TAS2R	taste receptor subfamily 2
TGR5	Takeda G-protein coupled receptor 5
TLCA	taurolithocholic acid
TM	transmembrane domain
TRP	transient receptor potential channel
TRPV1	transient receptor potential vanilloid-1
VDR	vitamin D receptor
VFTD/VFD	venus flytrap domain
VGNC	voltage-gated sodium channel
VRAC	volume-regulated anion channel

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Zusammenfassung

Die Erkennung und Signalweiterleitung der Geschmackssinne süß, umami und bitter findet im menschlichen Körper, aber auch in weiteren Säugetieren durch die Aktivierung von G-Protein-gekoppelten Rezeptoren statt. Neben den im Mund-Rachenraum befindlichen Rezeptoren, welche für eine erste Bewertung der aufgenommenen Nahrung verantwortlich sind, konnte die Expression dieser Rezeptoren in den vergangenen Jahren auch in zahlreichen weiteren Geweben, wie beispielsweise dem Gastrointestinaltrakt, dem Herzen und dem Respirationstrakt nachgewiesen werden. Diese Erkenntnis lässt auf weitere physiologische Funktionen der Geschmacksrezeptoren schließen. Allerdings sind zum jetzigen Zeitpunkt nur wenige dieser Funktionen, sowie die an der Aktivierung beteiligten Agonisten bekannt.

Geschmacksrezeptoren sind im Vergleich zu anderen G-Protein-gekoppelten Rezeptoren unempfindlich. Dennoch wird im Fall von Bitterrezeptoren, abgekürzt TAS2R, die Aufnahme von hohen Konzentrationen bitterer Substanzen bereits durch ihren bitteren Geschmack verhindert. Deshalb erreichen über die Nahrung aufgenommene Agonisten nicht-gustatorische Bitterrezeptoren vermutlich selten in Konzentrationen, welche für deren Aktivierung ausreichen. Somit stellt sich die Frage, welche Substanzen diese Bitterrezeptoren aktivieren. Das Ziel dieser Arbeit war daher die Klärung, ob endogene Substanzen oder Metabolite für eine Aktivierung von Bitterrezeptoren verantwortlich sein können.

Dabei wurden drei Bausteine in Betracht gezogen. Unter anderem spielt in der heutigen Forschung die Bioinformatik eine immer wichtigere Rolle und kann, verglichen mit in-vitro Experimenten, durch Modellierungsstudien dazu beitragen schnell die potentesten endogenen Agonisten zu identifizieren. Um diese Methode anwenden zu können, ist allerdings eine sehr gute Kenntnis der Rezeptorstruktur Voraussetzung. Nachdem bis vor kurzem noch keine experimentelle Struktur eines Bitterrezeptors bekannt war, basierten die Modelle lediglich auf Sequenzhomologien zu verwandten G-Protein-gekoppelten Rezeptoren, welche allerdings sehr gering sind. Um zur Aufklärung der Rezeptorstrukturen beizutragen, wurden alle durch frühere Studien bestätigten Aminosäurepositionen, welche an Agonistenbindung und Rezeptoraktivierung beteiligt sind in einem Review zusammengestellt.

Des Weiteren wurde die Konservierung von Rezeptorantworten untersucht. Da in der Evolution meist Individuen selektiert werden, welche einen Vorteil gegenüber ihren Artgenossen besitzen, kann eine Konservierung eines Agonisten in Spezies, welche sich vor mehreren Millionen Jahren in der Evolutionsgeschichte getrennt haben auf die Wichtigkeit der Erkennung dieses Agonisten hindeuten. So wurde in vergangenen Studien die Aktivierung des menschlichen TAS2R7 durch zwei- und dreiwertige Metallionen gezeigt. Durch Expression des eins-zu-eins Orthologs der Vampirfledermaus in Zellkulturmodellen, konnte die Konservierung dieser Antwort in dieser Spezies aufgedeckt werden. Diese Erkenntnis kann zum einen darauf hindeuten, dass eine übermäßige Aufnahme von Metallionen eine schädliche Wirkung auf Säugetiere haben kann und somit verhindert werden soll. Andererseits sind Metallionen im Körper auch an zahlreichen Prozessen beteiligt und fungieren beispielsweise als Co-Faktoren in Enzymen oder als sekundäre Botenstoffe. Von daher können lokal im Körper hohe Konzentrationen an Metallionen auftreten, welche eine Aktivierung des TAS2R7 zur Folge haben können.

Eine bereits seit langem für ihren Bittergeschmack bekannte Körperflüssigkeit ist die Galle. Um herauszufinden, welche Bittergeschmacksrezeptoren des Menschen auf die darin enthaltenen Gallensäuren reagieren, wurden 25 funktionelle menschliche TAS2Rs in einem Zellkulturmodell exprimiert und ihre Aktivierung nach Applikation verschiedenster Gallensäuren bestimmt. Dadurch konnten fünf dieser Rezeptoren als Gallensäure-Rezeptoren identifiziert werden. Durch die Aufklärung der Dosisabhängigkeit dieser Aktivierungen, konnte zudem eine Überschneidung von endogenen Gallensäurekonzentrationen und den Konzentrationsschwellenwerten für eine Rezeptoraktivierung festgestellt werden. Wie bereits erwähnt kann eine Konservierung von biologischen Prozessen auch Rückschlüsse auf die Relevanz dieses Prozesses für das Wohlbefinden von Lebewesen aufzeigen. Somit wurde dieses experimentelle Prozedere auch auf 34 Bitterrezeptoren der Maus angewandt. Hierbei konnten sechs Gallensäure-Rezeptoren entschlüsselt werden.

Diese Arbeit zeigt somit auf, dass extra-orale Bittergeschmacksrezeptoren durch essentielle endogene Substanzen aktiviert werden können, welche im menschlichen Körper in Konzentrationen auftreten, die für eine TAS2R Aktivierung ausreichen.

Dennoch muss zukünftig noch aufgeklärt werden, welche funktionelle Auswirkung eine solche Aktivierung zur Folge haben kann. Diese Erkenntnisse können sowohl für die Lebensmittelindustrie zur Entwicklung von Nahrungsergänzungsmitteln als auch für die Pharmaindustrie zur Identifizierung neuer pharmakologischer Targets von großer Bedeutung sein.

Summary

In humans and other mammals, the detection and signal transduction of the taste qualities sweet, umami and bitter is mediated by G protein-coupled receptors. Besides the oral cavity, where they are responsible for a first evaluation of ingested food, their expression was further proven in several non-gustatory tissues like the gastrointestinal tract, the heart, and the respiratory tract. This knowledge hints to further physiological functions of taste receptors beside taste perception. However, nowadays only a few of these functions, and the involved agonists, are known.

As taste receptors are rather insensitive and in the case of bitter taste receptors, called TAS2Rs, the ingestion of high concentrations of bitter substances is avoided by their bitter taste, it is questionable whether compounds that are ingested by food will frequently reach non-gustatory TAS2Rs in concentrations sufficient for activation. Therefore, the question arises by which substances extra-oral bitter taste receptors are activated. One aim of this thesis was the elucidation of the potential of endogenous substances or metabolites to activate bitter taste receptors.

Nowadays, bioinformatics is an important tool in research. Modeling studies can be an appropriate alternative assisting in-vitro experiments to speed up the identification of potent endogenous agonists. Nevertheless, knowledge of receptor structures is a key requirement for accurate modeling. Until recently, no experimental structure of bitter taste receptors was elucidated. Therefore, receptor models were based on homology modeling with related G protein-coupled receptors, even though sequence identities are not completely overlapping. To contribute to improvement of receptor models, amino acid positions that are important for agonist binding and receptor activation were summarized in a review.

Furthermore, the conservation of receptor responses was investigated. In evolution, individuals, which have an advantage over their conspecifics, are selected. Therefore, conservation of agonists in species that divided millions of years ago can hint to the importance of these agonists. In recent studies, the activation of human TAS2R7 by di- and trivalent metal ions was shown. The expression of the one-to-one orthologous receptor of the vampire bat in a cell culture model showed the conservation of metal

ion response in this species. This information may indicate that the excessive consumption of metal ions has a harmful effect on mammals and should therefore be avoided. Nevertheless, metal ions are involved in several processes in the body. For example, they act as cofactors in enzymes or as second messengers in cellular signaling cascades. Therefore, high metal ion concentrations can be present locally, which may result in activation of the TAS2R7 in non-gustatory tissues.

The bitter taste of the body fluid bile has long been known. To elucidate, which human bitter taste receptors are addressed by the compound class of bile acids, 25 functional human TAS2Rs were expressed in cell culture models and their activation was measured after application of several bile acids. In doing so, five of these receptors were identified as bile acid receptors. By measuring dose-response relationships, an overlap of endogenous bile acid concentrations and threshold concentrations for bitter taste receptor activation was detected. As already mentioned, conservation of biological processes can hint to the relevance of these processes for the well-being of animals. For this reason, this experimental design was applied to 34 mouse bitter taste receptors. Thereby, six receptors that responded to bile acids were deciphered.

Finally, this thesis shows that non-gustatory bitter taste receptors are activated by essential endogenous substances, which are present in the human body in concentrations that suffice to activate TAS2Rs. The task for future researchers will be to use this knowledge to elucidate biological effects of extra-oral bitter taste receptor activation. This information will be useful for the food to develop dietary supplements for human well-being and for the pharma industry to identify new pharmacological targets to address diseases.

1. Theoretical background

1.1. The mammalian taste system

Food intake is an essential requirement for all kinds of living animals and the first evaluation of food components takes place in the mouth. In general, mammals are able to distinguish five basic taste qualities including sweet, umami and salty with favorable characteristics like sensing energy content and sodium ions. In contrast, the senses of bitter and sour taste are rather perceived as unpleasant. As many toxic substances taste bitter, it serves as an important warning signal for mammals to avoid the ingestion of possibly harmful compounds. [1-3]

Taste sensation in the mouth takes place in the pore region of taste buds (Figure 1) [4-6]. These bulb-shaped structures are located in circumvallate, foliate and fungiform papillae on the tongue, as well as in the soft palate, epiglottis, larynx and pharynx [7-11]. Taste buds are composed of 50 – 120 taste bud cells that are divided into type I, II, III and IV and each of them fulfills its distinct function [2].

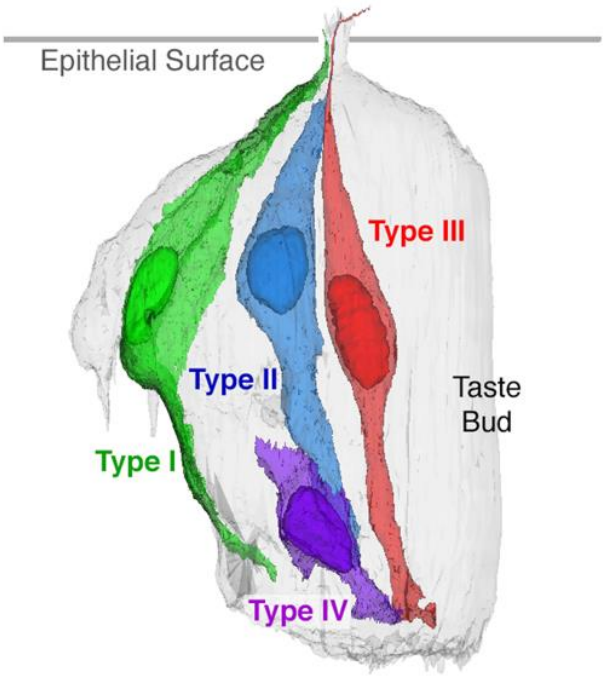

Figure 1: Cell type organization in taste buds. Four different cell types are housed in the taste buds. Type I, II and III cells are involved in taste sensation. Type IV cells are immature precursor cells [6].

With roughly 50 %, type I cells are the most abundant cell type in taste buds [12]. They appear to have glial-like function and are responsible for ion redistribution and clearance of extracellular neurotransmitters [13-16]. Type II cells are sensors for sweet, umami and bitter taste sensation. They express G protein-coupled receptors (GPCRs), called taste receptors type 1 (TAS1Rs) and taste receptors type 2 (TAS2Rs). [17-19] These receptors mediate the mentioned taste qualities and downstream signaling molecules transfer the signal to nerve fibers or type III cells via adenosine triphosphate (ATP) release [2,20,21]. To transmit the taste signal to the brain, type III cells have synaptic contacts with intragemmal nerve fibers [18]. Furthermore, otopetrin-1 (OTOP1), an ion channel for H^+, is present on the cell surface of type III cells, which is why they are responsible for sour taste [22,23]. In contrast to sweet, umami, bitter and sour tastes, which were shown to be recognized by a distinct cell type, salt taste perception was suggested to implicate all three cell types involving the epithelial sodium channel (ENaC), but exact mechanisms are still unknown [24-28]. The last group of type IV cells, also called basal cells, represent undifferentiated precursor cells that develop to type I, II or III cells [2,29].

Beside the five basic taste qualities, a sixth putative sense of taste, called the fat taste, is discussed in several studies. In addition to GPCRs, like GPR40, GPR41, GPR43 and GPR120, several non-GPCR proteins, like CD36 or inwardly rectifying potassium channels, are also associated with fat taste by detection of fatty acids. [30-35]

1.2. Sensation of sweet and umami taste

The first identified family of taste receptors was the group of TAS1Rs, which is composed of the three members TAS1R1, TAS1R2 and TAS1R3 [36-42]. They belong to the class C GPCRs, which have a common structure, containing the amino terminal venus flytrap domain (VFTD). This large extracellular domain is connected to the carboxyterminal seven transmembrane domain (7TM) by a cysteine-rich domain (CRD) (Figure 2). [43-45]

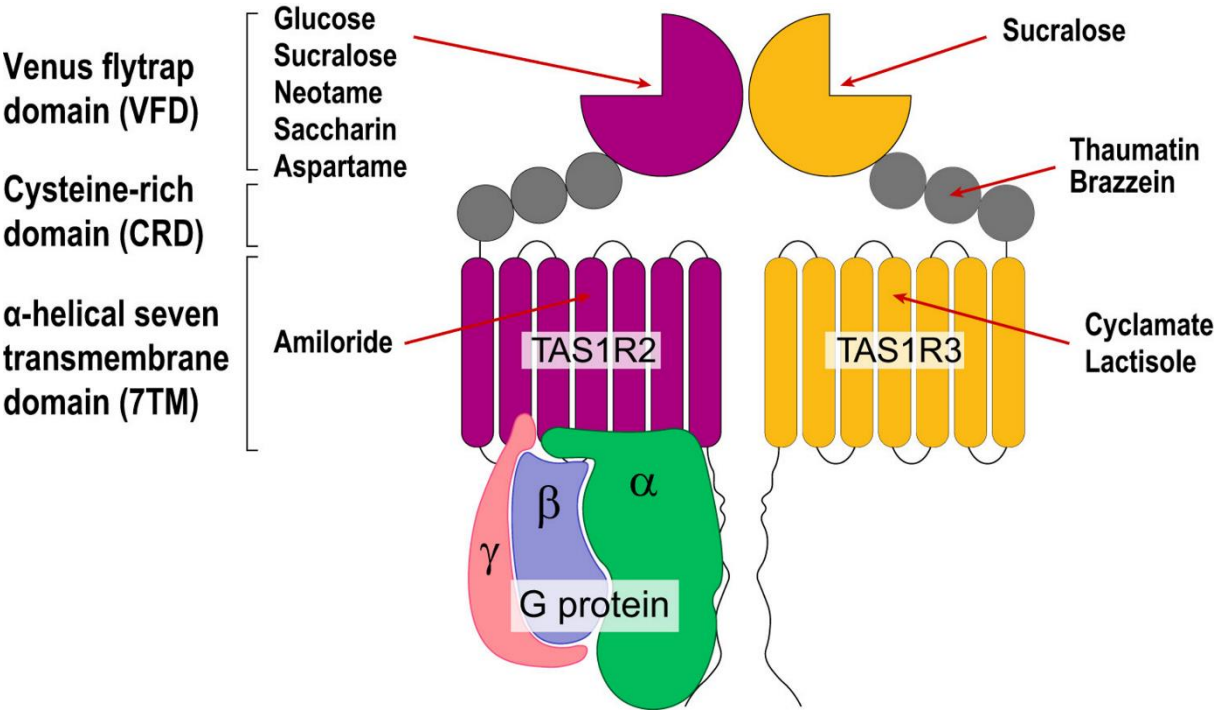

Figure 2: Schematic presentation of the sweet taste heterodimer composed of TAS1R2 and TAS1R3 including binding loci for different sweeteners. The typical domains of class C G protein-coupled receptors (GPCRs) with the large extracellular venus flytrap domain (VFD), which is connected to the 7-transmembrane domain (7-TM) via a cysteine-rich domain (CRD), are presented. Binding loci for different sweeteners are shown [45].

The TAS1Rs are responsible for sweet and umami tasting and are functional by forming heterodimers, with TAS1R3 as the common subunit of both taste receptors [40,41,46,47]. In humans, the heterodimer of TAS1R1 and TAS1R3 recognizes the amino acid L-glutamate, which is known as the stimulus for umami taste. The proposed binding site for this amino acid is the VFTD in the TAS1R1 subunit. [46,47] It was shown that there is a second binding site in close proximity for 5' ribonucleotides like inosine monophosphate (IMP) or guanosine monophosphate (GMP) [48,49]. These purinic acids are able to strongly enhance the intensity of umami taste by stabilizing the active confirmation of the receptor dimer [46,48-50]. The second heterodimer, which is composed of TAS1R2 and TAS1R3, is responsible for sweet tasting [41]. This receptor complex is able to perceive all kinds of sweet tasting compounds from mono- and disaccharides to natural and artificial sweeteners, as well as sweet proteins. This large number of ligands is recognized by different binding sites within both receptor subunits with relatively low sensitivity compared to other members of class C GPCRs (Figure 2).

[45,51-61] Beside the heterodimers, it was further suggested that a homodimer consisting of the TAS1R3 subunit is able to detect mono- and disaccharides in rodents [62].

In addition to the mentioned sweet and umami taste receptors, further alternative sensation pathways are discussed. There are three GPCRs known, which are activated by L-amino acids, called extracellular-calcium-sensing receptor (CaSR), GPCR, class C, group 6 subtype A (GPRC6A) and GPR92. [63-65] All of them were shown to be expressed in mouse taste bud cells indicating their role in taste sensation [66,67]. The unique taste of L-glutamate as umami is further detected by truncated forms of the glutamate receptor type 1 and 4 (mGluR1/4), which were also shown to be expressed in gustatory tissues [68-70].

An alternative sweet taste sensation pathway includes the glucose transporters sodium/glucose-cotransporter 1 (SGLT1) and glucose transporters (GLUT) [71-74]. The glucose entry into the cell via these transporters leads to an increase in ATP, followed by the blocking of K_{ATP} channels. This, in combination with a putative activation of volume-regulated anion channels (VRAC) due to cell swelling, might depolarize the cell and induce further downstream processes. [75-78] Beside taste bud cells, these transporters are also expressed in extra-oral tissues like enterocytes, enteroendocrine cells or brain neurons [79-82].

1.3. Bitter taste is mediated by TAS2Rs

The second family of taste GPCRs, called TAS2Rs, mediates bitter taste sensation. In contrast to the umami and sweet taste receptors, bitter taste receptors only possess a short N-terminal domain. Beside monomers, they can further form homomers, as well as heteromers. Recent studies showed no influence of heteromer formation on plasma membrane localization and functionality of TAS2Rs, which is why they are proposed to sense as mono- or homomers. [83-87]

Because of its unpleasant taste, bitterness is thought to warn mammals against the ingestion of toxic food components, but there is no direct correlation between bitterness and toxicity [88]. However, a mild bitter taste can also be appreciated and associated with favorable health effects [89-91]. In contrast to sweet and umami taste receptors

where only one TAS1R heterodimer is known, the family of TAS2Rs is composed of several subtypes, differing a lot in number from species to species. In humans, around 25 functional bitter taste receptors were identified. [92] Recently, the functionality of a 26th human TAS2R was elucidated [93]. In contrast, chickens only have three, mice 35 and coelacanth almost 80 bitter taste receptors [94,95]. Some mammals, like the bottlenose dolphin, do not express any bitter taste receptor indicating the complete loss of bitter taste [96]. Until today, there are still four human bitter taste receptors, named TAS2R42, TAS2R45, TAS2R48 and TAS2R60, which agonists are still unknown [97,98]. The remaining TAS2Rs are able to detect a broad range of bitter substances. To facilitate this, some of the TAS2R family members, called generalists, can detect a broad range of agonists. For humans, the three broadly tuned receptors TAS2R10, TAS2R14 and TAS2R46 are able to detect more than half of all known bitter substances. In contrast, some other receptors only have a small number of agonists, which are then called specialists, whereas the biggest group of TAS2Rs shows an intermediate agonist spectrum. [97] Due to the broad tuning of their bitter taste receptors, as found for chickens, a small amount of bitter taste receptors does not necessarily indicate a reduced perception of bitter taste [94]. Whether bitter taste receptor repertoires of different species are adapted due to consumption of bitter compounds because of eating habits or habitats is still not fully clarified [20,96,99].

Compared to several other GPCRs that detect second messengers like hormones or neurotransmitters, TAS2Rs are rather insensitive with potencies mostly between high nanomolar and low millimolar concentrations [97]. As toxic effects of most bitter compounds are dependent on concentration and a mild bitterness might have beneficial effects, harmless concentrations are well accepted, which broadens the availability of foods with low amounts of bitter substances like vegetables [20].

1.4. Signaling cascade of human taste GPCRs

In general, taste GPCRs share a common signaling cascade to transmit the activation signal (Figure 3) [100,101]. For bitter taste, ligand binding to the receptors induces conformational changes followed by the dissociation of the intracellularly bound heterotrimeric G-protein, consisting of Gα-gustducin and Gβ1γ13 [102,103]. The β and γ

subunits are activating the membrane-bound phospholipase C isotype β2 (PLCβ2) [100,104]. Activated PLCβ2 cleaves phosphatidylinositol-4,5-bisphosphate into diacylglycerol and inositol-1,4,5-triphosphate (IP_3), which activates the type 3 IP_3 receptor (IP_3R3), located in the membrane of the endoplasmic reticulum (ER) [105-107]. This receptor releases Ca^{2+}-ions from the ER to the cytosol. The increase in cytosolic Ca^{2+} concentration results in the opening of the membrane-bound monovalent-selective cation channels transient receptor potential channels M4 (TRPM4) and M5 (TRPM5), leading to the depolarization of the cell by influx of Na^+ [100,108-111]. Depolarization then triggers the activation of voltage-gated Na^+ channels (VGNC) [112]. The resulting action potential results in the opening of the voltage-gated ATP release channels calcium homeostasis modulator 1 and 3 (CALHM1/3) [113-116]. Both interact with each other to form a hexameric channel [116]. The secreted ATP is demonstrated to activate afferent nerve fibers in the taste buds [21].

A second pathway is elicited by the α-subunit of the G-protein. It is proposed that it is activating a phosphodiesterase, which hydrolyses cAMP and consequently results in a reduced activity of protein kinase A (PKA). In the absence of the α-subunit, PKA phosphorylates signaling compounds of the PLC pathway, leading to their inhibition. [117] The precise mechanism of this signaling cascade is still unknown, but an increase in intracellular Ca^{2+} by activation of cyclic nucleotide inhibited channels is proposed [118].

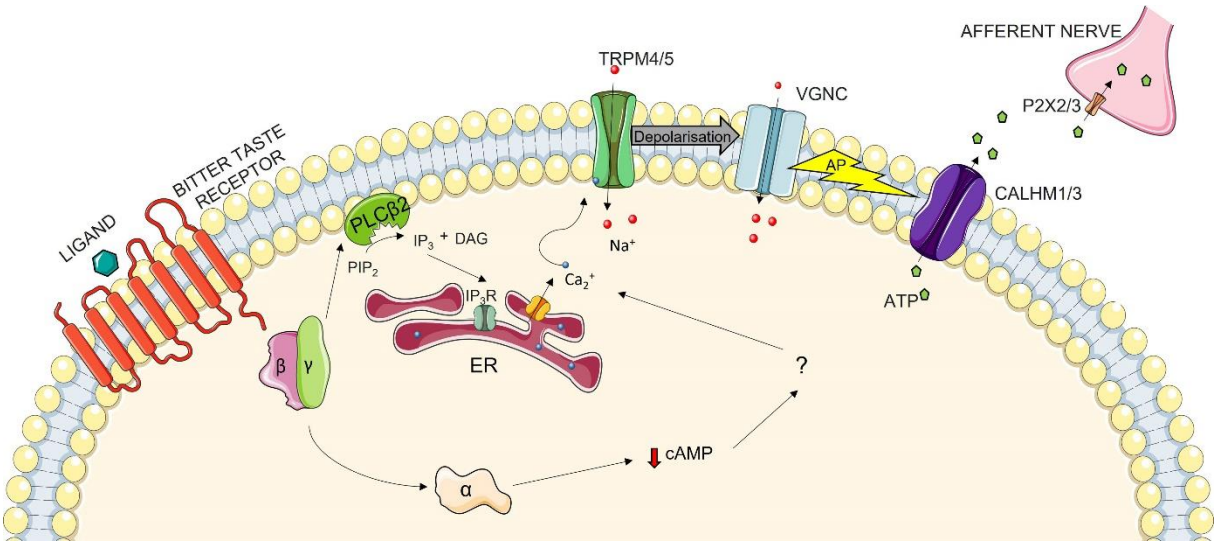

Figure 3: Downstream signaling cascade of bitter taste receptor activation. The bitter taste receptor in the plasma membrane binds extracellular agonists. Subsequent conformational changes of the receptor mediate the dissociation of the intracellular G-protein consisting of the three subunits G_{α}, G_{β} and G_{γ}. The β and γ subunits are activating the Phospholipase C subtype β (PLCβ). This activation leads to the production of inositol-1,4,5-triphosphate (IP_3), which is activating the IP_3 receptor (IP_3R). The opening of this calcium channel results in the efflux of Ca^{2+}-ions from the endoplasmic reticulum. This increased Ca^{2+} concentration triggers the opening of the transient receptor potential cation channels M4 and M5 (TRPM4/5), which leads to the depolarization of the cell, opening of voltage-gated Na^+ channels (VGNC) and the ATP release via the calcium homeostasis modulator 1 and 3 (CALHM1/3). The released ATP is taken up by afferent nerves via P2X ionotropic purinergic receptors 2 and 3 (P2X2/3) for further signal transduction. The α-subunit of the G-protein triggers hydrolysis of cAMP [101].

1.5. Structure-function correlation of bitter taste receptors

Bitter taste receptors are classified as G protein-coupled receptors and therefore, they possess seven transmembrane domains linked by three extracellular and three intracellular loops and ending in an extracellular amino and an intracellular carboxyl terminus [119]. However, bitter taste receptors only share low sequence homologies with other GPCRs, whereby a classification in a distinct group of GPCRs is difficult. Former publications showed that bitter taste receptors share the most similarities with the groups of frizzled-receptors or class A/Rhodopsin-like GPCRs. [119,120] Nowadays, they are classified as separate class T GPCRs [121]. Until recently, no experimental structure of bitter taste receptors was available and therefore knowledge of structural characteristics that are important for agonist binding and transmission of receptor signal was based on functional experiments or modeling approaches [122].

Even though some bitter taste receptors are able to respond to a broad range of agonists, former experimental studies confirmed the existence of one single binding pocket. By point mutation of amino acids that are responsible for strychnine responsiveness in TAS2R46, in TAS2R31 and TAS2R43, which are members of the same subfamily, the full agonist spectrum of TAS2R46 was transferred to TAS2R31 and TAS2R43. [123] The other way around, one specific point mutation in TAS2R46 led to the inhibition of TAS2R46 by GIV3727, which was originally identified as an antagonist of TAS2R31 and TAS2R43 [124]. However, later a second vestibular binding site was detected in TAS2R46, which is only transiently occupied and might play a role as a filter unit in agonist selectivity [122,125].

Depending on the bitter taste receptor, positions of all seven transmembrane domains were proposed to be involved in ligand binding by mutagenesis experiments and homology modeling approaches [123,126-138]. However, some studies suggest extracellular loops also play a role in receptor-agonist interaction. These findings are in conformity with the suspected vestibular binding pocket at the extracellular part of the TAS2R46. [125,139-142]

Recently, the first cryo-electron microscopy structure of a bitter taste receptor was elucidated. Thereby, the binding mode of the most potent TAS2R46 agonist strychnine was investigated in more detail. The binding pocket of TAS2R46 is built from positions of TM2, TM3, TM5 and TM7 forming a wide-open funnel. The two positions W88$^{3.32}$ and E265$^{7.39}$ turned out to be of special importance for recognition and placement of strychnine and other TAS2R46 agonists. [143] Both positions were already identified as crucial in previous mutagenesis and *in silico* modeling experiments [123].

A common feature of all 25 human bitter taste receptors that was shown to be important for bitter taste receptor functionality, is a highly conserved Asn-linked N-glycosylation in the center of the second extracellular loop (ECL) region, which is co-translationally added by the oligosaccharyltransferase. However, it was shown that glycosylation is not affecting acute receptor function, but its maturation or membrane trafficking, as co-expression of receptor transporter protein 3 (RTP3) and 4 (RTP4) with non-glycosylated TAS2R16 partially recovered the functionality of this bitter taste receptor. [144,145]

Beside agonist binding, former studies revealed some important structural requirements for TAS2R activation. There are similar conserved motifs of class A GPCRs found in bitter taste receptors. The $N^{1.50}xxI^{1.53}$ motif in TM1 and the $H^{7.49}S^{7.50}xxL^{7.53}$ motif in TM7 were proposed to be involved in stabilization of the inactive form of bitter taste receptors, as hyperactive receptors were generated by point mutagenesis of position 1.53 in TAS2R1, as well as of position 7.50 in TAS2R4. [119,146-148] The knowledge of features that are important for functionality of other GPCRs is also applicable for TAS2R38 [133]. Two amino acid positions in the bovine rhodopsin are responsible for structural rearrangements upon agonist binding by forming hydrophobic interactions [149]. In the TAS2R38 the corresponding positions are F255 and V296 and point mutations of both positions to amino acids that possess similar physical properties maintain the functionality of this receptor [133].

More insights into the activation process of bitter taste receptors are now available because of the structure elucidation of TAS2R46. There were four amino acid positions identified that are crucial for the stabilization of the active state of TAS2R46 by forming hydrophobic and π-π stacking interactions. These residues are $R55^{2.50}$, $F188^{5.51}$, $Y241^{6.51}$ and $Y271^{7.45}$, all well conserved in bitter taste receptors, indicating their general involvement in active TAS2R stabilization. A further well conserved motif among TAS2Rs, that was found in TAS2R46 and is the counterpart of $D^{3.49}R^{3.50}Y^{3.51}$ in class A GPCRs, is $F^{3.49}Y^{3.50}L^{3.51}$ [143,150]. The amino acid tyrosine in this motif is forming a hydrogen bond with the Gα gustducin subunit, which is why the motif is supposed to be involved in G protein binding. [143]

In former studies a point mutation at position 3.41 to tryptophan is used to conformationally stabilize class A GPCRs [151]. Indeed, in human bitter taste receptors this W3.41 is fully conserved, and structural information suggests the importance of this amino acid position for stabilization of the connection between transmembrane domains 3, 4 and 5 [143].

1.6. Bitter taste receptors in non-gustatory tissues

1.6.1. Tissue expression of G protein-coupled bitter taste receptors

Taste receptors were initially detected in gustatory tissues. Consequently, they were believed to be sensory receptors of food compounds and serve for quality evaluation of foods. However, since the beginning of this century, more and more studies elucidate the expression of taste GPCRs in several non-gustatory tissues outside the oral cavity indicating their role in further biological functions. One of the first extra-oral locations, where bitter taste receptor expression was identified, were epithelial cells in the gastrointestinal tract and the nasal cavity. [152,153] In the following years, more and more tissues that express bitter taste receptors were discovered (Figure 4) [154].

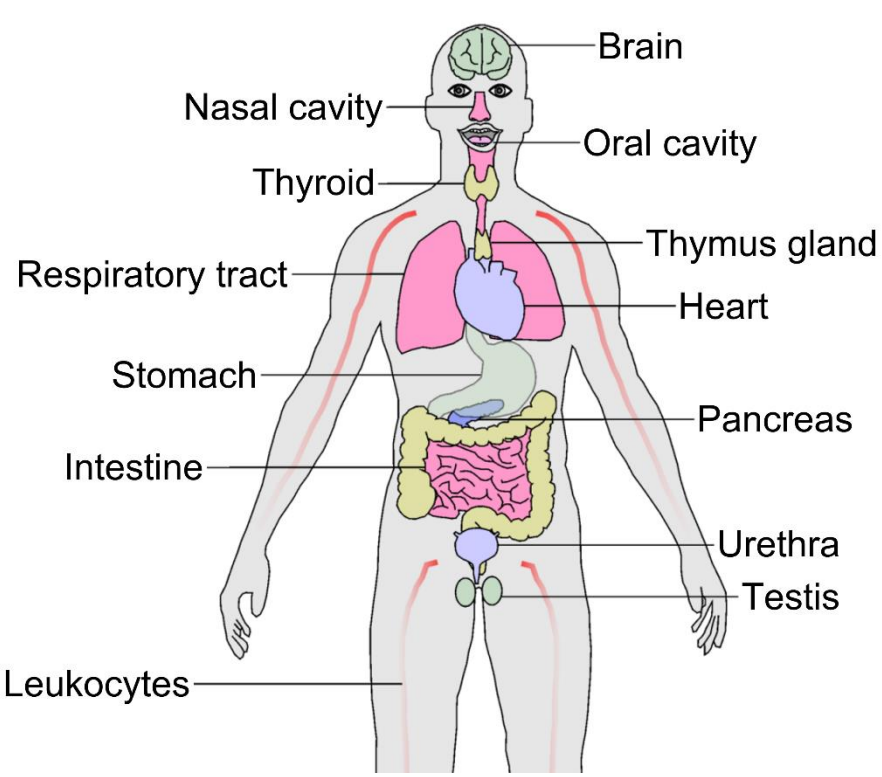

Figure 4: Bitter taste receptor expression throughout the human body. An overview of tissues and cells that were identified to express human bitter taste receptors is presented (modified with the permission of Behrens and Meyerhof [154]).

Nowadays, TAS2R expression is evident in tissues distributed all over the human body including the respiratory tract, the gastrointestinal tract, the nervous system, the reproductive system, the cardiovascular system, as well as the skin [152,155-159]. The expression of bitter taste receptors in non-gustatory tissues indicates their essential physiological functions. Evolution would have eliminated functionality in these tissues for example by suppressing transcription, if it is useless for human life and well-being. Besides, overexpression of bitter taste receptors was detected in disease related tissues like adipose and tumor tissues indicating a further involvement of TAS2Rs in disease development. [160-162] To elucidate the physiological relevance of extra-orally expressed TAS2Rs, they are an issue of current research addressing their agonists and the triggered biological functions.

1.6.2. Physiological roles of non-gustatory bitter taste receptors

Once bitter taste receptors are activated by their agonists, the downstream signaling cascade is initiated and in case of non-gustatory bitter taste receptors a distinct physiological function is triggered. Former studies already proposed several biological processes, where TAS2R activation is involved. A well-known example is the presence of TAS2Rs in solitary chemosensory cells and ciliated epithelial cells of the nasal cavity. It was shown that these cells express the human bitter taste receptors TAS2R4, TAS2R43 and TAS2R46, which in case of their activation, increase the ciliary beat frequency leading to the accelerated clearance of microorganism. [163] Activation of TAS2R38 leads to the release of nitric oxide, which is known as a bactericide. These results indicate that taste receptors in extra-oral tissues can modulate the innate immune response. [164]

Taste sensation varies between individuals because of single nucleotide polymorphisms (SNPs) that influence receptor function. A very well investigated receptor is the mentioned human TAS2R38, which is known to be present in two major genotypes that differ in three distinct amino acid positions. The mutations of proline to alanine at position 49, alanine to valine at position 262 and valine to isoleucine at position 296 lead to a complete loss of the response of TAS2R38 to its prime agonist phenylthiocarbamide (PTC). [165,166] Beside their influence on taste sensation, these

SNPs were further shown to play a role in innate immunity of humans. The TAS2R38 is expressed in the nasal cavity and individuals possessing the functional homozygous form PAV/PAV are less affected by infections caused by gram-negative bacteria. [164] In accordance with this finding was the observation that 90 % of all patients, who suffer from chronic rhinosinusitis, are homozygote AVI/AVI or heterozygote AVI/PAV representing the non-functional, respectively the less-functional form of TAS2R38 [167].

In the respiratory tract, bitter taste receptors are not only participating in innate immune response, but expression was further shown in airway smooth muscle (ASM) cells [168]. The activation of these TAS2Rs mediates a relaxing effect on human ASMs making them a promising target for bronchial asthma therapy [168,169].

Beside their role in homeostasis and immune response, TAS2Rs are further postulated to be involved in disease related processes like cancer development. On the one hand, bitter taste receptors like TAS2R4 and TAS2R14 are expressed in breast cancer cells and their activation elicits anti-proliferating and anti-migrating effects on cancer cells [170]. On the other hand, the activation of the expressed TAS2R38 in lipid droplets of pancreatic cancer cells triggers the activation of key transcription factors and indicates the involvement of bitter taste receptors in cancer progression [171].

These are only some examples of the multitude of physiological processes involving the activation of bitter taste receptors showing the importance of TAS2Rs beside taste sensation in the oral cavity. Due to their roles in several immune responses and development of diseases, they are a promising target for future drug design.

1.7. Endogenous compounds as promising agonist candidates for extra-oral bitter taste receptors

In former research, several synthetic and mostly plant derived natural agonists of human bitter taste receptors were identified [97]. Consequently, these exogenous compounds must be taken up from the environment and some of the identified non-gustatory tissues expressing TAS2Rs will not get in direct contact with these substances, as high amounts of bitter substances will already be excluded by the strong bitter taste in the oral cavity. Furthermore, some of these tissues like the brain

by the blood-brain barrier are well protected. For these reasons, it is hypothesized that agonists of TAS2Rs are of endogenous origin generated by synthesis or metabolism. [122]

1.7.1. Metal ions

Metal ions are essential in several biological processes in plants, animals, and humans. That includes mono-valent metal ions like Na^+ and K^+, which are important for the cell membrane potential and action potentials that are necessary, among other things, for transmission of neuronal signals [172]. Ca^{2+} is found in high concentrations in bones responsible for their stability [173]. Intracellularly, it is further involved in downstream cell signaling processes [174]. Additionally, metal ions are found as co-factors of several proteins. As co-factors of enzymes, they are critical for catalytic reactions. Iron for example as part of catalase, which is decomposing hydrogen peroxide, supports the detoxification of human cells. [175] In case of hemoglobin, iron is further participating in oxygen transport [176].

These examples indicate the importance of the permanent presence of metal ions in the human body and the diversity of biological processes they are involved in. For proper functionality, metal ion concentrations in the body must be leveled in a distinct range [177]. This means, concentrations, that are too low or too high, have negative effects on health. In the case of excessive concentrations, metal ions can be associated with toxicity [178-180]. Consequently, the intake of excessive amounts of metal ions should be avoided. The previously described taste of divalent metal salts as metallic, bitter and astringent would suggest, that the bitter taste should warn against ingestion. [181] In rodents, the involvement of TRPM5 and the transient receptor potential vanilloid-1 (TRPV1) in sensation of divalent metal salts was shown [182]. Whereas the TRPV1 activation may be responsible for the astringent mouthfeel, the TRPM5 is a downstream signaling component of G protein-coupled taste receptors, indicating a role of TAS2Rs in detection of divalent metal ions [100,109,182]. Indeed, two former studies identified the human TAS2R7 as bitter taste receptor for di- and trivalent metal ions like Mg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+}, which may explain the proposed bitter taste [130,183]. A well-known example for bitter tasting metal ions are the water springs in the British city

Epsom, which contain high concentrations of magnesium sulfate also called Epsom salt [184]. Whether bitter taste response to these salts is important to avoid the ingestion of too high concentrations of metal ions, which may influence the well-balanced body concentrations or TAS2R7 is extra-orally involved in non-gustatory functions or even both has still to be clarified.

1.7.2. Bile Acids

1.7.2.1. Synthesis and metabolism of bile acids in the human body

The bitter taste of the body fluid bile is already known for a long period of time [185]. A major functional component of bile is the class of bile acids, which are endogenously produced by the classical and the alternative pathway in hepatocytes of the liver and stored in the gallbladder [186,187].

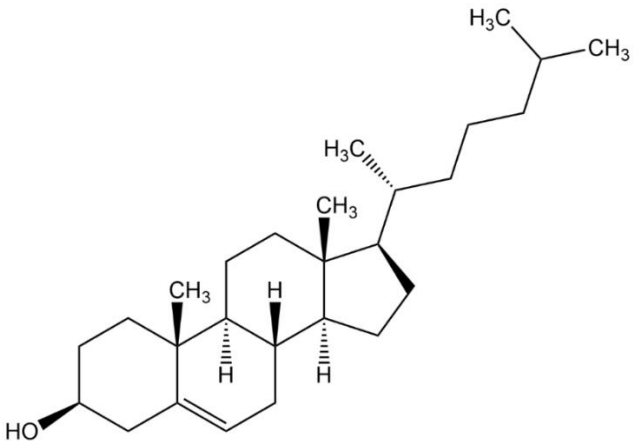

Figure 5: Cholesterol, the scaffold structure of bile acids. The structure was generated with ChemDraw 21.0.

The primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are produced in the liver from cholesterol as scaffold structure (Figure 5). Prior to their secretion into the gallbladder, these primary bile acids are conjugated to the amino acid moieties taurine or glycine by the liver enzyme human bile acid–CoA:amino acid N–acyltransferase (hBAT). [188] During the synthesis of bile acids 17 cytochrome P450 enzymes (CYPs) are involved [189]. In the classical pathway, the liver specific 7α – hydroxylase CYP7A1 and CYP8B1 are the most important ones. CYP7A1 triggers the

bile acid synthesis from cholesterol to form 7α–hydroxycholesterol. [190] Consequently, CYP7A1 is responsible for the bile acid pool size. In contrast, CYP8B1 is necessary for the synthesis of CA and determines the ratio of the primary bile acids CA and CDCA. For the synthesis of both bile acids from 7α – hydroxycholesterol another CYP enzyme, named CYP27A1 is necessary. [191] This enzyme is furthermore the major component of the alternative synthesis pathway, as it catalyzes the oxidation of cholesterol resulting in 27–hydroxycholesterol. From this precursor the primary bile acid CDCA is formed by hydroxylation by CYP7B1. In contrast to the classical pathway, the alternative pathway is not restricted to the hepatocytes, as its enzymes are also present in other tissues. [192] The majority of bile acids is synthesized by the classical pathway and only a small part is produced by the alternative pathway [193] (Figure 6).

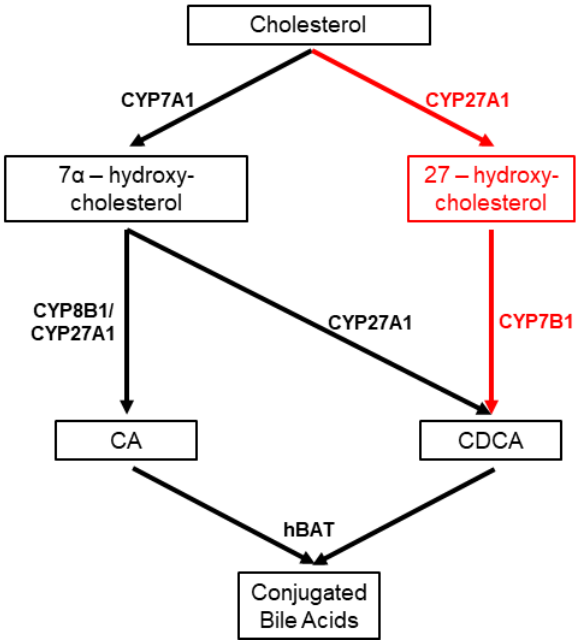

Figure 6: Simplified presentation of the synthesis of bile acids in the liver. Bile acids are synthesized in the liver from the scaffold structure cholesterol**.** During the synthesis via the classical pathway (black), 17 cytochrome P450 enzymes (CYPs) are involved. The three most important ones are presented here. CYP7A1 drives cholesterol to the bile acid production resulting in 7α–hydroxycholesterol. This compound is transformed to the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA). For both, CYP27A1 is necessary. The ratio of CA and CDCA is determined by CYP8B1. Before the secretion into the gallbladder, bile acids are conjugated to either taurine or glycine by the liver enzyme human bile acid – CoA:amino acid N–acyltransferase (hBAT). In red, the alternative pathway is presented. This pathway is not restricted to the liver. 27–hydroxycholesterol is built from cholesterol. This compound is then further modified to the primary bile acid CDCA by CYP7A1.

As bile acids fulfill nutritional functions, like solubilization and supporting the digestion of lipophilic food compounds, they are released into the small intestine after food intake [186]. There, the released conjugated bile acids are exposed to modifications by the gut microbiota. First, they are deconjugated back to primary bile acids by the microbial bile salt hydrolase (BSH) by cleaving the amide bond at C24. [194,195] In the colon, these bile acids are further modified to secondary bile acids, generating an enormous amount of different bile acid variations [194]. Therefore, gut microbiota play a significant role for the pool size and diversity of bile acids in the human gut. All changes in the composition of gut microbiota may lead to an influence on bile acid composition and consequently trigger downstream effects like different diseases. [196]

In the final step of the enterohepatic circulation, bile acids are reabsorbed into the enterocytes of the intestine, by either passive diffusion or active transport via specific transporters like apical sodium-dependent bile acid transporter (ASBT) [197,198]. By organic solute transporter alpha and beta (OSTα/OSTβ) bile acids are released into the portal venous blood and transported back to the liver, where they are recycled and enter the enterohepatic circulation again [199,200]. It was shown that bile acids can pass through this circulation four to twelve times per day (Figure 7) [201,202].

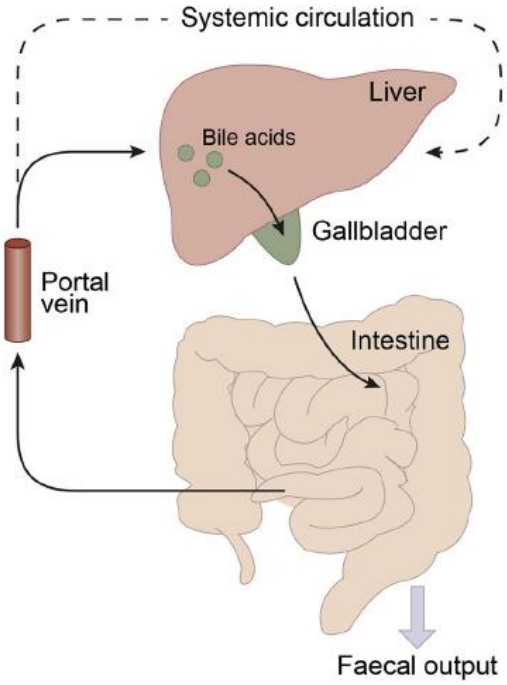

Figure 7: Schematic presentation of the enterohepatic circulation. Bile acids are synthesized in the liver and stored in the gallbladder in their conjugated forms. After food intake, bile acids are released into the small intestine for nutritional functions. There bile acids are modified by the gut microbiota to unconjugated and secondary bile acids. Bile acids are reabsorbed by the intestinal enterocytes and transported back to the liver via the portal vein blood. In the liver bile acids are recycled and enter the enterohepatic circulation again. Only a small proportion of the total amount of bile acids is excreted by the feces or is released to the systemic circulation [202].

95 % of the bile acid pool are recycled by the enterohepatic pathway and only 5 % are excreted with the feces and newly synthesized [187]. To facilitate the homeostasis of a constant amount of bile acids in the body, the bile acid synthesis in healthy people is controlled via negative feedback regulation. In hepatocytes, the binding of bile acids to the farnesoid X receptor (FXR) leads to a reduction of the CYP7A1 expression and in enterocytes this interaction triggers the production of fetal growth factor 19 (FGF19), which migrates to the liver and binds to the specific cellular receptor FGFR4 leading to the repression of the *cyp7a1* transcription. [203,204]

1.7.2.2. The potential of bile acids as bitter taste receptor agonists

It was shown in former works, that bile acids are able to activate different human cellular receptors like the FXR, the vitamin D receptor (VDR) and Takeda G-protein

coupled receptor 5 (TGR5) [205-209]. Similar to sweet, umami and bitter taste receptor, the TGR5 is a GPCR, which indicates that bile acids may also be ligands for human taste receptors [209]. As it was described above, taste receptors are expressed in several tissues throughout the human body [154]. Because of the enterohepatic circulation of the bile acids and their release in the blood stream, they also reach regions, where the expression of taste receptors was shown [202,210].

Following their binding to their cellular receptors, bile acids trigger different downstream signaling events. It was published, that the activation of TGR5 by taurolithocholic acid (TLCA) leads to a reduction of the expression of proinflammatory cytokines and an induction of the expression of the anti–inflammatory cytokine IL-10 [211]. Because of different sensitivities or specificities of the cellular bile acid receptors for different bile acids, the bile acid composition in the gut may play a crucial role for the maintenance of the physiological homeostasis [196]. For this reason, changes in the composition of gut microbiota, which have a big impact on the modification of bile acids, or failures in the synthesis of bile acids, may influence this homeostasis and trigger diseases, like colorectal cancer or small intestinal bacterial overgrowth [212]. For this reason, interest in pharmacology of bile acids is increasing more and more. The high quantity of 48 produced bile acids by the human body, and multiple modification sites for microbiota in the gut, expanding the number of possible derivatives of bile acids to nearly 400, complicates this research issue [213]. In this context, the occurring concentrations of bile acids within the human body are of main interest. Depending on the location, these concentrations can vary a lot. In the gallbladder, where the bile acids are stored, concentrations can reach up to 300 mM, whereas concentrations in blood plasma are below 5 µM [205]. But it was shown, that bile acid concentrations in the blood plasma can increase up to 100 fold in humans during liver failure [214]. In this case, bile acid receptor activation in extra–oral tissues may be of interest.

As mentioned above, it was published that taste receptors are also expressed in the brain tissue and the activation mechanism of these receptors is mostly unknown [156]. Former studies were able to show the occurrence of bile acids in this region [215]. To reach brain tissue by systemic circulation bile acids have to cross the blood-brain barrier. For conjugated and unconjugated bile acids it was shown that they are able to

cross this barrier, possibly by diffusion through phospholipid bilayers or active transport mechanisms. [216-218] The fact that unconjugated bile acid concentrations in the rat brain and serum are correlated is a further indication for diffusion processes [219].

1.8. Functional cell-based assay for bitter taste receptor agonist identification

The elucidation of bitter taste receptor agonists and the establishment of dose-response relationships to identify the potency of agonists is based on an established heterologous expression system (Figure 8). Therefore, the cell line HEK293T is transfected to stably express the G protein chimera Gα16gust44, which can be recruited for signal transduction by bitter taste receptors. [220,221] The transient transfection of an expression vector containing a bitter taste receptor coding cDNA elongated by the N-terminal part of the rat somatostatin receptor subtype 3 (sst3-tag) for proper membrane trafficking, results in a functional receptor cell assay [222-224]. The application and subsequent binding of agonists of the transfected TAS2R triggers the downstream signaling cascade via Gα16gust44 to result in a Ca^{2+}-release from intracellular calcium stores. Using a fluorescent calcium indicator, this increase in Ca^{2+} and consequently in fluorescence can be measured quantitatively by an automated fluorescence plate reader. [94,225] To exclude any unspecific cellular effects, the cells transfected with the empty expression vector serve as a negative control [94]. An appearing amplitude compared to the negative control is interpreted as receptor activation and the applied substance is identified as agonist of the transfected bitter taste receptor. Application of descending concentrations of the agonist reveals the activation threshold concentration, which is defined as the lowest concentration that triggers a significant receptor response. [226]

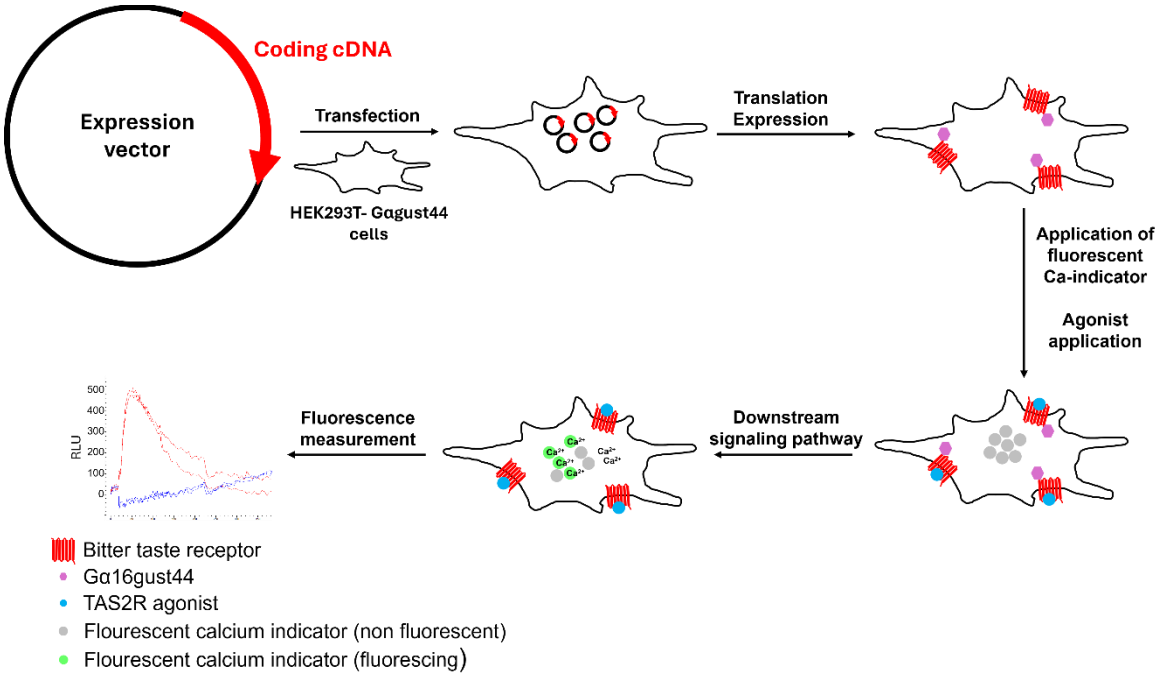

Figure 8: Schematic presentation of heterologous expression system for identification of bitter taste receptor agonists. An expression vector carrying the full coding cDNA of a distinct bitter taste receptor (TAS2R) is transiently transfected into HEK293T cells, which express the G protein Gα16gust44. The TAS2R cDNA is then translated intracellularly and the TAS2R is expressed and trafficked to the cell membrane. Afterwards, a fluorescence Ca-indicator and a TAS2R agonist is applied to the transfected cells. Agonists are activating the membrane-located TAS2Rs and the downstream signaling cascade (see chapter 1.4) is initiated via Gα16gust44. The released calcium ions form a complex with the Ca-indicator. The resulting fluorescence can be measured, and a significantly increased fluorescence compared to the negative control (empty vector transfection without TAS2R cDNA) indicates the activation of the transfected bitter taste receptor by the applied agonist.

2. Objectives of the thesis

As described above, bitter taste receptor expression was detected in non-gustatory tissues. To elucidate their assumed physiological relevance, the search for agonists and the elucidation of their biological functions are tasks of the present research. Compared to numerous other GPCRs, bitter taste receptors are relatively insensitive receptors. Therefore, it is assumed that many of the substances that are ingested, will not reach peripheral tissues in concentrations sufficient for receptor activation. Consequently, we hypothesize that agonists can include endogenous compounds and previous research already gave hints to compound classes present in the circulation including amino acids, metal ions and bile acids. [183,225,227]

Within this work, we provide a deeper insight into the activation profiles of human bitter taste receptors by bile acids and link our results to known endogenous concentrations to clarify the potential for the activation of non-gustatory bitter taste receptors. In this context, the conservation of bitter taste responses to metal ions and bile acids was investigated to conclude the importance of the maintained bitter taste response in evolutionary distantly related species. Finally, we also performed molecular modeling studies to clarify the binding mode of bile acids to their corresponding receptors.

3. Results

3.1. Structure-Function Analyses of Human Bitter Taste Receptors – Where Do We Stand?

The review *"Structure-Function Analyses of Human Bitter Taste Receptors – Where Do We Stand?"* (https://doi.org/10.3390/molecules25194423) was published in "Molecules" in 2020. Maik Behrens and Florian Ziegler participated equally in writing the manuscript and revising it according to the reviewers' suggestions.

For the discovery of bitter taste receptor agonists, which may serve as drug candidates, and the understanding of signal transduction, the structure elucidation of bitter taste receptors is of major interest. Until the publication of this review, no experimental structure of any TAS2R was described, which is why structural information on bitter taste receptors were solely based on functional experiments and homology modeling studies. This review gives an overview of the current knowledge of structural requirements for proper bitter taste receptor activation and function. First, some general characteristics of TAS2Rs including their categorization in the family of GPCRs, their expression and membrane trafficking in heterologous expression systems, their agonist tuning breadth and potency, as well as the general signaling pathway of TAS2Rs are described.

In the second section, the common methods to elucidate receptor structures are illustrated. These methods include the experimental procedures to obtain protein structures. As no experimental structures of bitter taste receptors were known until recently, current data are based on the described homology modeling approach and the functional heterologous expression in combination with in vitro mutagenesis. Subsequently, all previously published data on the location of ligand binding pockets of the receptors TAS2R1, TAS2R4, TAS2R7, TAS2R10, TAS2R14, TAS2R16, TAS2R20, TAS2R38, TAS2R40 and TAS2R46 are summarized and all known amino acid positions that are involved in ligand binding are shown. Finally, we gave insights into the current knowledge of structural receptor characteristics, which are important for receptor activation and signal transduction.

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Review

Structure-Function Analyses of Human Bitter Taste Receptors—Where Do We Stand?

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Abstract: The finding that bitter taste receptors are expressed in numerous tissues outside the oral cavity and fulfill important roles in metabolic regulation, innate immunity and respiratory control, have made these receptors important targets for drug discovery. Efficient drug discovery depends heavily on detailed knowledge on structure-function-relationships of the target receptors. Unfortunately, experimental structures of bitter taste receptors are still lacking, and hence, the field relies mostly on structures obtained by molecular modeling combined with functional experiments and point mutageneses. The present article summarizes the current knowledge on the structure–function relationships of human bitter taste receptors. Although these receptors are difficult to express in heterologous systems and their homology with other G protein-coupled receptors is very low, detailed information are available at least for some of these receptors.

Keywords: bitter taste receptor; TAS2R; GPCR; molecular modeling

1. Introduction

The human sense of taste is indispensable for the rapid assessment of the chemical composition of food. The concentrations of sodium ions and protons as well as the energy content in the form of carbohydrates and proteins, by means of their building blocks, mono- and disaccharides and L-glutamic acid, respectively, are sensed as salty, sour, sweet and umami. The fifth basic taste quality, bitter, is elicited by a large number of chemically diverse substances of which a considerable proportion is rather poisonous, and hence, their ingestion needs to be avoided [1]. For each of these five basic taste qualities specific receptors or receptor families exist, which are expressed in sensory cells located in the oral cavity [2]. Whereas the sour taste receptor, otopetrin-1 [3–5], and the salt taste receptor, ENaC (epithelial sodium channel) [6], represent ion channels, sweet, umami (the taste of L-glutamic acid in humans) and bitter are sensed by G protein-coupled receptors (GPCRs) [7]. The three members of the taste 1 receptor (TAS1R) gene family assemble to form the functional sweet taste receptor heteromer, TAS1R2/TAS1R3 [8–10] and the corresponding umami taste receptor, TAS1R1/TAS1R3 [11,12], respectively. The taste 2 receptor (TAS2R) gene family is devoted to the detection of bitter substances [13–15].

In humans, the TAS2R gene family consists of ~25 putatively functional members [16]. The number of bitter taste receptor genes is not conserved among vertebrates [17]. Whereas primates and rodents possess similar or slightly higher gene numbers compared to humans, respectively, the gene numbers in other vertebrates can fluctuate between 0 to 3 at the low end and 56 to 74 at the high end [17]. There are initial hints that a low number of functional bitter taste receptors could be partially compensated by elevated average tuning breadths [18]. While the functional profiling of bitter taste receptors in the early years after their discovery was somewhat biased towards human TAS2Rs [16], more recently other vertebrates' receptors were more avidly investigated. To date, we know at least one bitter agonist

for 21 of the 25 human TAS2Rs [16], 21 of 35 mouse Tas2rs [19] and we have such information for several bird species [18], the domestic cat [20,21], rat [22,23], frog [18], fish [24], bat [25] and some primates [26–31].

After the successful characterization of about half of the human TAS2Rs and the finding that TAS2Rs can be classified according to the number of agonists into broadly tuned receptors with numerous agonists, narrowly tuned receptors with very few agonists, receptors recognizing selective chemical classes and intermediately tuned receptors [16], the structural features determining the tuning characteristics especially of broadly tuned TAS2Rs moved into the focus of research. However, it was, and still is, a major obstacle that bitter taste receptors only exhibit very minor homology with other GPCR families [32,33] and that experimental structures of TAS2Rs are lacking. Hence, homology modeling was necessary despite the fact that homology was very low. The first attempts to elucidate the structures of TAS2Rs were, therefore, done either by functional experiments combined with mutagenesis of receptor positions believed to play an important role in agonist interaction only [34] or by pure homology modeling and docking experiments [35,36]. The first publication using a combination of functional experiments, point mutageneses and in silico analyses was done with human TAS2R46 [37] and this approach quickly was adopted for subsequent investigations, because it allowed the iterative refinement of the obtained structures. To date, a considerable number of studies have been performed on multiple human and non-human bitter taste receptors, providing insight into the architectures of bitter taste receptor binding sites and how a diverse set of compounds can be accommodated.

Yet, all models published to date still have to be considered low-resolution models [38]. This potential lack in accuracy will likely continue until experimental crystal structures of bitter taste receptors become available. Because the heterologous expression of chemoreceptors, including bitter taste receptors, is considered particularly difficult [14,39–41], it is not surprising that these receptors are trailing in the list of successful structure elucidations. Moreover, since bitter taste receptors also share very limited homology among each other, a single crystal structure will likely not fully solve the resolution issues. Another problem that affect the prediction of bitter taste receptor structures and agonist interactions is the difficulty to model extracellular loop (ECL) regions even with low precision [32]. Since the ECL of TAS2Rs are close to the experimentally predicted agonist binding sites, their participation in agonist interactions, although frequently proposed, requires confirmation. This is even more challenging because it was shown that TAS2Rs are glycoproteins exhibiting a highly conserved site for asparagine-linked glycosylation in the center of the second ECL [42], a feature that has not yet been incorporated in homology modeling and docking experiments thus far.

The present article summarizes the current knowledge regarding bitter taste receptor biochemistry and cell biology as well as the methods that are currently applied to investigate their structural features. Then, the localization and architecture of the receptor binding pockets will be detailed before the receptor activation mechanism is discussed. Finally, open questions and future directions are highlighted.

2. General Features of TAS2Rs

Although the typical seven transmembrane domains, linked by three intra- and three extracellular loops, an extracellular amino terminus and an intracellular carboxyl terminus clearly earmark the TAS2Rs as members of the GPCR superfamily, the low homology with any other GPCR class (e.g., the amino acid sequence of chicken Tas2r1 shares about 10% identity and 30% similarity at the most with currently crystallized GPCRs [38]) makes the appropriate integration into the superfamily difficult. Whereas some researchers propose a common branch with frizzled-receptors based on few conserved amino acid sequence signatures [33], others favor the grouping with class A/Rhodopsin-like GPCRs because of the similarities shared with respect to the localization and architecture of the ligand-binding pockets [32].

Similar to the large odorant receptor (OR) family, TAS2Rs are difficult to express in heterologous mammalian cell lines. A successful strategy to overcome this problem was to connect the receptors'

coding sequences with N-terminal residues of other GPCRs such as the N-terminal 20 or 39 amino acids of bovine rhodopsin [14,43] (named "rho-tag") or the N-terminal 45 amino acids of the rat somatostatin receptor subtype 3 [22,44] (named "sst3-tag") and to use these chimeric receptors for functional heterologous expression assays. These amino terminal sequences have been coined "export-tags" to highlight their roles in the routing of receptors to the plasma membrane. As the necessity to generate fusion proteins for functional expression in vitro indicated that chemosensory cells may possess factors that facilitate functional expression in vivo, which are lacking from heterologous cells, researchers have been searching for these factors. For ORs, specific members of two small gene families of auxilary factors, the receptor transporting proteins (RTP) 1–4 and the receptor expression enhancing proteins (REEP) 1–6, were identified [45]. Later, it turned out that some of the bitter taste receptor subtypes also benefited from the co-expression of distinct members of the RTP family, namely RTP3 and RTP4 [46]. Whereas some TAS2Rs become trapped in intracellular compartments of human embryonal kidney cells when expressed without sst3-tag, RTP3- and likewise RTP4-coexpression resulted in elevated levels of these receptors at the cell surface [46]. Another important feature contributing to the levels of functional TAS2Rs at the cell surface in heterologous cell lines is the existence of an N-linked oligosaccharide moiety in the center of the second ECL, which has been predicted by the presence of a highly conserved consensus sequence in all 25 human TAS2Rs [42]. Similar to the observations of native TAS2Rs without export-tags being insufficiently expressed at the cell surface, TAS2Rs without an oligosaccharide-side chain attached to their second extracellular loop also show functional deficits when expressed in heterologous cells. Intriguingly, co-expression of RTP3 and RTP4 partially restored the function of non-glycosylated TAS2R16, suggesting that ECL2-glycosylation is not required for acute receptor function, but rather for proper folding and/or receptor routing to the plasma membrane [42]. Surprisingly, also the presence of certain agonists may promote cell surface localization of TAS2Rs. This has been shown at the example of quinine, a bitter and membrane-permeable natural compound, and TAS2R4 as well as four additional human TAS2Rs, indicating that quinine may act as pharmacochaperone [47]. Other test substances, including the likewise amphiphilic, membrane-permeable substance dextrometorphan did not show a comparable effect, indicating that this activity of quinine might be rather exceptional.

Similar to numerous other GPCRs (for a review see [48]), TAS2Rs can form oligomers [49]. As the 25 human TAS2Rs co-expressed in vitro have been demonstrated to readily form homomers as well as heteromers with each other, one can assume that the population of bitter taste receptor cells (TRC) in vivo could possess ~325 distinct receptor dimers. Indeed, it was assumed that such a large number of different receptors might be necessary to detect all the diverse bitter substances present in nature. However, it turned out that no functional changes that could be attributed to heteromeric TAS2Rs could be observed. Instead, the existing agonist profiles of TAS2Rs were sufficiently explained by the homomeric receptors [49,50]. In light of the extremely broad tuning of especially TAS2R10 [22], -R14 [51] and -R46 [52], each recognizing about one-third of all bitter substances and combined about half of them [16], heteromerization may not be necessary to broaden the receptive range of the TAS2R repertoire [17]. Not all TAS2Rs are broadly tuned; in fact, several receptors are very narrowly tuned responding only to one–three bitter compounds, whereas the majority of the TAS2Rs exhibit intermediate tuning breadths. At present, four TAS2Rs remain orphan [16,53].

Compared to other GPCRs, TAS2Rs are rather insensitive, detecting their agonists at concentrations between the mid nanomolar and the low millimolar range [54]. As taste receptors in the oral cavity are confronted with concentrated mixtures of chemicals from food items, an elevated sensitivity would not necessarily be beneficial, but could rather lead to the rejection of edible food, which would be an evolutionary disadvantage. It has to be noted though that the expression of bitter taste receptors is not limited to the oral cavity. The list of non-gustatory tissues that possess bitter taste receptors is long and the extraoral expression and function of taste receptors has been subject to several comprehensive reviews recently [55–60]. Therefore, it should just be mentioned here that the function of TAS2Rs is not limited to gustation, but extends to roles in innate immunity and respiratory function, regulation of

digestive function and metabolism as well as male fertility. Hence, the physiological roles of TAS2Rs clearly go beyond taste and tastant detection, an interaction with endogenous agonists and metabolites must be taken into account.

The canonical signal transduction cascade in human bitter taste receptor cells has been described in much detail in a variety of review articles [2,61–63]. Briefly, the activation of TAS2Rs by bitter agonists result in the activation of a heterotrimeric G protein composed of Gα-gustducin, a Gαi-type subunit identified first in the gustatory system [64], Gβ3 (Gβ1) [65] and Gγ13 [66]. Upon dissociation of the activated G protein, the βγ-subunits activate phospholipase Cβ2 (PLCβ2), resulting in the production of the second messenger inositol 1,4,5-trisphosphate (IP_3) along with diacylgylcerol from the signaling precursor phosphatidylinositol 4,5-bisphosphate (PIP_2) [67]. Next, IP_3 binds to its ER-membrane-resident receptor, IP_3R3, which, upon opening, allows the flux of calcium ions stored in the ER-lumen into the cytoplasm [68,69]. The increased cytosolic calcium level in turn triggers the opening of the transient receptor potential channel TRPM5 [70] in the plasma membrane, leading to an influx of extracellular sodium ions causing the cell to depolarize, which is followed by action potentials generated through voltage-gated sodium channels [71]. Finally, the neurotransmitter adenosine 5′-triphosphate (ATP) [72] is secreted through the calcium homeostasis modulator 1 (CALHM1) [73], a pore-forming voltage-gated channel, and purinergic afferent nerve fibers are activated [72] to transmit the signal to the brainstem.

3. Different Approaches to Investigate TAS2Rs

3.1. Obtaining Experimental Structures

To assess ligand binding to GPCRs and the existence of intramolecular networks that result in structural transformations involved in receptor activation, experimental structure determination would be extremely valuable. Even though the number of experimentally solved structures by crystallization and X-ray diffraction as well as cryo-EM approaches almost 70 (https://zhanglab.ccmb.med.umich.edu/GPCR-EXP/, accessed August 2020), not a single bitter taste receptor is included. Since TAS2Rs were not considered as relevant drug targets until very recently, one could imagine that these receptors were not ranked as a top priority for such kind of analyses. The findings that some bitter compounds represent highly efficient drugs for asthma treatment [74] may serve as alternative anti-diabetic drugs [75], and could even become relevant for the treatment of cancer [76], may result in a shift of priorities.

Ideally, the necessary material to prepare receptors for structure determination can be accessed from natural tissue sources in which the receptor of interest is strongly expressed at high concentrations. Not surprisingly, early receptors successfully subjected to initial structure determinations or even X-ray crystallography were the nicotinic acetylcholine receptor from the electrical organ of the electric ray Torpedo marmorata [77] and bovine rhodopsin that could be purified from bovine retinas [78], respectively. Unfortunately, chemosensory tissues, such as main olfactory epithelia, epithelia of vomeronasal organs or gustatory papillae, represent perhaps the worst source for such purification attempts, because of their small sizes, mixed cell and receptor populations interspersed with numerous non-sensory cells and with high, lifelong turnover [79–81].

To obtain large quantities of chemoreceptors, researchers relied on prokaryotic and eukaryotic overexpression systems. Thus far, prokaryotic overexpression and purification has not convincingly resulted in properly folded and functional chemoreceptors, although some publications reported that bacterially-produced taste receptors specifically interact with their agonists in particular as part of biophysical sensors [82]. However, the rather disappointing outcome of bacterial overexpression of chemoreceptors applies only to full length receptors including the integral membrane-associated 7-TM region. Globular extracellular domains have been successfully produced and used to assess ligand binding characteristic with excellent results [83,84]. Most researchers favor the use of eukaryotic cells ranging from yeast to mammalian cells to overexpress chemoreceptors (for a recent review, see [39]).

Yet, as outlined above, a plethora of problems make chemoreceptor expression in eukaryotic cells demanding, and this is likely among the reasons for the absence of experimental structures to date.

3.2. Homology Modeling

To get first insights into the structure and function of bitter taste receptors, in silico modeling of the peptide sequences has been performed using two different approaches. A pretty straightforward method relies on the homology of a peptide sequence with another peptide sequence for which a crystal structure exists [36]. It is assumed that sequence similarities translate into structural similarities, and hence, the query sequence is more or less simply folded onto the structure determined for the template sequence. This can be done with the help of commercial software packages or free online tools. Depending on the degree of homology, the initial alignment of the query and template sequences can be very demanding or rather easy if homology is extensive. Unfortunately, in addition to the initial resolution of the structurally analyzed template receptor, a low degree of homology will result in low resolution models. This is certainly the case for bitter taste receptors which exhibit very low sequence similarity with any other GPCR-family [38,85]. Knowing that the homology of TAS2Rs with other GPCRs is low, and thus that homology modeling can only provide low-resolution models, another approach to model TAS2Rs, called ab-initio modeling, was suggested. Here, the entire model was prepared in silico without using a specific template. Only few models were generated using this method [35,86].

After molecular modeling, the generated receptor structure can be used for in silico docking experiments that usually require the suitable preparation of ligand structures, a feature that is included in the above-mentioned software packages. It should be noted that ligand docking into a receptor model typically requires the researcher to decide whether and which receptor residues are designated as "flexible" and where in the receptor model the docking should take place. Ideally, this is done after determining potential binding pockets with sufficient volumes to accommodate the ligand(s), a feature also commonly available in commercial software packages. After docking of cognate ligands, the results ideally correlate with observed experimental data for the modeled receptor on both, the qualitative level as well as the quantitative level, such that only ligands that have been shown to interact with the receptor are docked with high scores and that the experimentally observed rank order of potencies is mirrored by the observed docking scores. Here, another problem with bitter taste receptors has to be mentioned; compared to other GPCRs, bitter taste receptors show low affinity between ligands and receptors [87]. In general, one needs to anticipate rather low docking scores, which may be close to, or overlapping with, alternative docking poses, leaving the determination of the most likely docking pose to the scientist.

3.3. Functional Heterologous Expression in Combination with In Vitro Mutagenesis

Because of a lack of experimental structures and the problems that arise from low resolution homology models, the current gold standard for the determination of receptor structures is composed of a combination of molecular modeling and docking with wet lab experiments, such as functional expression assays and in vitro mutagenesis of the investigated receptors. The combination of these experiments can be performed in an iterative fashion resulting in progressive improvements of in silico models. Again, chemoreceptors, including bitter taste receptors, possess specific problems that arise from their generally low-affinity interactions with their ligands; real experimental binding studies have not been successfully performed, and hence, binding is only assumed as a prerequisite for the commonly-monitored receptor activation. Therefore, wet lab researchers and in turn bioinformaticians using the data to refine their models have to be very cautious when interpreting data arising from functional receptor assays (see Figure 1).

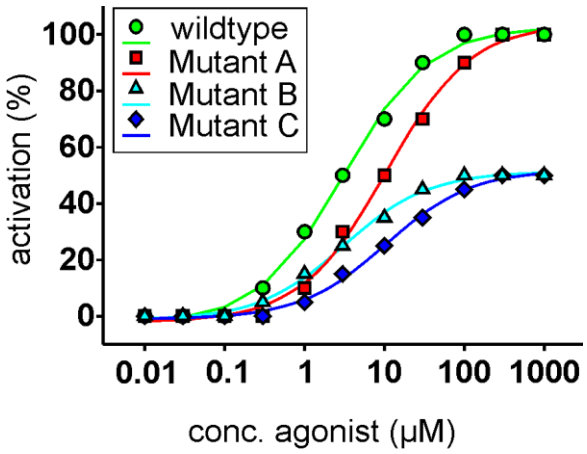

Figure 1. Idealized dose-response relationships of functional receptor assays. The unmodified receptor (circles, green) responses (x-axis, set to 100%) are plotted as a function of the agonist concentration (x-axis, logarithmically scaled). The fictitious receptor mutant A (squares, red) shows a right shift of the curve; however, it reaches the maximal response magnitude of the unmodified receptor. Since only the agonist concentration needs to be raised to achieve an activation similar to the unmodified receptor, the interaction of the agonist with the receptor's binding pocket seems to be weakened by this mutation. The fictitious receptor mutant B (triangles, cyan) exhibits only maximal amplitudes of 50% of the unmodified receptor; however, the EC_{50}-concentration (concentration at which half-maximal receptor activation is reached) is identical. In this case, one can assume that the residue mutated in mutant B is important for the activation by the agonist but not in the binding, since identical agonist concentrations result in similar proportional receptor activities. Finally, fictitious receptor mutant C (diamonds, blue) shows a drop in maximal signal amplitude as well as a shift in the EC_{50}-concentration, making interpretations of the underlying reason(s) difficult to impossible. In fact, this type of behavior is sometimes observed when in vitro mutagenesis is combined with functional assays.

In fact, the number of potential effects resulting in functional changes of receptors by far exceeds the few mentioned in the legend of Figure 1. The number of functional receptors at the cell surface can be modified by point mutations, which result in misfolding and mis-routing of the newly synthesized protein. Tentatively, and if properly folded and routed receptors are still formed, one would assume that the magnitude of signaling rather than the threshold or EC_{50}-concentrations should change; however, in cases of severe depletion of functional and cell surface associated receptors, shifts in these parameters are conceivable as well. Moreover, a plethora of additional complications (e.g., protein stability, G protein-coupling, desensitization, improper posttranslational modification, etc.), which cannot be discussed here, could be envisaged as underlying causes for functional changes of mutated bitter taste receptors. Hence, astounding and sapid effects caused by receptor mutations can be rapidly generated and, with a low-resolution model at hand, also acceptably illustrated and explained; however, experimental controls are crucial for the reliability of the mapped interactions and, ultimately, the receptor model and the generated docking poses. The finding that a point-mutated receptor exhibits reduced or even a complete loss of responses per se may only serve as a first hint for a possible involvement of the modified residue in agonist interaction. Partial or complete misfolding of the receptor or associated/related problems during biosynthesis and trafficking could also explain this observation. There are some general hints for well performed structure-function studies (see Figure 2).

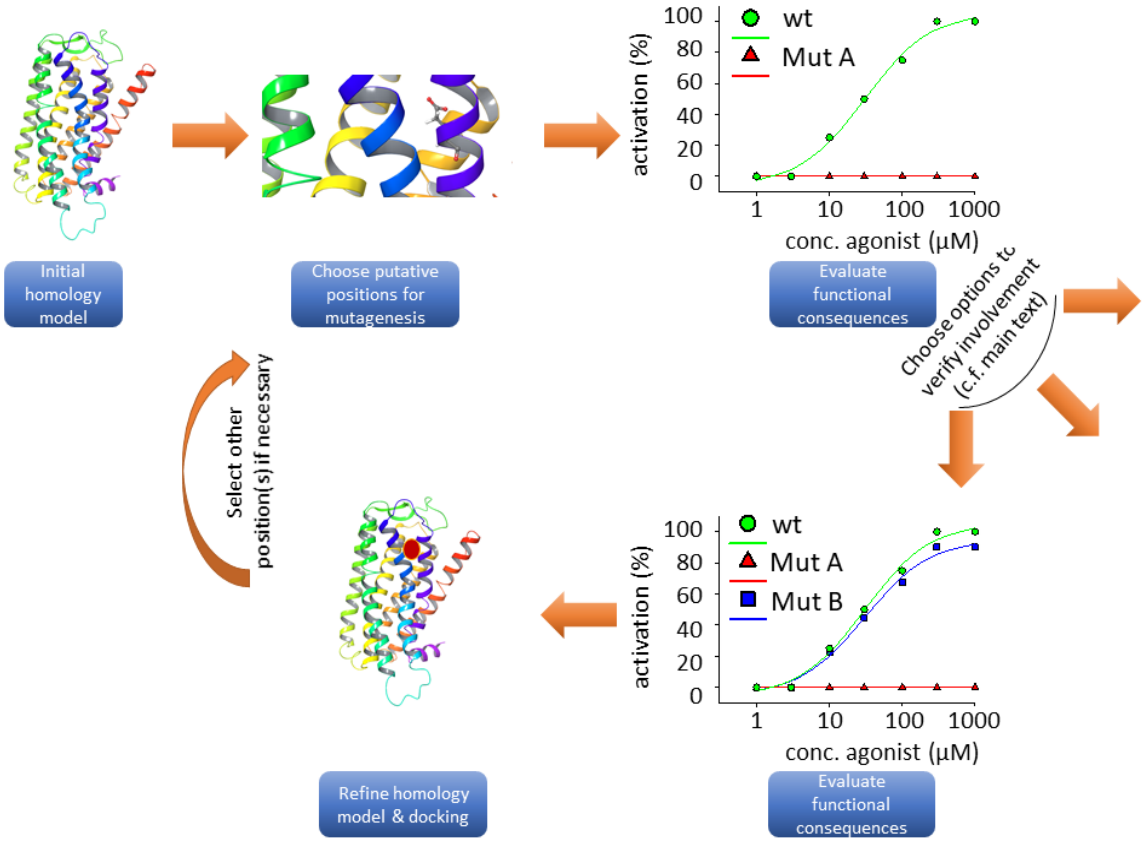

Figure 2. Flow chart illustrating the typical steps involved in structure-function studies. The taste 2 receptor (TAS2R) ribbon model was taken from bitterDB [88,89]. The graphs do not rely on experimental data; they were drawn for illustration purposes only.

(1) A very laborious but convincing control has been published by Brockhoff et al., who identified by point mutagenesis and functional experiments all residues in TAS2R46 that contributed to strychnine sensitivity. Next, the identified residues were transferred onto the recipient receptors TAS2R31 and TAS2R43, which share considerable amino acid sequence homologies with TAS2R46 but do not respond to strychnine. Subsequent functional experiments confirmed that strychnine sensitivity was also established in the recipient receptors. Hence, it was not only demonstrated that the lack of specific contact points caused reduced strychnine responsiveness, but that the presence of these residues were required and sufficient for strychnine interaction [37]. However, later, it was realized that not all identified strychnine-contacting positions must necessarily interact simultaneously with the agonist, but that some contacts occur in a so-called vestibular binding pocket, which is only transiently occupied, whereas other contacts are limited to the orthosteric binding site of TAS2R46 [90]. (2) The intense and constant search for bitter agonists of human TAS2Rs has resulted in large arrays of cognate agonists for many TAS2R subtypes, in particular for the receptors with broad agonist spectra. A well-performed structure-function study will not rely on single agonists, but select several agonists, favorably a chemically diverse set of agonists. A wider range of test substances bears the chance that mutations that result in reduced receptor responses for one agonist will not affect responses of other agonists. Hence, full functionality of the receptor mutant is demonstrated with one (set of) agonist(s), whereas selectivity of interactions between the mutated position and another agonist (set) is demonstrated. In fact, at least the broadly tuned bitter taste receptors possess binding sites, which are tailored to accommodate multiple diverse bitter compounds at the expense of potentially higher sensitivities for individual agonists by providing different contact points, a feature discovered at the example of the TAS2R10 [91], which exhibited strongly improved responses for some of its agonists caused by point-mutations. Recently, a comprehensive structure-function study performed with the most

broadly tuned human TAS2R, the TAS2R14, identified that almost all receptor positions that contribute to the ligand binding site of this receptor, exhibited agonist-selective effects [92]. As some of these positions were considered highly conserved among the TAS2R-family, a drop in agonist activation by point-mutating these positions have been seen with caution, because potential misfolding was suspected. The full functionality of TAS2R14 mutations at these conserved positions with some agonists suggests that misfolding may not be the most likely reason for reduced responsiveness in other receptors as well, and hence, an involvement in agonist interaction appears more likely. (3) Typically, the observation that a mutated receptor position might be crucial for agonist interaction will be the starting point for additional mutations, introducing more subtle changes of the residue in the position of interest. These could also contribute to the reliability of the assumed contact points with the agonist, e.g., if response properties are not negatively affected. (4) If functionality of the receptor cannot be confirmed by one of the above-mentioned tests, different methods to assess proper expression and/or cell surface localization of the investigated receptor mutants should be performed. If an initial receptor model has been generated to, e.g., guide mutagenesis, it is imperative that the model is adjusted in the end to incorporate all knowledge gained from the functional experiments.

4. Localization of TAS2R Binding Pockets

As mentioned already, bitter taste receptors are difficult to categorize due to their low amino acid sequence homology with other GPCR families. The ligand binding pocket of class A GPCRs is located at the extracellular side of TM III, V, VI and VII [32]. Indeed, the majority of structure-function studies with bitter taste receptors confirmed the contribution of these TMs in the formation of the ligand binding pocket, although exceptions exist. This may not be too surprising since TAS2Rs are not only rather distantly related to other GPCR-families, but also considerably differ among each other.

In light of the extraordinary broad tuning of the bitter taste receptors TAS2R10, -R14 and -R46, a valid question is if these receptors possess only a single or multiple ligand binding sites to accommodate all the various agonists arose. To address this question, Brockhoff et al. took advantage of the existence of a primate-specific subfamily of eight TAS2Rs, which share vast amino acid sequence homologies but exert very different agonist profiles [37]. The analyses of the residues involved in strychnine responsiveness of TAS2R46, one member of this subfamily, revealed multiple positions affecting the binding of strychnine. The transfer of the identified residues onto the same positions of two other subfamily members, TAS2R31 and TAS2R43, which did not show strychnine sensitivity, resulted not only in the transfer of strychnine responsiveness onto the recipient receptors, but in the transfer of the entire (tested) agonist profile of TAS2R46 [37]. The finding that the bitter taste receptor antagonist 4-(2,2,3-trimethylcyclopentyl)butanoic acid (GIV3727), which inhibits TAS2R31 and TAS2R43 via the interaction with one of the same receptor positions, could interact and inhibit the TAS2R46 modified in a single position confirmed the existence of a single binding pocket [93]. In fact, the binding of such structurally diverse compounds is enabled by the involvement of different subsets of residues within the binding pocket with individual agonists. For the three most broadly tuned receptors, TAS2R10, -R14 and -R46, an involvement of residues in TM III, V, VI and VII has been experimentally confirmed [37,91,92,94–96], which is in perfect agreement with the localization of the ligand binding pocket of class A GPCRs. In addition, for TAS2R14 [92] and TAS2R46 [37], the involvement of TM II in agonist binding has been suggested, a fact that might be attributed to the rather spacious shape of the pocket as shown for the TAS2R14 [97]. A modeling and docking study done without in vitro mutagenesis localized the contact points of the antagonist enterodiol in TM III, IV, ECL2, V, VI and VII of the TAS2R10, which is in pretty good agreement with the study published by Born et al. [91]; however, residues in TM IV and ECL2 were exclusively proposed to be involved in enterodiol binding [98]. Whether this could provide a hint on more general differences in the binding of agonists and antagonists to this receptor remains to be determined. Among the more recently discovered agonists of the receptor TAS2R7 are bitter salts [99,100]. Using point mutageneses the contact points for this rather unusual type of stimuli were also mapped to TM III and TM VII [100],

which is somewhat different from the study by Liu et al., who investigated the activation of the more conventional organic compound agonists of this receptor and reported instead that residues in TM III, TM V and ECL2 mainly contribute to ligand binding [101]. Compared to most other TAS2Rs, agonist binding to TAS2R16 tentatively should be less complex, because this receptor exhibits a strong bias for structurally similar βD-glucopyranosides [22]. However, it turned out that several structure-function studies showed discrepancies with regard to the binding modes of agonists and, consequently, the receptor positions involved in agonist binding [102–105]. The most recent report by Fierro et al. concluded that, depending on the agonist, residues in TM II, III, V, VI and VII are responsible for ligand binding [106]. For the human TAS2R38, TM III, V and VI are proposed to be involved in the binding of its agonists phenylthiocarbamide (PTC) and 6-n-propyl-thiouracil (PROP) [107–109]. For the TAS2R1, in three autonomous studies, different ligand binding pockets are suggested. Whereas Upadhyaya et al. incorporated experimental mutagenesis studies and assumed TM I, II, III and VI to be involved in ligand binding [110] and Stoeger et al. suggested positions in TM III, TM VI and ECL2 as contact points for L-arginine [111], Dai et al. proposed the putative binding pocket within TM III, V, VI, VII and the extracellular loop 2 (ECL2) [112]. Combining all results of the latest investigations of ligand binding pockets of the human TAS2Rs, TM I and IV seem to be the only transmembrane domains rarely participating in ligand binding. As was already mentioned for the human TAS2R1, besides the transmembrane domains, also the extracellular loops (ECL) are suggested to be involved in ligand binding [34,113]. In TAS2R14, the amino acid residue Arg160, which is located in ECL2, is proposed to participate in the binding of the agonist aristolochic acid [114]. Furthermore, the exchange of ECL1 between TAS2R43 and TAS2R31 leads to the loss of receptor response for TAS2R43 and the gain of responsiveness for TAS2R31 triggered by n-isopropyl-2-methyl-5-nitrobenzenesulfonamide (IMNB) [87].

Besides the orthosteric binding site, for some class A GPCRs, and recently for TAS2R46, the existence of a second vestibular binding site, which is involved in ligand selectivity, is proposed [90]. This vestibular binding site is located at the extracellular part of the receptor and only transiently occupied by agonists. To manage the high amount of different and highly concentrated bitter compounds, this binding site may function as filter for the orthosteric binding site. As extracellular loops are part of this vestibular binding site, these findings are in good agreement with the suggested involvement of extracellular loops in agonist selectivity. For an overview of receptor positions implicated in ligand binding, see Table 1.

Table 1. Positions involved in TAS2R ligand binding. Amino acid residues involved in agonist binding of human TAS2R1, -4, -7, -10, -14, -16, -20, -40 and -46 are presented. Positions are indicated according to Ballesteros-Weinstein numbering (BW pos.). The interacting amino acid, its position and the corresponding references are displayed.

BW Pos.	TAS2R									
	1	**4**	**7**	**10**	**14**	**16**	**20**	**38**	**40**	**46**
2.53						L59 [105 #, 106 *]				
2.57						S63 [106 *]				
2.60			D65 [101 #]				H65 [95 #]			N65 [90]
2.61	N66 [110 *, 115]				W66 [92,94 *]	N67 [106 *]				W66 [37, 90, 96 *]
2.65										E70 [37, 90, 96 *]
2.66										L71 [37, 90, 96 *] a
ECL1	E74 [110 *, 115] b						T74 [95]			
3.24						V77 [105 #]				
3.25		S81 [95]								I82 [37, 90, 96 *] c
3.28					L85 [92]	L81 [105 #]				
3.29	C82 [111 *]		D86 [101 #]	S85 [91, 96 *]	T86 [92, 95 #]	T82 [105 #]			K98 [94 *]	Y85 [90]
3.30					N87 [92]					N86 [90]
3.32	L85 [94 *]		W89 [101 #]	W88 [91]	W89 [92, 114 *]	W85 [106 *]	W88 [95 #]		T101 [94 *]	W88 [37, 90, 96 *]
3.33	L86 [94 *, 112 *]			V89 [91, 96 *]	T90 [92]	E86 [103, 106 *]				A89 [90]
3.35						F88 [106 *]				
3.36	N89 [94 *, 110 *, 115 *d]			N92 [91, 96 *]	N93 [92, 94 *]	N89 [106 *]		N103 [107, 108]	N105 [94 *]	N92 [37, 90, 96 *]
3.37	E90 [110 *, 112 *]		H94 [100]	Q93 [96 *]	H94 [92]	I90 [105 #]				H93 [90]
3.39						T92 [106 *]				
3.40						F93 [103 #, 106 *]				N96 [90]
3.41						W94 [103 #]		W108 [109 *#]		
3.42								L109 [109 *#]		
3.45								C112 [109 *#]		
4.60	I140 [94 *]								F156 [94 *]	I147 [90]
4.62						S144 [105 #]				
4.64	H144 [94 *]				I148 [94 *, 95 #]					
4.65										N150 [37, 96 *]
ECL2						N148 [105]				
ECL2		Q152 [95]								
ECL2		S154 [95]								
ECL2					R160 [95]					
ECL2					R161 [95]					N161 [96 *]
ECL2	N163 [110 *]	R163 [95]			K163 [95]					
ECL2	A164 [111 *]						W164 [95]			
ECL2		N165 [95]								

Table 1. *Cont.*

BW Pos.	TAS2R									
	1	**4**	**7**	**10**	**14**	**16**	**20**	**38**	**40**	**46**
ECL2		T166 [95]					I166 [95]			
ECL2			N167 [101]							
ECL2	K168 [112 *]									
ECL2			T169 [101]							
ECL2			W170 [101]							
5.38			S181 [101 #]		L178 [94 *]					S175 [90]
5.39	Q175 [94 *]			K174 [96 *]	I179 [95 #]	Q177 [103 #]			L194 [94 *]	N176 [37, 90, 96 *]
5.40				Q175 [91, 96 *]						
5.42	S178 [94 *]			L177 [96 *]	T182 [92, 95 #]			F197 [108]		V179 [96 *]
5.43				L178 [91, 96 *]	S183 [92]	H181 [103 #]				T180 [90]
5.44								Y199 [109 *#]		
5.46	E182 [94 *, 112 *]				F186 [92]	A184 [105 #]		W201 [108]		
5.47					I187 [92]					N184 [90]
5.48								V203 [109 *#]		
5.49								P204 [109 *#]		
6.48	Y237 [112 *]				Y240 [92]	F236 [106 *]				
6.49					A241 [92]					
6.51				Y239 [91]	F243 [92, 94 *]	Y239 [106 *]				Y241 [37, 90, 96 *]
6.52						F240 [103 #]		S260 [108]		F242 [90]
6.54	I243 [94 *]							A262 [109 *#]		S244 [90]
6.55	K244 [94 *, 112 *]				F247 [92, 94 *]	I243 [103#]		A263 [109 *#]	L263 [44 *]	I245 [90]
6.56								F264 [108]		
6.57								I165 [109 *#]		
6.58		Q249 [95]						S266 [109 *#]		S248 [90]
6.59			T255 [101 #]		V251 [95 #]		F249 [95#]			V249 [90]
6.60	S248 [111 *]									
6.63										E253 [37, 96 *]
7.32			E264 [100]							
7.35	I258 [94 *]	K262 [95]			I262 [94 *]	L258 [105 #]				F261 [90]
7.36					I263 [92]					
7.38	F261 [94 *]					W261 [105 #]				
7.39	F262 [94 *]		E271 [101 #]	M263 [96 *]	Q266 [92]	E262 [105 #, 106 *]	Q265 [95 #]		K282 [94 *]	E265 [37, 90, 96 *]
7.42				T266 [96 *]	G269 [92]	V265 [106 *]				A268 [37, 90, 96 *]
7.43						Y266 [106 *]				F269 [37, 90, 96 *]
7.45						F268 [106 *]				
7.46						I269 [106 *]				

(a) assigned ECL1 in [90], (b) assigned BW 2.73 in [115], (c) assigned BW 3.26 in [90], (d) assigned BW 3.45 in [110,115]. * no experimental validation provided in reference, # lacking BW numbering supplemented according to gpcrdb.org.

5. Receptor Activation

The activation mechanism of bitter taste receptors is a poorly investigated challenge. However, most of the conserved motifs of class A GPCRs were shown to have a corresponding counterpart in bitter taste receptors. An $N^{1.50}$xx$I^{1.53}$ motif replaces the $N^{1.50}$xx$V^{1.53}$ motif in TM I and the $N^{7.49}P^{7.50}$xx$Y^{7.53}$ in TM VII is changed to $H^{7.49}S^{7.50}$xx$L^{7.53}$ [32]. For GPCRs in general, conformational changes by ligand binding to the inactive receptor are thought to trigger a receptor response [116]. It is assumed that the conserved motifs may be involved in stabilization of this inactive conformation until agonist binding, as the mutations of isoleucine at position 1.53 in TAS2R1 and serine at position 7.50 in TAS2R4 to alanine result in hyperactive receptors [115,117,118].

Further important amino acid residues for receptor activation were suggested for the human TAS2R38 in analogy to rhodopsin [107]. By X-ray crystallography, two very different structural arrangements in the G protein-bound and unbound state of the bovine rhodopsin were identified [119]. By forming a hydrophobic interaction, the two amino acid residues at positions 6.43 and 7.52 were proposed to be responsible for these structural rearrangements [119]. Transferring this knowledge on the related TAS2R38 suggests an involvement of the amino acids F255 and V296 in receptor activation [107]. By introducing a double mutant, which maintains the putative interaction between F255 and V296, Biarnés et al. were able to show an TAS2R38 response to PTC, which is comparable to the wildtype receptor. These results indicate a critical role of F255 and V296 in TAS2R38 activation [107].

Molecular modeling studies showed the involvement of inter- and intrahelical H bonds in TAS2R1 activation. The amino acid residue $N24^{1.50}$ was shown to establish an H bond network connecting TM I, TM II and TM VII, which is only present in the agonist bound state and absent in the unbound TAS2R1 [115]. Dai et al. further proposed a control switch between the intracellular loop 2 (ICL2) and the cytoplasmic end of TM III in the human TAS2R1. Upon ligand binding, a combination of the opening of this switch and the formation of a helix in the ICL2 are assumed to be involved in TAS2R1 activation. The conservation of amino acid residues involved in the control switch in TAS2Rs suggests a conserved bitter taste receptor activation mechanism [112]. These molecular modeling studies give first insights in putative activation mechanisms of human bitter taste receptors, but experimental evidence is still scarce.

As already mentioned, mutations of amino acid residues of broadly tuned bitter taste receptors have agonist-specific effects [37,91,92]. Most of the identified residues in the human TAS2R14 impaired the potency, as well as the efficacy, of the investigated agonists, but a drop in sensitivity is sometimes correlated with an increase in signal amplitude. These results indicate that amino acid residues, which are involved in ligand binding, may have an additional function in receptor activation [92].

6. Outlook

Despite the numerous issues associated with structure–function analyses of bitter taste receptors, research has made considerable progress towards a better understanding of the receptors' interactions with their various agonists in particular for the broadly tuned generalist receptors. At present, the field is urgently awaiting the availability of experimental models to compare the existing models with experimental data and to facilitate the transition from low to high resolution models, although the authors anticipate that low resolution models supported by vigorous experimental confirmation would likely also allow the de novo prediction of novel agonists and antagonists as well as a better understanding of the receptors' activation mechanism in the future. However, it will certainly speed up the process if these endeavors could be started with experimental structures in hand. As TAS2Rs are not only very distantly related to other GPCRs, but also among each other, a single crystal structure may not be sufficient to serve as template for the high-resolution modeling of all 25 TAS2Rs. As TAS2Rs, in light of their expression in multiple extraoral tissues and with presumed roles numerous important physiological processes, are considered important drug targets, the development of small molecules for their activity modulation will soon become a very active research field benefiting from structure–function research.

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3.2. Bitter taste receptors of the common vampire bat are functional and show conserved responses to metal ions in vitro

The article "*Bitter taste receptors of the common vampire bat are functional and show conserved responses to metal ions in vitro"* (https://doi.org/10.1098/rspb.2021.0418) was published in "Proceedings of the Royal Society B" in 2021. Florian Ziegler developed the concept in consultation with Maik Behrens. Florian Ziegler performed the experiments, evaluated the data and prepared the original draft of the manuscript, and all co-authors contributed to revision. Revision according to the reviewers' suggestions was performed by Florian Ziegler in collaboration with Maik Behrens.

Vampire bats are sanguivorous members in the order of chiroptera. Therefore, it was hypothesized that their bitter taste receptors can detect bitter substances in mammalian blood. Three of these receptors, called TAS2R1, TAS2R4 and TAS2R7, are conserved in all 3 vampire bat species, indicating their importance. Receptor genes of the common vampire bat *Desmodus rotundus* were cloned into an established expression vector. Because of their origin, nomenclature for the three receptors was set as drTAS2R1, drTAS2R4 and drTAS2R7. As these were orphan receptors, first a set of 57 natural and synthetic compounds, known to be agonists of bitter taste receptors of other species, was tested for its ability to activate them. This was performed using a heterologous expression system and detecting Ca^{2+} release upon bitter taste receptor activation. In doing so, 3 agonists for drTAS2R1, 4 agonists for drTAS2R4 and 9 agonists for drTAS2R7 were identified. In former studies, the activation of the human TAS2R7 by metal ions was shown [130,183]. As the drTAS2R7 is a one-to-one orthologous receptor, activation of drTAS2R7 by this compound class was further investigated. Indeed, the *Desmodus rotundus* receptor showed a quantitative and qualitative conserved response to di- and trivalent metal ions. Despite their only distant evolutionary relationship with a divergence ~94 million years ago, the response to metal ions of one-to-one orthologous TAS2R7 is still present in both species. In the human body, metal ions are important cofactors for several enzymes and are involved in further biological processes. Therefore, a role of metal ions as endogenous agonists of extra-oral bitter taste receptors can be considered.

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Bitter taste receptors of the common vampire bat are functional and show conserved responses to metal ions *in vitro*

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The bitter taste sensation is important to warn mammals of the ingestion of potentially toxic food compounds. For mammals, whose nutrition relies on highly specific food sources, such as blood in the case of vampire bats, it is unknown if bitter sensing is involved in prey selection. By contrast to other bat species, vampire bats exhibit numerous bitter taste receptor pseudogenes, which could point to a decreased importance of bitter taste. However, electrophysiological and behavioural studies suggest the existence of functional bitter taste transmission. To determine the agonist spectra of the three bitter taste receptors that are conserved in all three vampire bat species, we investigated the *in vitro* activation of *Desmodus rotundus* T2R1, T2R4 and T2R7. Using a set of 57 natural and synthetic bitter compounds, we were able to identify agonists for all three receptors. Hence, we confirmed a persisting functionality and, consequently, a putative biological role of bitter taste receptors in vampire bats. Furthermore, the activation of the human TAS2R7 by metal ions is shown to be conserved in *D. rotundus*.

1. Introduction

The mammalian sense of taste provides important information about the food constituents prior to consumption [1]. Typically, mammals are able to detect and distinguish the five basic taste qualities: salty, sour, sweet, umami and bitter [2]. Whereas salty and sour tastes are mediated via ion channels [3,4], the remaining three taste qualities are based on G protein-coupled receptors [5]. These receptors belong to two different gene families, named taste 1 receptors (note the use of species-specific gene symbols: *TAS1R*, human; *Tas1r*, mouse; *T1R*, common for other vertebrates) and taste 2 receptors (*TAS2R*, *Tas2r*, *T2R*). The group of *TAS1Rs* consists of the three members *TAS1R1*, *TAS1R2* and *TAS1R3*, which are responsible for sweet and umami tasting, and thus mediate the sensing of energy-rich food items [6–9]. By contrast, bitter taste, which is facilitated by *TAS2Rs*, is proposed to prevent the ingestion of potentially poisonous compounds, although a general correlation between toxicity and bitterness is lacking [10–13]. Instead, a mild bitterness can be appreciated by humans and may further serve to guide a range of mammals to pharmacologically active food items for self-medication purposes [14–16]. Mostly bitter compounds are rather complex organic molecules with an extraordinary chemical diversity [17]. However, inorganic molecules such as magnesium sulfate, which is known as 'bitter salt' or 'Epsom salt', also activate the human TAS2R7 [18,19]. Magnesium ions can profoundly affect human physiology, as evidenced by their effects as a laxative [20], suppressant of premature labour (= tocolytic) [21] and by their interference with cardiac parameters [22]. Given these varied and drastic effects, magnesium sensation by bitter taste receptors, similar to the more 'classical' organic bitter compounds, might be broadly selected for as a mechanism to regulate ingestion.

The repertoires of functional bitter taste receptors differ widely between species (for a recent review see [23]) and might be related to the dietary habits [24]. However, the mere number of bitter taste receptors does not allow

conclusions about their importance for individual species as their average tuning breadths can fluctuate considerably such that few receptors may enable the detection of a large array of bitter compounds [25]. With more than 1000 different species, bats (Chiroptera) represent one of the largest mammalian clades [26]. Bats exhibit a wide range of dietary habits with herbivorous, insectivorous, frugivorous, carnivorous, piscivorous and sanguivorous species [27]. The differential endowment of bat species with taste receptors reflects these very distinct feeding habits. The *T1R1* gene is absent or non-functional in all investigated bat species, supposing the general loss of canonical umami taste in Chiroptera [28]. To date, rather little information about the *T2R* repertoires in bats exists. Based on the hypothesis that the taste receptor repertoire is evolutionary modified according to the feeding habits, it has been suggested that herbivorous and insectivorous bats might have more functional *T2Rs* compared to others [24,29,30]. While the insectivorous *Myotis* species indeed showed an increased number of *T2Rs*, the hypothesis could not be confirmed for all vertebrates including bats in general [24,31,32]. The three members of the *Myotis* genus, *Myotis davidii*, *Myotis brandtii* and *Myotis lucifugus*, are spread over largely diverse habitats [33,34]. Therefore, the high number of potentially functional *T2Rs* is considered important for the adaptation to different prey insect repertoires [32].

Vampire bats represent a special group among the Chiroptera, as their nutrition relies exclusively on blood [27]. There are only three species known, named *Desmodus rotundus*, *Diaemus youngi* and *Diphylla ecaudatus*. In vampire bats all three *T1R* genes including the already mentioned *T1R1* are absent from the genomes or became pseudogenes, suggesting that not only umami, but also sweet taste perception has been lost [28,35,36]. The bitter taste sensation is also speculated to play a minor role in food selection, since blood is unlikely to contain a high quantity of toxic compounds and as it was shown that vampire bats primarily use their sense of odour, echolocation and infrared sensors for prey detection and the identification of capillary-rich regions [27,37,38]. These facts correlate with an evolutionary reduction of taste receptors in vampire bats. Indeed, the number of *T2R* pseudogenes in vampire bats is significantly higher than in non-blood-sucking bats. Whereas 13 of the 21 *T2Rs* of the common vampire bat *D. rotundus* are pseudogenes and only 8 *T2Rs* are considered functional, the pseudogene ratio for the above-mentioned *Myotis* species is below 25% [32]. However, three intact *T2Rs*, named *T2R1*, *T2R4* and *T2R7*, are conserved in all three vampire bat species indicating a persisting function [24] (figure 1*b*). Moreover, functional bitter taste signal transduction components such as CALHM1 exist [36]. Interestingly, vampire bats avoid blood adulterated with bitter compounds, indicating a remaining role of *T2Rs* for taste perception [42]. Furthermore, anatomical and electrophysiological studies identified normal taste buds and functional taste receptor cells in vampire bats, although vallate papillae, one of three common types of taste papillae, are absent in *D. rotundus* [43–45]. The existence of these structures and molecules suggests an intact gustatory system in vampire bats. A recent study confirmed the activation of *D. rotundus* T2Rs by mainly synthetic bitter compounds *in vitro* [46].

Beside the gustatory system, *T2Rs* are expressed in extra-oral tissues, indicating a role beyond taste sensation [47]. Specific agonists for taste receptors present in non-gustatory tissues are largely unknown. However, recent studies presented the activation of the human TAS2R7 by bi- and trivalent metal ions, which were discussed to have a putative biological function as endogenous agonists as they are released upon excitation of cells like Zn^{2+} in β-cells [18,19]. Interestingly, the three *D. rotundus* receptors, T2R1, T2R4 and T2R7 are one-to-one orthologues to human TAS2R1, TAS2R4 and TAS2R7 (figure 1*c*), raising the possibility for a functional conservation of these orthologues including metal ion responses.

In this work, we aimed to identify the spectrum of bitter compounds detected by vampire bats by functional analyses of the three highly conserved bitter taste receptors T2R1, T2R4 and T2R7 of *D. rotundus*. We hypothesize that they may play a role in feeding decisions and, perhaps, detect bitter components in the blood or other materials relevant to their behaviour, ecology and physiology.

2. Material and methods

(a) Bioinformatics

To highlight the great evolutionary distance between human and *D. rotundus*, we obtained a phylogenetic tree of Boreoeutheria using the TimeTree webserver (www.timetree.org, [39]). Amino acid sequences of the 25 human TAS2Rs were taken from Meyerhof *et al*. [48], the amino acid sequences of *D. rotundus* T2R1 (XP_024434157.1), T2R4 (XP_024410857.1) and T2R7 (XP_024431669.1) were retrieved through NCBI resources (www.ncbi.nlm.nih.gov). As an outgroup, the amino acid sequences of the small zebrafish pheromone receptor family ORA (olfactory receptor class A-related, [41], ORA1 (NP_001124140.1), ORA2 (NP_001091865.1), ORA3 (XP_009294044.1), ORA4 (XP_005168339.1), ORA5 (XP_002663506.1) and ORA6 (XP_003200835.2) were included and fetched from the NCBI database. The phylogenetic tree of the 25 human and three *D. rotundus* bitter taste receptors was established using the phylogeny.fr webserver (www.phylogeny.fr). Briefly, sequences were aligned with ClustalW (v. 2.1), the phylogenetic tree was reconstructed using the maximum likelihood method implemented in PhyML (v. 3.1) and reliability of the internal branch was assessed using aLRT (χ^2-based parametric, v. 3.0). Tree rendering was done with TreeDyn (v. 198.3). For the determination of orthogroups the phylogenetic tree construction was done as before including now, in addition to the 25 human TAS2Rs, all 8 putatively functional *D. rotundus* T2Rs [36], all 35 mouse Tas2rs [49], the 12 cat and 15 dog T2Rs [50]. The resulting Tree file was then subjected to orthogroup detection using the software UPhO [40]. Estimation of the rates of non-synonymous/synonymous substitutions (dN/dS) for human and *D. rotundus* orthologous bitter taste receptor pairs was done using the program PAL2NAL (www.bork.embl.de/pal2nal [51]).

(b) Chemicals

For the functional screening of *D. rotundus* T2R1, T2R4 and T2R7, we used a set of 57 bitter compounds. The substances (see electronic supplementary material, table S1) were selected based on previous successful screenings covering a large range of vertebrate bitter taste receptors, including human [48,49,52], mouse [49], rat [53], chimpanzee [54], various bird and frog [25], zebrafish and coelacanth [55] receptors, using a wide range of diverse natural and synthetic bitter compounds. Stock solutions were prepared either in the assay buffer C1 (130 mM NaCl, 10 mM HEPES pH 7.4, 5 mM KCl, 2 mM $CaCl_2$, 0.18% glucose) or in DMSO. The final DMSO concentration in the screening procedure was kept below 0.5% to prevent unspecific cellular responses. The highest concentrations of substances employed

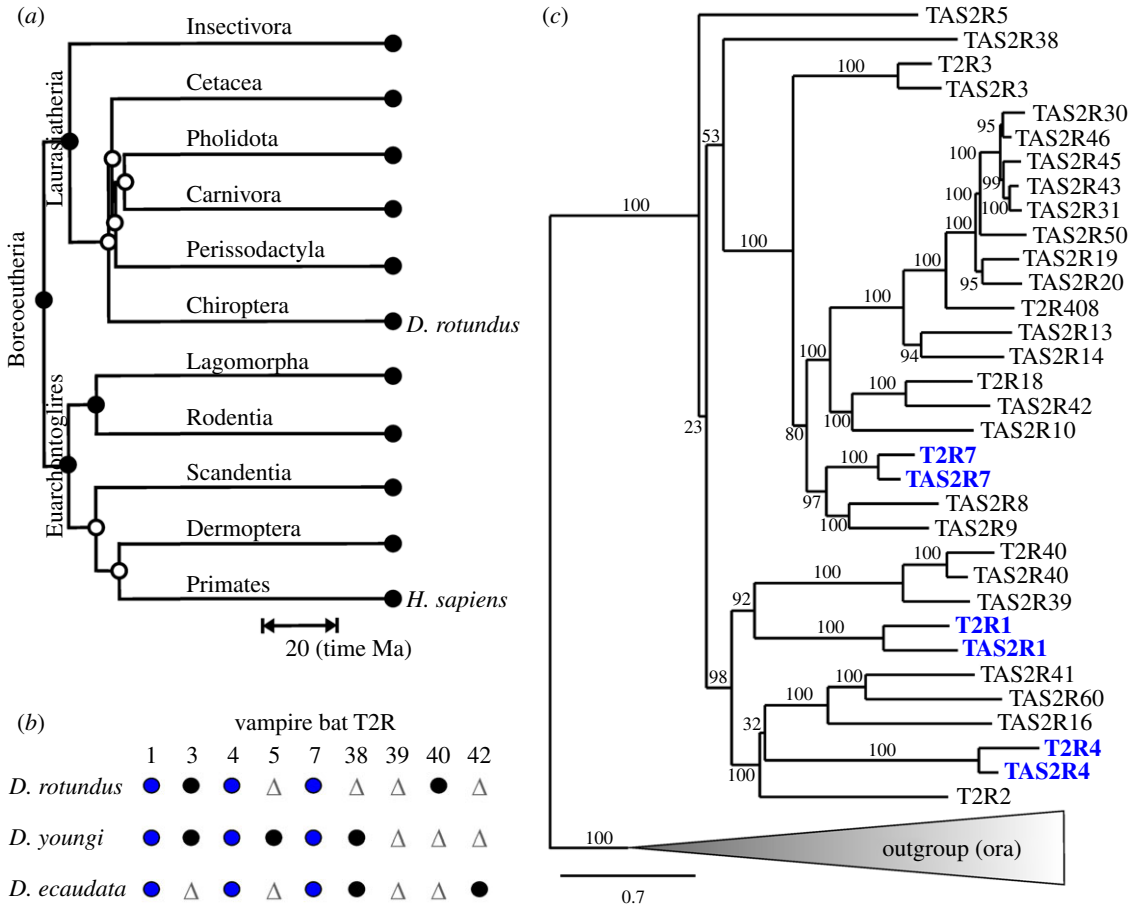

Figure 1. The 3 extant T2Rs of vampire bats are direct one-to-one orthologues of human TAS2Rs. (*a*) The phylogenetic tree of Boreoeutheria demonstrates the evolutionary distant relationship of the vampire bat *Desmodus rotundus* with humans. The scale bar indicates the time of divergence in million years (computed with TimeTree (www.timetree.org) [39]). (*b*) Extant and pseudogenized T2R genes in the 3 vampire bat species. T2R pseudogenes (triangles, grey), extant T2R genes (circles, black) and T2R genes that are extant and conserved among vampire bats (circles, grey/blue online) are shown (taken from [32]). (*c*) Phylogenetic tree showing the *D. rotundus* T2Rs together with the 25 potentially functional human TAS2Rs. The one-to-one orthologues (confirmed with the software UPhO [40]) are highlighted in bold type/blue online. The six zebrafish pheromone receptors, ORA1 to 6, were included as an outgroup [41]. Branch support (%) is indicated by grey numbers, scale bar = amino acid substitutions per site. (Online version in colour.)

in the receptor screening were chosen based on previous experiments to avoid receptor-independent cellular responses or solubility issues.

The finding of rather strong negative selective pressure on *D. rotundus* T2R7 and its human orthologue TAS2R7 prompted us to screen the two receptors additionally with metal salts, a compound group recently found to elicit responses specifically on human TAS2R7 [18,19].

(c) Cloning

The predicted cDNAs covering the entire coding regions of *D. rotundus* bitter taste receptors T2R1 (XM_024578389), T2R4 (XM_024555089) and T2R7 (XM_024575901) (electronic supplementary material, appendix S1), elongated by 5′- and 3′-restriction sites, were synthesized (BioCat GmbH, Heidelberg, Germany) and cloned into the pcDNA 5 FRT T/O expression vector (Thermo Fisher Scientific, Waltham, USA). The open reading frames of the receptor genes were elongated by an N-terminal sst3-tag for effective cell surface expression and a C-terminal HSV-tag, which is necessary for immunocytochemical detection. The tags were already present in the expression vector or in case of T2R4 the synthetic cDNA was elongated by the sequence. The vector and the synthetic cDNAs were cleaved using the restriction enzymes EcoRI (Thermo Fisher Scientific) and NotI (Thermo Fisher Scientific) for T2R1 and T2R7 and BamHI (Thermo Fisher Scientific) and NotI for T2R4. Subsequently, linearized vector DNA was dephosphorylated using FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) and, after purification, ligated using T4 DNA ligase (New England Biolabs, Frankfurt, Germany) with the receptor cDNAs. The integrity of the final constructs was confirmed by sequencing (Eurofins Genomics GmbH, Ebersberg, Germany).

(d) Cultivation of HEK293T–Gα16gust44 cells

HEK293T–Gα16gust44 cells [56] were grown in Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Sigma Aldrich, St Louis, USA), 2 mM L-glutamine (Sigma Aldrich), 100 units ml^{-1} penicillin (Sigma Aldrich) and 100 µg ml^{-1} streptomycin (Sigma Aldrich) at 37°C and 5% CO_2.

(e) Transient transfection

The day before transfection, HEK293T–Gα16gust44 cells were seeded onto 96-well plates coated with 10 µg ml^{-1} poly-D-lysine to reach a confluence of 40–60% the next day. Transient transfection was performed with 150 ng of the cloned plasmid DNA and 0.3 µl lipofectamine 2000 in serum-free DMEM per well. For negative controls empty vector DNA (mock) was transfected. Five hours post transfection, the media was changed to DMEM supplemented as described above.

Table 1. Identified agonists of the *Desmodus rotundus* bitter taste receptors T2R1, T2R4 and T2R7. Natural and synthetic bitter substances, which showed receptor activation in Ca^{2+}-imaging experiments are presented. Multiple activators per receptor were identified, and their maximal applied concentrations (c_{max}) are presented. No activation is indicated by (—) and for agonists efficacies and the corresponding standard deviations are given as relative fluorescence ($\Delta F/F$) ($n = 3$). Further, activation threshold concentrations are given in parenthesis for compounds, which activation threshold concentration is different from the maximal concentration.

	c_{max}	T2R1	T2R4	T2R7
artemisinin	100 µM	—	0.12 ± 0.01	0.13 ± 0.01
chloramphenicol	1 mM	0.08 ± 0.02	—	0.09 ± 0.02
chloroquine	10 mM	0.16 ± 0.03	0.29 ± 0.08	0.34 ± 0.10 (3 mM)
coumarin	1 mM	—	0.13 ± 0.03	0.16 ± 0.01
denatonium benzoate	10 mM	—	0.32 ± 0.02	0.32 ± 0.04 (300 µM)
ethylhydrocupreine	30 µM	—	—	0.10 ± 0.02
picrotin	1 mM	—	—	0.08 ± 0.02
picrotoxinin	1 mM	0.08 ± 0.02	—	0.13 ± 0.03
quinine sulfate	30 µM	—	—	0.11 ± 0.01

(f) Calcium imaging

Twenty-four hours after transfection HEK293T–Gα16gust44 cells were loaded with the fluorescence dye Fluo-4-AM (Invitrogen) in the presence of 2.5 mM probenecid (Sigma Aldrich) for 1 h as described previously [25,49]. Cells were washed with buffer C1 using a BioTek Cell Washer, incubated in the dark for half an hour and washed again. Measurement of fluorescence changes upon automated application of different agonist concentrations was done using a $FLIPR^{TETRA}$ system (Molecular Devices, San Jose, USA). Somatostatin 14 (final concentration 100 nM) (Bachem, Bubendorf, Switzerland) was used as cell viability control.

(g) Data analysis

For data export, the FLIPR software ScreenWorks was used. Data were negative control corrected using the data of mock-transfected cells and exported. Exported fluorescence intensities were standardized to basal fluorescence and normalized to the buffer-only control to obtain the relative changes in fluorescence ($\Delta F/F$) using the Microsoft Excel software. Statistical evaluation was done using SigmaPlot. Statistical significance ($p < 0.01$) was determined using Student's *t*-test.

3. Results

(a) Screening of *Desmodus rotundus* T2Rs with a set of known bitter compounds

Prior to performing functional receptor assays with transiently transfected cells, the expression and membrane localization of the T2Rs was confirmed (electronic supplementary material, appendix S2). As described in the material and methods section, we initially used a set of 57 natural and synthetic bitter compounds with diverse chemical structures to cover a large chemical space for receptor characterization (electronic supplementary material, table S1). These compounds were tested for their activation of the *D. rotundus* bitter taste receptors T2R1, T2R4 and T2R7. In total, we detected 3 agonists for T2R1 (approx. 5%), 4 for T2R4 (approx. 7%) and 9 for T2R7 (approx. 16%) (table 1 and figure 2) indicating different tuning breadth. All of the receptors exhibited responses to substances of different compound classes. Among these, common structural motifs were not detected, similar to what has been observed for the human orthologous receptors TAS2R4 and TAS2R7 [48]. The group of agonists include natural, as well as synthetic compounds, with different toxicities among these substances. When comparing the agonists of the three receptors, we observed overlapping agonist profiles. Chloroquine was found to activate all three tested receptors. T2R1 and T2R7 responded to chloramphenicol and picrotoxinin and T2R4 and T2R7 to artemisinin, coumarin and

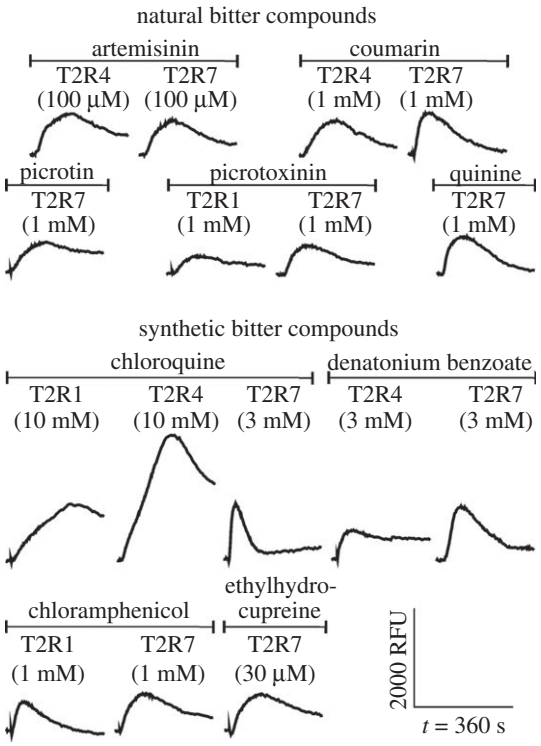

Figure 2. Cellular responses to application of organic bitter agonists. HEK293T–Gα16gust44 cells transfected with *D. rotundus* T2R1, T2R4 and T2R7 were loaded with a Ca^{2+}-indicator (Fluo-4-AM) and fluorescence emission after ligand application was measured. The panels show the FLIPR recordings after negative control (mock) correction. All identified agonists of *D. rotundus* T2Rs and their corresponding concentrations are presented. Relative light units (from −200 to 3000) are plotted against the time (6 min).

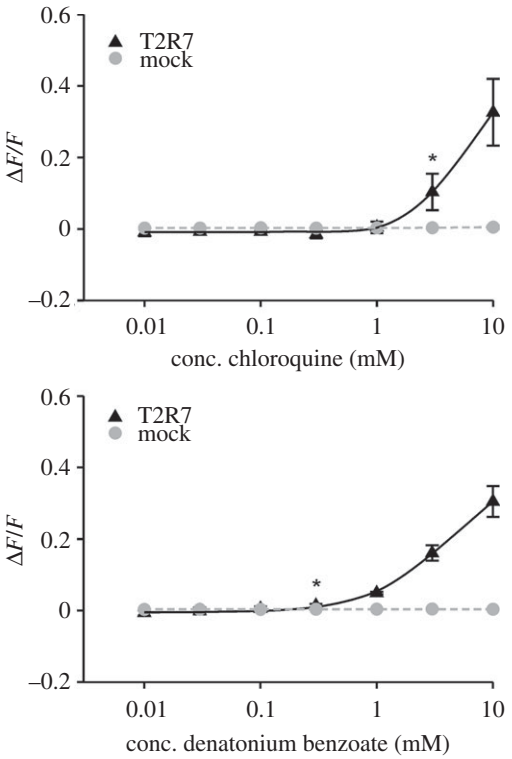

Figure 3. Concentration–response relationships of the activation of *Desmodus rotundus* T2R7 by chloroquine and denatonium benzoate. HEK293T–Gα16gust44 cells were transiently transfected with *D. rotundus* T2R7 and the empty vector control (mock). Fluorescence intensities, according to the Ca^{2+}-release upon receptor activation, were measured with an automated fluorometric imaging plate reader ($FLIPR^{Tetra}$). For dose–response curves seven different concentrations of chloroquine (upper panel) and denatonium benzoate (lower panel) ($c_{max} = 10$ mM) were used. The relative fluorescence intensities were mock subtracted and plotted against the agonist concentration in mM ($n = 3$). Statistical significance ($p < 0.01$) is presented by (*).

denatonium benzoate. Only T2R7 was activated by ethylhydrocupreine, quinine and picrotin (table 1). For 48 of the 57 compounds (approx. 84%) we observed no responses (electronic supplementary material, table S1).

(b) Determination of activation threshold concentrations by Ca^{2+}-imaging

For the description of receptor–agonist relationships, potencies and efficacies are important parameters. The lowest agonist concentration, which resulted in a statistically significant fluorescence increase ($p < 0.01$), compared to the empty vector control, is defined as activation threshold concentration (table 1). For most of the agonists, activation was solely detectable at the maximal applied concentration. As T2R7 responded in a wider range to its agonists chloroquine and denatonium benzoate, we established dose–response relationships. However, the limited solubility of the test compounds and the occurrence of receptor-independent artefacts prevented us from using higher compound concentrations necessary for achieving signal saturation. Thus, the determination of EC_{50}-concentrations was not possible (figure 3).

The efficacy of an agonist determines the extent of receptor activation. The efficacies observed for the activation of T2R7 by chloroquine (0.34 ± 0.10) and denatonium benzoate (0.32 ± 0.04) were almost identical (table 1). Similarly, we observed comparable efficacies for all other compounds that activated two receptors (table 1).

(c) The *Desmodus rotundus* bitter taste receptor T2R7 responds to metal ions

Comparing the rates of non-synonymous to synonymous substitutions among the investigated orthologous human and *D. rotundus* bitter taste receptors revealed that the strongest purifying selection exists for the TAS2R7/T2R7 pair of receptors with a dN/dS ratio of 0.52, whereas the TAS2R1/T2R1 ($dN/dS = 0.73$) and TAS2R4/T2R4 ($dN/dS = 0.67$) pairs exhibited lower ratios. Former works showed the activation of the human bitter taste receptor TAS2R7 by bitter salts [18,19]. In the light of the strong negative selective pressure identified for the *D. rotundus* orthologue T2R7, we further tested a set of bitter salts for their activation of the three *D. rotundus* receptors (table 2). While, as anticipated, no responses were evident for cells expressing T2R1 and T2R4 (data not shown), we observed responses for T2R7. All tested di- and trivalent salts showed an activation of the T2R7, whereas the monovalent salt potassium chloride did not. Because of the use of a Ca^{2+}-imaging assay, the activation by $CaCl_2$ cannot be fully confirmed, however, T2R7 transfected cells showed a higher response than mock-transfected cells suggesting the activation by this bitter salt as well (figure 4).

To compare the human and the *D. rotundus* receptor orthologues in potency and efficacy, we monitored dose–response relationships for the tested bitter salts (figure 5; electronic supplementary material, figures S1 and S2). Considering the determined activation threshold values, human TAS2R7 and *D. rotundus* T2R7 showed similar sensitivities with some minor deviations (table 2). By contrast, the receptors showed different efficacies for the metal ions, except for $MgCl_2$ and $FeCl_2$. We were not able to determine most of the EC_{50} values, because of lacking receptor signal saturation in the range of applicable agonist concentrations. Only for $CuCl_2$ an EC_{50} value for both receptors was calculated. With 1.62 ± 0.11 mM for the *D. rotundus* T2R7 and 2.03 ± 0.03 mM for the human TAS2R7, these values were in a comparable range, indicating a similar potency of $CuCl_2$ to activate both receptors (table 2).

4. Discussion

Vampire bats' nutrition is solely based on blood and their prey selection is suggested to be primarily facilitated through smell, echolocation and infrared sensors [27,37,38]. Therefore, the functionality of T2Rs and their involvement in prey selection and sensing of bitter substances has been debated in former works [32]. We found that 84% of the tested natural and synthetic bitter compounds did not activate the investigated *D. rotundus* receptors, which may indeed hint at a more restricted perceptual space of *D. rotundus*. For comparison, human receptors did not respond to approximately 12%, frog T2Rs to 25%, chicken T2Rs to 32%, mouse Tas2rs to 34%, turkey T2Rs to 43%, domestic cat T2Rs to 50% and zebra finch T2Rs to 75% of a similar set of natural and synthetic bitter substances [25,48–50]. It has to be considered that in the case of frog, zebra finch and *D. rotundus* not all of the

Table 2. Activation of human TAS2R7 and *Desmodus rotundus* T2R7 by metal salts. The tested metal salts are presented including their maximal applied concentration (c_{max}). No activation is indicated by (x). As a Ca^{2+}-imaging assay was used, the activation of the TAS2R7 by $CaCl_2$ cannot be confirmed with certainty. Dose–response relationships were established for the metal salts $CuCl_2$, $FeCl_3$, $MgSO_4$, $MgCl_2$, $FeCl_2$, $MnCl_2$ and $ZnSO_4$, which activated the human TAS2R7 and the *D. rotundus* T2R7. For comparison of both receptors, the activation threshold concentrations, the EC_{50}-concentrations in mM and the efficacies in relative light units (RLU) are presented. EC_{50}-concentrations, that were not determinable, are indicated by (—). The compound concentrations triggering the strongest receptor responses are given in parenthesis.

		threshold		EC_{50}		efficacy	
	c_{max}	T2R7	TAS2R7	T2R7	TAS2R7	T2R7	TAS2R7
$CaCl_2$	30 mM	?	?	?	?	?	?
KCl	100 mM	x	x	x	x	x	x
$CuCl_2$	30 mM	1.3	1.3	1.62 ± 0.11	2.03 ± 0.03	0.81 ± 0.15 (10 mM)	0.35 ± 0.02 (10 mM)
$FeCl_3$	30 mM	0.75	0.9	0.77 ± 0.09	—	0.82 ± 0.17 (3 mM)	0.40 ± 0.04 (1 mM)
$MgSO_4$	300 mM	30	10	—	10.45 ± 4.02	0.19 ± 0.05 (100 mM)	0.37 ± 0.02 (100 mM)
$MgCl_2$	100 mM	10	30	12.74 ± 8.66	—	0.17 ± 0.06	0.21 ± 0.04
$FeCl_2$	50 mM	3	1	4.51 ± 0.46	—	0.21 ± 0.05	0.14 ± 0.04 (10 mM)
$MnCl_2$	100 mM	10	3	—	3.94 ± 0.32	0.14 ± 0.04 (30 mM)	0.36 ± 0.09 (30 mM)
$ZnSO_4$	15 mM	3	0.3	—	0.38 ± 0.02	0.23 ± 0.01	0.42 ± 0.05

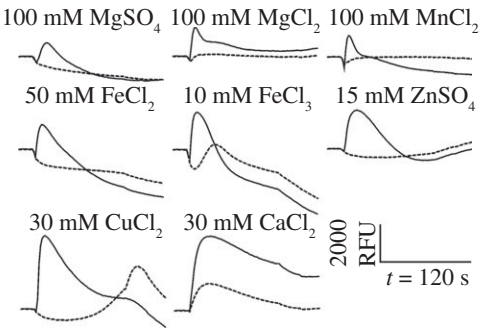

Figure 4. Identification of bitter salts as agonists for the *Desmodus rotundus* bitter taste receptor T2R7. HEK293T–Gα16gust44 cells were transiently transfected with the *D. rotundus* bitter taste receptor T2R7 and the empty vector control (mock). Fluorescence intensities, according to the Ca^{2+}-release upon receptor activation, were measured with an automated fluorometric imaging plate reader ($FLIPR^{Tetra}$). Responses of receptor (solid lines) and mock-transfected (dashed lines) cells to the exposure of $MgSO_4$, $MgCl_2$, $MnCl_2$, $FeCl_2$, $FeCl_3$, $ZnSO_4$, $CuCl_2$ and $CaCl_2$ are presented.

putatively functional T2Rs were included in the studies and that the bitter compound sets used in the different studies are slightly varying. Consequently, the real percentage of undetected bitter compounds by these T2Rs may differ from those indicated. Moreover, it is important to point out that the bitter compound libraries used by us and others are biased towards humans, and thus species tailored libraries may result in different fractions of activating bitter substances. It is further hypothesized that the bitter taste sensation abilities of mammals are correlated to nutritional requirements [57]. Vertebrates with limited nutrition spectra, like blood for vampire bats, might have a decreased or more specialized ability to taste bitter substances [24,31]. Hence, important agonists for vampire bat T2Rs may not have been included in our set of bitter compounds.

However, genetic data showed evolutionary conserved T2Rs in vampire bats and some electrophysiological and behavioural studies already confirmed their functionality [32,42,44]. The characterization of the three *D. rotundus* T2Rs in our work as well as in the recent report by Lu *et al.* [46], suggests the persistence of function for these receptors, which may, therefore, be involved in taste sensation by responding to bitter compounds in blood. Except for the synthetic compounds ethylhydrocupreine, chloroquine and denatonium benzoate, a substance known as a very strong bitter agent for humans, all of the identified agonists are natural. Whether these or other yet to be discovered agonists appear in the blood in concentration ranges able to activate bitter taste receptors of vampire bats remains elusive.

An independent and concurrent study recently reported the ligands of some *D. rotundus* T2Rs [46]. Lu and colleagues screened a set of 19 mostly synthetic bitter compounds selected from past research conducted by members of the present authorship at a single concentration and identified agonists for 5 of the 8 investigated *D. rotundus* T2Rs. Surprisingly, some differences in the activation of the tested T2R1, T2R4 and T2R7 were evident. Considering these 3 T2Rs, Lu and colleagues showed activation of all receptors by denatonium benzoate and activation of T2R1 and T2R4 by chloramphenicol, whereas we could not confirm T2R1 responses to denatonium benzoate and found T2R1 and T2R7 responding to chloramphenicol instead. One additional compound, the natural bitter substance yohimbine was reported to activate T2R7, although our screening efforts could not confirm a T2R7 response to this substance. Despite intrinsic similarities in the two screening approaches (both performed calcium-imaging analyses), we speculate that the observed differences reflect the numerous variations in the experimental setups including the use of different cell lines, different screening strategies (single compound concentrations versus high and low concentrations) and different sensitivities of the employed fluorometric imaging devices. By contrast to Lu *et al.* [46], we identified quinine, which was shown to be avoided by *D. rotundus* when added to blood samples, as an agonists of *D. rotundus* T2R7 [42].

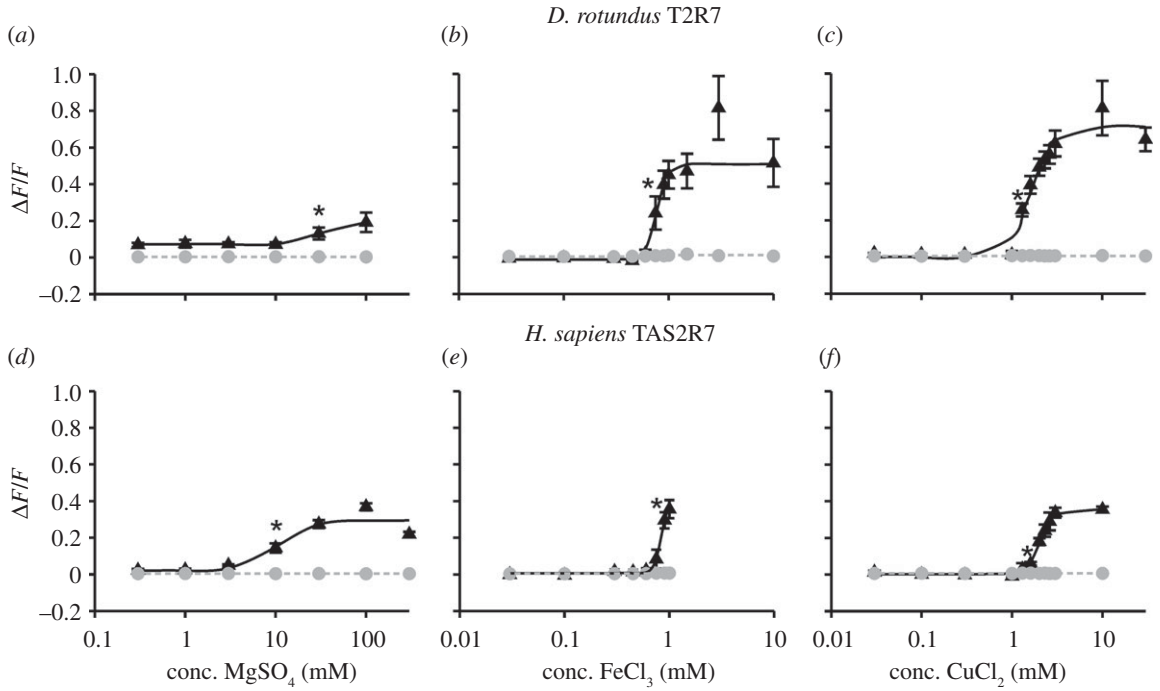

Figure 5. Concentration–response relationships of the activation of human TAS2R7 and *Desmodus rotundus* T2R7 by selected bitter salts. HEK293T–Gα16gust44 cells were transiently transfected with human TAS2R7 (*d–f*) and *D. rotundus* T2R7 (*a–c*) and the empty vector control (mock). Fluorescence intensities, according to the Ca^{2+}-release upon receptor activation, were measured with an automated fluorometric imaging plate reader (FLIPR^{Tetra}). Different concentrations of $MgSO_4$ (*a*,*d*), $FeCl_3$ (*b*,*e*) and $CuCl_2$ (*c*,*f*) were used. The relative fluorescence intensities (black) were mock (grey) subtracted and plotted against the ligand concentration in mM ($n = 3$). Statistical significance ($p < 0.01$) is presented by (*).

The behavioural experiments with quinine were performed in a concentration range between 0.1 and 30 mM, with a statistically significant avoidance at 30 mM. In our screening, the T2R7 response to quinine was already detectable at a concentration of 30 µM, which is one thousandth of the published data. As the bitter substances in our assay are dissolved in a simple buffer system, whereas blood is very complex and rich in carrier proteins for rather hydrophobic substances such as quinine, we speculate that this has prevented a more sensitive detection in the *in vivo* experiment [58]. Assuming that this is also true for other bitter compounds potentially present in blood, vampire bats may generally require T2Rs whose threshold concentrations are able to detect their agonists despite quenching by blood components. Further influences, like the composition of the vertebrate's saliva, can manipulate the sensitivity of bitter-tasting *in vivo* [59–61].

Artemisinin and quinine represent typical members of sesquiterpene lactone and alkaloid bitter compounds and even chloroquine, albeit a synthetic compound, shows chemical similarities with quinine, hence, these activators may represent prototypical plant bitter substances. Therefore, in light of the assumption that the common ancestor of phyllostomid bats, including Desmodontinae, exhibited an insectivorous lifestyle with slight frugivory prior to the massive adaptive radiation giving rise to multiple nutritionally specialized bat clades [62], the responses to plant-derived bitter substances may represent retained functionality inherited from a insectivorous/frugivorous ancestor.

The alignment of *D. rotundus* T2R7 and human TAS2R7 (figure 1; electronic supplementary material, appendix S3) revealed an amino acid sequence identity of approximately 77%. It was reported previously that the human TAS2R7 is responding to bitter-tasting salts, with H94$^{3.37}$ and E264$^{7.32}$ as crucial residues for the interaction with metal ions [18,19]. These residues are conserved in *D. rotundus* T2R7 (electronic supplementary material, appendix S3). Interestingly, we demonstrated that the *D. rotundus* T2R7 is indeed also activated by these substances and activation threshold concentrations of human TAS2R7 and *D. rotundus* T2R7 are in similar ranges (table 2). However, these concentrations are in a high µM to low mM range and bitter salts are, therefore, unlikely to appear in taste relevant concentrations in the blood of prey animals of *D. rotundus* (table 2) [19]. Occasionally, vampire bats do drink water [63] and some springs (e.g. hot volcanic springs in Cauca valley, Colombia, a habitat of *D. rotundus* [64]) contain water highly enriched in bitter metal salts with magnesium ion concentrations of up to 15.5 mM [65]. This concentration is above the half-maximal activating (EC_{50}-) concentration for *D. rotundus* T2R7. As metal salts were shown to interact with heparin [66], the digestibility of blood meals might become changed by water from bitter springs, providing a possible explanation for this T2R function.

Mammalian bitter taste receptors are also present in various extra-gustatory tissues, where they might play important roles in fulfilling significant endogenous biological functions [47]. A putative extra-oral function of the activation of the *D. rotundus* T2R7 by bitter salts, as was proposed for the human orthologue [19], may also be possible and is a promising avenue for future research.

In conclusion, we were able to characterize the three T2Rs common to all extant vampire bat species, with T2R7 as the most broadly tuned receptor. In combination with previously published data, our results confirm a persisting functionality of *D. rotundus* T2Rs [36,42,44,46]. In particular, *D. rotundus* T2R7 shares di- and trivalent metal ions as

common agonists with human TAS2R7. To fully understand the role of *D. rotundus* T2Rs in taste sensation and signalling in extra-gustatory tissues, further research concerning the functionality and activation mechanisms of these receptors *in vivo* is necessary.

Data accessibility. All data of this study are provided in the main text and electronic supplementary material.

Authors' contributions. M.B. and F.Z. designed the work. F.Z. performed the experiments. F.Z. and M.B. analysed the data. M.B. and F.Z. wrote the article. All authors have read and agreed to the published version of the manuscript.

Competing interests. We declare we have no competing interests.

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3.3. Physiological activation of human and mouse bitter taste receptors by bile acids

The article *"Physiological activation of human and mouse bitter taste receptors by bile acids"* (https://doi.org/10.1038/s42003-023-04971-3) was published in "Communications Biology" in 2023. Florian Ziegler developed the concept in consultation with Maik Behrens. Florian Ziegler generated and evaluated experimental data and prepared the original draft of the manuscript. All co-authors contributed to the revision. Alexandra Steuer and Antonella Di Pizio performed the modeling studies and wrote the respective section. Revision according to the reviewers' suggestions was performed by all authors of the manuscript.

Bitter taste receptors are expressed in several non-gustatory tissues in mammals. Compared to numerous other GPCRs, bitter taste receptors sensitivity to its agonists is rather weak. Therefore, exogenous compounds taken up by ingestion will presumably not reach most of these tissues in concentrations that suffice to activate bitter taste receptors. For this reason, endogenously synthesized compounds are of major research interest for their potential to activate bitter taste receptors. Here, we showed a comprehensive functional characterization of human and mouse bitter taste receptor responses to a set of eight different bile acids, including primary, secondary, and tertiary, as well as conjugated bile acids. Using an established heterologous expression system, bitter taste receptor activation was measured by detecting the Ca^{2+} release with an automated fluorometric plate reader. In doing so, we identified five human and six mouse bitter taste receptors that responded to bile acids. To conclude a biological relevance of bitter taste receptor activation by bile acids, we further established dose-response relationships. By comparison of the identified activation threshold concentrations, meaning the lowest concentration that showed bitter taste receptor activation, with published bile acid concentrations in the human body, we were able to show that in some cases endogenous concentrations exceed measured threshold concentrations. This indicates the potential of bile acids to activate non-gustatory bitter taste receptors. For the activation of bile acids to the human TAS2R1 we were able to identify hydroxyl groups at position 7 and 12 as critical moieties for sensitivity in experimental studies. To verify these results and get a closer look into the

binding mode of bile acids to TAS2R1, we further performed modeling studies of bile acid interactions with the binding pocket of TAS2R1. These studies showed that the hydroxyl groups do not have any influence on the pose of binding of bile acids but affect the hydrophobic interactions with phenylalanines at position 179 and 183.

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Physiological activation of human and mouse bitter taste receptors by bile acids

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Beside the oral cavity, bitter taste receptors are expressed in several non-gustatory tissues. Whether extra-oral bitter taste receptors function as sensors for endogenous agonists is unknown. To address this question, we devised functional experiments combined with molecular modeling approaches to investigate human and mouse receptors using a variety of bile acids as candidate agonists. We show that five human and six mouse receptors are responsive to an array of bile acids. Moreover, their activation threshold concentrations match published data of bile acid concentrations in human body fluids, suggesting a putative physiological activation of non-gustatory bitter receptors. We conclude that these receptors could serve as sensors for endogenous bile acid levels. These results also indicate that bitter receptor evolution may not be driven solely by foodstuff or xenobiotic stimuli, but also depend on endogenous ligands. The determined bitter receptor activation profiles of bile acids now enable detailed physiological model studies.

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The mammalian taste system is generally able to distinguish the five basic taste qualities salty, sour, sweet, umami and bitter[1,2]. Among the four different cell types housed in taste buds, the type II cells express the G protein-coupled receptors (GPCRs) mediating sweet, umami and bitter taste[3]. Taste GPCRs are divided into the two subfamilies TAS1Rs and TAS2Rs[4]. The three TAS1R members, TAS1R1, TAS1R2, and TAS1R3, form the heterodimers TAS1R1/TAS1R3 for the functional umami and TAS1R2/TAS1R3 for the functional sweet taste receptor[5–10]. In contrast, the group of TAS2Rs consists of ~25 known functional bitter taste receptors in human, but this number varies considerably among species[11]. Despite the relatively few bitter taste receptors, mammals are able to sense hundreds of bitter tasting compounds[12–14]. This is ensured by the presence of broadly tuned receptors, which can detect high numbers of chemically diverse agonists[12]. In humans, the three broadly tuned receptors TAS2R10[15,16], TAS2R14[17] and TAS2R46[14] recognize together more than half of all tested bitter substances[12]. Narrowly and intermediately tuned bitter taste receptors were shown to have more restricted agonist profiles[12]. A physiological relevance for the observed differences in tuning breadths remains to be determined.

In general, strong bitter taste is perceived as unpleasant and as many noxious substances are known to taste bitter, it was thought to have its main function in warning mammals of the ingestion of toxic substances[18], although no strict correlation between bitterness and toxicity has been observed[19,20]. Moreover, the expression of bitter taste receptors also in non-gustatory tissues has been confirmed. The detection in tissues like the gastrointestinal tract, the respiratory tract and the heart hints at further biological functions beyond taste[21–23]. Beside the gastrointestinal tract, where the swallowed food compounds may directly activate the expressed bitter taste receptors, research is ongoing to uncover agonists of extra-oral bitter taste receptors. Activation of TAS2Rs in airway epithelial cells for example, was already shown to induce increase in ciliary beat frequency to speed up mucociliary clearance with bacterial quorum-sensing molecules as suggested agonists[22].

It has long been known, that the body fluid bile containing the endogenously produced compound class of bile acids tastes extremely bitter[24]. In human, bile acids are produced as the primary bile acids cholic acid and chenodeoxycholic acid in the liver from cholesterol as scaffold structure, secreted into the gallbladder after conjugation to taurine or glycine and released into the small intestine in response to food intake as they primarily fulfill nutritional functions, like solubilization of lipophilic food compounds[25–27]. In the intestinal lumen, they are exposed to the gut microbiota, which further modify them to secondary bile acids[28,29]. By reabsorption, bile acids are released into the portal venous blood and transported back to the liver to start the circulation again[30,31]. Only a small proportion of bile acids is excreted by the feces or enters the systemic circulation[32].

Besides their role in digestion, bile acids are already known to fulfill further physiological functions by activation of receptors like the GPCR TGR5[33]. The stimulation of this receptor by taurolithocholic acid in human macrophages induces the expression of anti-inflammatory cytokine IL-10 and reduces the expression of proinflammatory cytokines, indicating the importance of bile acids in immune responses[34]. Therefore, the question arises if also extra-oral bitter taste receptors can mediate biological functions by stimulation with bile acids.

In fact, recent studies demonstrated the bile acid taurocholic acid representing an agonist for bitter taste receptors of mouse, human and bony fish[11,13]. For bony fish furthermore, chenodeoxycholic acid, deoxycholic acid, glycocholic acid and taurolithocholic acid were shown to activate the T2R02 of *Latimeria chalumnae* with activation threshold concentration for taurolithocholic acid between 0.3 and 1 μM[11]. Up to now, there are no data about the activation thresholds for human receptors when stimulated with bile acids, however, to conclude a physiological function for endogenous bile acids activating non-gustatory bitter taste receptors (periodic) supra-threshold concentrations are essential.

The human metabolome database (HMDB) revealed the detection of several endogenously occurring bile acids and provides quantitative data for almost 30 of them in various body fluids[35]. To determine if they might be relevant for the activation of non-gustatory TAS2Rs, their bitter taste receptor activation profile has to be investigated in more detail. Therefore, we employed a set of eight bile acids that were detected and quantified in the human body[35] to perform a complete functional characterization of the activation of human and mouse bitter taste receptors. The chosen bile acids covered a broad range, including primary, secondary and tertiary bile acids.

Results

Screening of human and mouse bitter taste receptors for their activation by bile acids. To gain a comprehensive insight in the activation profiles of human and mouse bitter taste receptors by bile acids, we tested a set of 8 different bile acids for their activation of 25 human (TAS2R1, −R3, −R4, −R5, −R7, −R8, −R9, −R10, −R13, −R14, −R16, −R19, −R20, −R30, −R31, −R38, −R39, −R40, −R41, −R42, −R43, −R45, −R46, −R50, −R60) and 34 mouse bitter taste receptors (Tas2r102, −r103, −r104, −r105, −r106, −107, −r108, −r109, −r110, −r113, −r114, −r115, −r117, −r118, −r119, −r120, −r121, −r122, −r123, −r124, −r125, −r126, −r129, −r130, −r131, −r134, −r135, −r136, −r137, −r138, −r139, −r140, −r143, −r144). The set of bile acids included the primary bile acids cholic and chenodeoxycholic acid, the secondary bile acids lithocholic and deoxycholic acid, the tertiary bile acid ursodeoxycholic acid, as well as the conjugated bile acids taurolithocholic, glycocholic and taurocholic acid (Fig. 1).

By performing a screening using Ca^{2+}-imaging assay, we were able to identify 5 human bitter taste receptors responding to bile acids (Fig. 2).

The human bitter taste receptors TAS2R1, TAS2R4, TAS2R14, TAS2R39 and TAS2R46 responded to at least three of the tested bile acids. Of those, TAS2R1 was the least selective as it was stimulated by all eight bile acids. Additionally, TAS2R4 was activated by 6, TAS2R14 by 5, and both TAS2R39 and TAS2R46 by 3 of the bile acids.

To assess the potential phylogenetic conservation of the responses observed for the human receptors, we performed the identical screening procedure using mouse Tas2rs (Fig. 3).

For mouse bitter taste receptors Tas2r105, Tas2r108, Tas2r117, Tas2r123, Tas2r126 and Tas2r144 responses to bile acids were observed. Compared to the human receptors, none of them responded to all tested bile acids and CDCA did not activate any of the tested mouse Tas2rs.

Establishment of dose-response relationships with activated bitter taste receptors. To elucidate the potency of bile acids to activate human bitter taste receptors and thus get a first hint if endogenous bile acid concentrations could suffice to activate bitter taste receptors, dose-response relationships were established (Fig. 4, Supplementary Figures 1–4). For this purpose, different concentrations of the bile acids were applied to the cells expressing the receptors. The lowest concentration eliciting a significantly increased fluorescence response ($p < 0.01$) compared to the empty vector control was defined as activation threshold

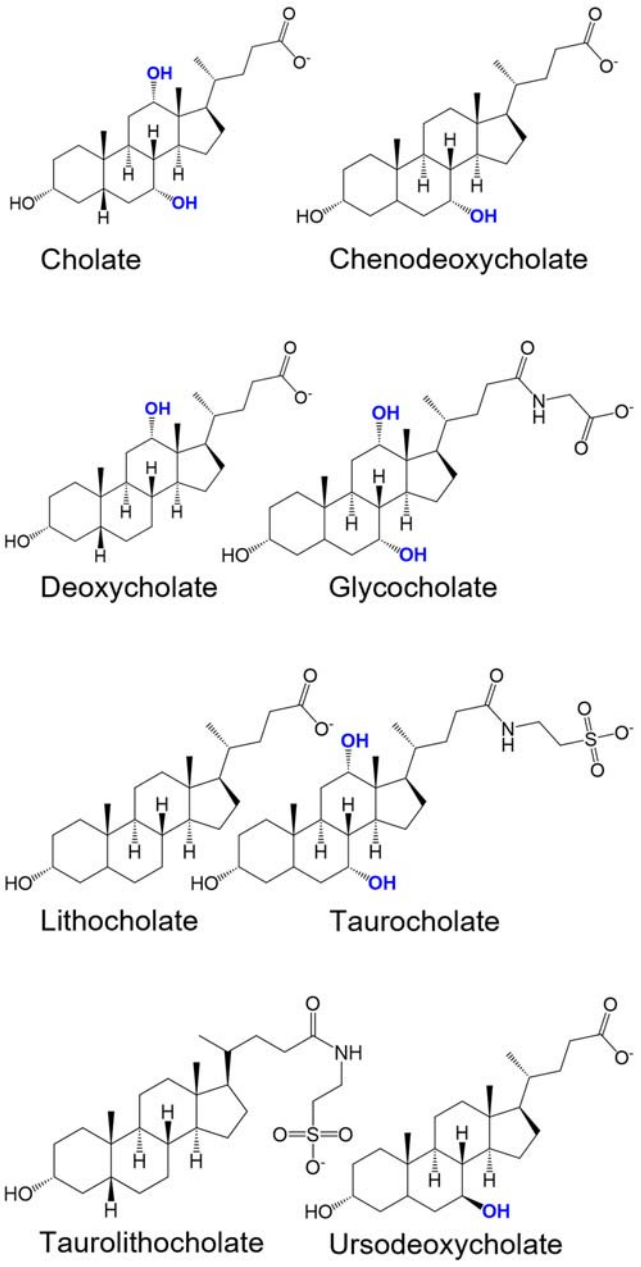

Fig. 1 Chemical structures of the investigated bile acids. The structural formulas of the eight bile acids cholic acid, chenodeoxycholic acid, deoxycholic acid, glycocholic acid, lithocholic acid, taurocholic acid, thaurolithocholic acid, and ursodeoxycholic acid in their deprotonated form at physiological conditions (pH ~ 7) are presented. Hydroxyl groups at positions 7 and 12 are highlighted in bold and blue. Structures were generated with ChemDraw software.

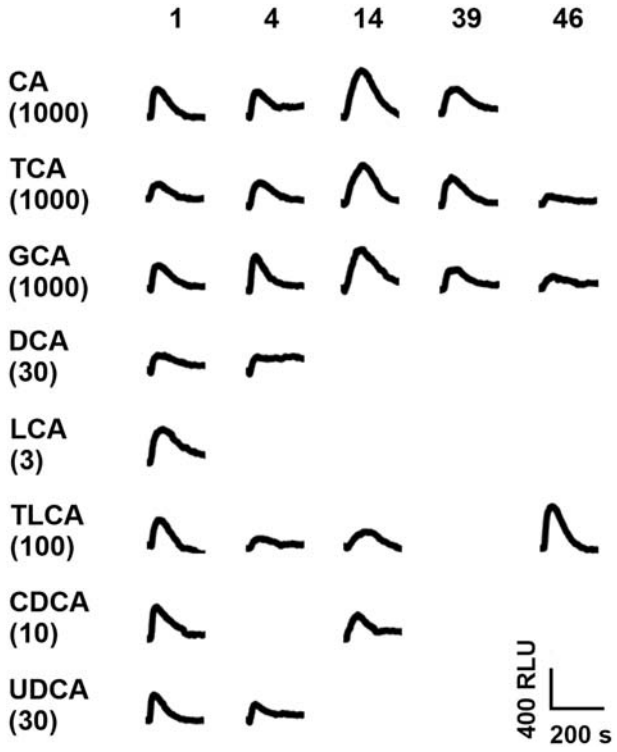

Fig. 2 Human bitter taste receptor responses to bile acids. Fluorescence traces of HEK293T-Gα16gust44 cells transiently transfected with expression constructs of the human bitter taste receptors TAS2R1 (1), TAS2R4 (4), TAS2R14 (14), TAS2R39 (39), and TAS2R46 (46). Cells were exposed to cholic acid (CA), taurocholic acid (TCA), glycocholic acid (GCA), deoxycholic acid (DCA), lithocholic acid (LCA), taurolithocholic acid (TLCA), chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA). Fluorescence changes were measured with an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). Fluorescence traces are negative control corrected. Applied bile acid concentrations are given in μM in brackets. Only traces of responsive receptors are shown. A scale bar is provided at the bottom right.

concentration (Table 1). In case receptor saturation was reached with the highest applied bile acid concentration, we further calculated the EC_{50}-value (Supplementary Table 1).

In doing so, the secondary bile acid lithocholic acid and its taurine-conjugated form taurolithocholic acid were identified as the most potent bile acids. For lithocholic acid, the determined threshold concentration for the activation of the TAS2R1 was 0.3 μM. Same threshold values were detected for the activation of TAS2R1, TAS2R14, and TAS2R46 by taurolithocholic acid. For TAS2R1, also the EC_{50}-values of these two bile acids were in the high nanomolar and low micromolar range, respectively (Supplementary Table 1). In total, the activation threshold concentrations of all tested bile acids varied considerably and ranged between high nanomolar values for the mentioned bile acids and low millimolar values for the activation of TAS2R46 by taurocholic acid and glycocholic acid (Table 1).

To evaluate the agonistic efficacy of bile acids for human TAS2Rs, we compared the signal amplitudes of well-known TAS2R agonists with bile acids activating TAS2R1, TAS2R4, TAS2R14, TAS2R39 and TAS2R46 (Fig. 5).

In case of TAS2R1, measured efficacies of all 8 bile acids were comparable with the control stimulus picrotoxinin. For TAS2R14, the agonist aristolochic acid elicited responses about twice as high as the most effective bile acids and the response of TAS2R46 to taurolithocholic acid is about equal to that obtained for strychnine. Bile acids responses of TAS2R4 and TAS2R39 were considerably stronger than for the control stimuli colchicine and denatonium benzoate, respectively.

The same workflow for the evaluation of potencies was applied for the 34 mouse Tas2rs. Again, we calculated activation threshold concentrations (Table 2) and generate dose-response relationships (Fig. 6, Supplementary Figures 5–10).

As it was already shown for the human receptors, lithocholic acid and taurolithocholic acid are also the most potent bile acids for mouse Tas2rs (Table 2).

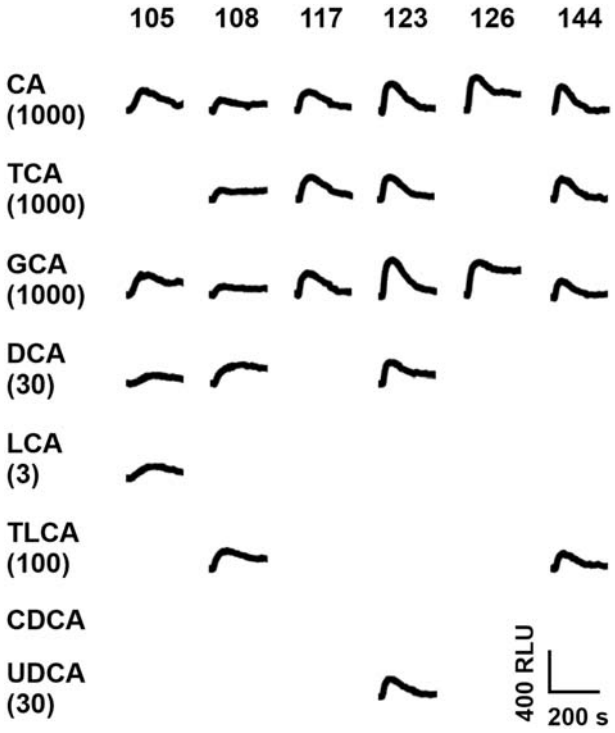

Fig. 3 Mouse bitter taste receptor responses to bile acids. Fluorescence traces of HEK293T-Gα16gust44 cells transiently transfected with expression constructs of the mouse bitter taste receptors Tas2r105 (105), Tas2r108 (108), Tas2r117 (117), Tas2r123 (123), Tas2r126 (126), and Tas2r144 (144). Cells were exposed to cholic acid (CA), taurocholic acid (TCA), glycocholic acid (GCA), deoxycholic acid (DCA), lithocholic acid (LCA), taurolithocholic acid (TLCA), chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA). Fluorescence changes were measured with an automated fluorescence plate reader (FLIPR^{TETRA}). Fluorescence traces are negative control corrected. Applied bile acid concentrations are given in μM in brackets. Only traces of responsive receptors are shown. A scale bar is provided at the bottom right.

By generating dose-response relationships, the threshold concentration for the activation of the Tas2r108 by taurolithocholic acid was determined as 1 μM. With 3 μM the activation threshold concentration of lithocholic acid for Tas2r105 and taurolithocholic acid for Tas2r144 were in a similar range (Fig. 6). Compared to the mouse bitter taste receptors, the human receptors are more sensitive for these two bile acids.

Predicted binding modes of bile acids within the TAS2R1 binding site. The 3D structure of TAS2R1 was obtained with homology modeling using the recently solved structure of TAS2R46[36] as a template (sequence identity = 27%). Interestingly, the first solved structures of TAS2R46 suggest a high flexibility of the EC loops, specifically of the ECL2 domain, which is not resolved in the bound state conformation of the receptor, and for which the folding obtained in the unbound states overlaps with the ligand position. The ECL2 connects transmembrane helices 4 and 5 and is diverse in length and composition in currently solved GPCRs[37]. It was demonstrated that docking performance could be insensitive to or even improved by excluding ECL2 from the calculations[16,38,39] Therefore, because of the uncertainness of the ECL2 folding, we modelled TAS2R1 without the ECL2.

To predict the binding modes of bile acids within the orthosteric TAS2R1 binding site, we ran molecular docking simulations, and generated thirty different poses for each compound. Among all poses, we selected a consensus binding mode, namely the most frequent pose observed for all ligands that is also the best pose for lithocholic acid. The ligands insert into the orthosteric binding site by anchoring to TM3 and TM5. These poses have docking scores that correlate well with activation thresholds, but to further optimize ligand-receptor interactions, the poses were rescored with MM/GBSA minimization. The resulting binding modes were not affected but the scoring improved even more (Table 3, Supplementary Figures 11 and 12), suggesting that the model can capture key ligand-receptor interactions and supporting the assumption that bile acids bind to the orthosteric binding site.

In Fig. 7, we show the 2D and 3D representations of the binding mode of lithocholic acid within TAS2R1. The ligand forms hydrogen bonds with the side chain of N89$^{3.36}$ and the main chain of Q175$^{5.39}$ and it is accommodated in a hydrophobic cavity generated by F179$^{5.43}$, F183$^{5.47}$, L247$^{6.51}$ and I266$^{7.42}$.

The binding modes of all other ligands are reported in Supplementary Figure 11. Lithocholic, chenodeoxycholic, ursodeoxycholic, deoxycholic and cholic acids differ mostly in the presence/absence of hydroxyl groups in position 7 and 12. Interestingly, we found that these hydroxyl groups do not affect the pose of binding but point to F179$^{5.43}$ and F183$^{5.47}$, affecting the hydrophobic interactions observed for lithocholic acid (Fig. 7). Taurolithocholic acid, with a similar activity threshold to lithocholic acid, also misses the hydroxyl groups in positions 7 and 12, suggesting that hydrophobic complementarity is important for receptor binding and activation. Glycocholic and taurocholic acids are the ligands that differ most in the binding mode. The methyl groups of glycocholic acid push the ligand higher in the binding site, we lose the interaction with N89$^{3.36}$, and the ligand anchors instead to Y9 in TM1.

Discussion

The detection of bitter taste receptors in tissues beside the gustatory system has resulted in an increased interest in the biological role(s) of these receptors in non-gustatory tissues and in the nature and putative origins of the bitter substances that activate the receptors outside the oral cavity. Therefore, one hypothesis is the existence of endogenous agonists[40] and previous studies already confirmed the activation of human, mouse and bony fish bitter taste receptors by bile acids[11,13]. In general, the group of bile acids is a very complex compound class. As they are released into the small intestine upon food uptake, they are exposed to the gut microbiota. Thereby bile acids are modified and finally a mixture of hundreds of different bile acids is present[28,29]. To get a deeper look into the activation of bitter taste receptors by bile acids, we investigated a diverse group of bile acids, including primary, secondary, and tertiary bile acids. For human and mouse receptors activated by taurocholic acid, we could confirm previously published data according to activation threshold concentrations and EC_{50}-values in this study[13]. Besides, we further identified the human TAS2R1, TAS2R14, TAS2R39 and TAS2R46, as well as mouse Tas2r108 as receptors for taurocholic acid. The different outcomes of both studies may occur due to the rather high activation threshold concentrations of this bile acid (30–1000 μM), which is near the highest applied concentration (1000 μM), whereby a weak activation could have been missed in the previous study in which the concentration used for the screening was limited to 300 μM. Moreover, all newly identified taurocholic acid responsive receptors (mouse Tas2r108, human TAS2R1, −R14, −R39, and −R46) exhibited quite high threshold concentrations of 100–1000 μM and, in case of Tas2r108 and TAS2R1, also low signal amplitudes (cf. Figures 2 and 3). For mouse Tas2r105, the lack of taurocholic acid responsiveness

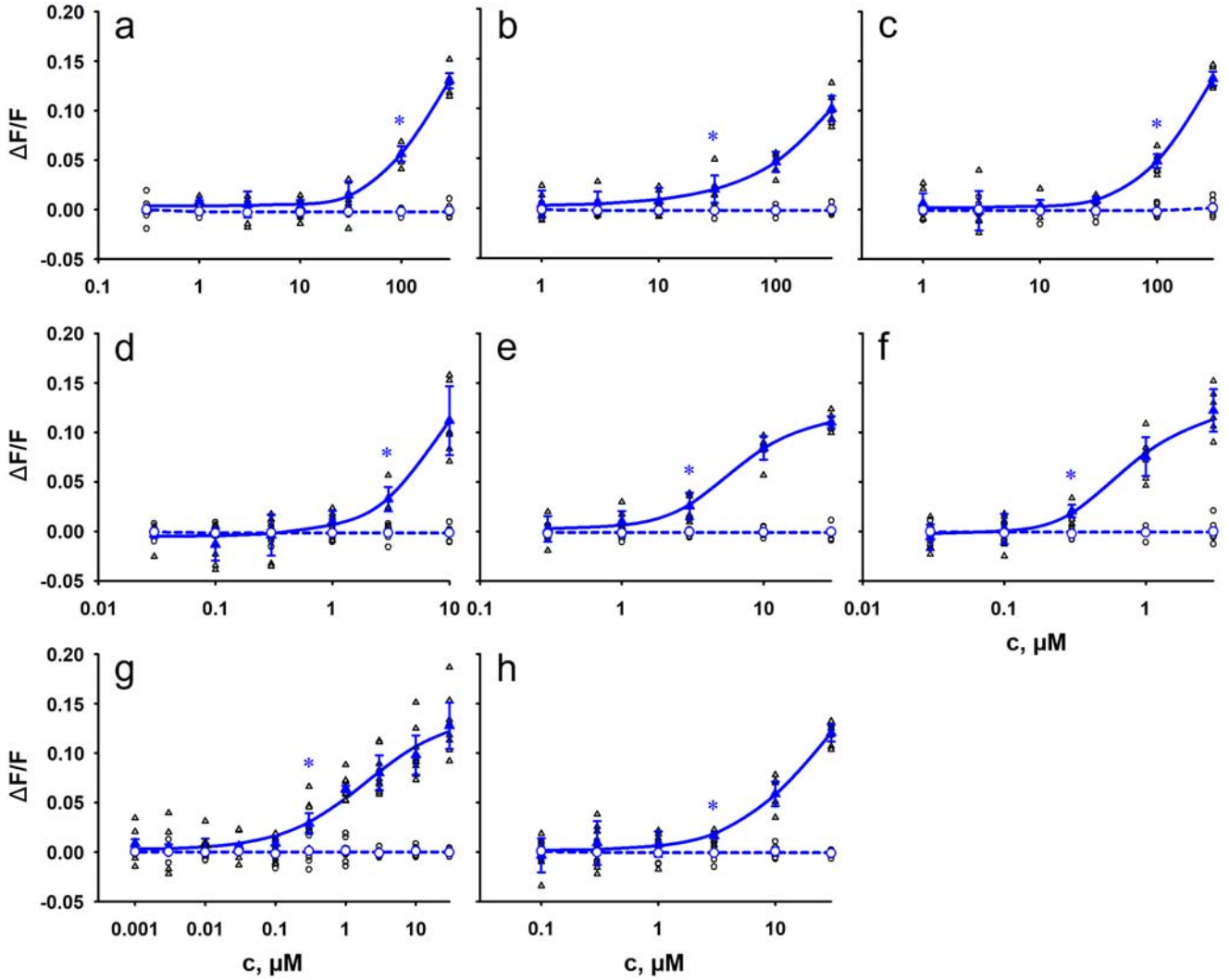

Fig. 4 Concentration-response relationships of eight tested bile acids with human TAS2R1. HEK293T-Gα16gust44 cells were transiently transfected with human TAS2R1 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was recorded by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, increasing concentrations of the bile acids cholic acid **a**), taurocholic acid **b**), glycocholic acid **c**), chenodeoxycholic acid **d**), deoxycholic acid **e**), lithocholic acid **f**), taurolithocholic acid **g**) and ursodeoxycholic acid **h**) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM ($n = 3$ biologically independent experiments). Data are presented as the mean ± standard deviation (STD). Beginning statistical significance ($p < 0.01$) is indicated by (*).

Table 1 Activation threshold concentrations of bile acids with human bitter taste receptors.

	C_{blood}	Max c	TAS2R1	TAS2R4	TAS2R14	TAS2R39	TAS2R46
Cholic Acid	0.1–1.7[61, 62]	1000	100	30	300	300	
Chenodeoxycholic Acid	0.2–1.8[61, 62]	30	3		30		
Lithocholic Acid	0.08–0.33[55, 62]	3	0.3				
Deoxycholic Acid	0.33–0.57[55, 63]	30	3	3			
Taurocholic Acid	0.1–0.38[61, 63]	1000	100	100	300	300	1000
Glycocholic Acid	0.6–0.88[61, 64]	1000	100	30	100	300	1000
Taurolithocholic Acid	0.61–1.81[65]	100	0.3	1	0.3		0.3
Ursodeoxycholic Acid	0.16[55]	30	3	3			

Presentation of TAS2Rs that were activated by bile acids. Determined threshold concentrations ($p < 0.01$) for receptor activation and maximum applied bile acid concentrations (Max c) are given in µM. Published bile acid concentration ranges in human blood (c_{blood};in µM) as summarized in the human metabolome database are listed.

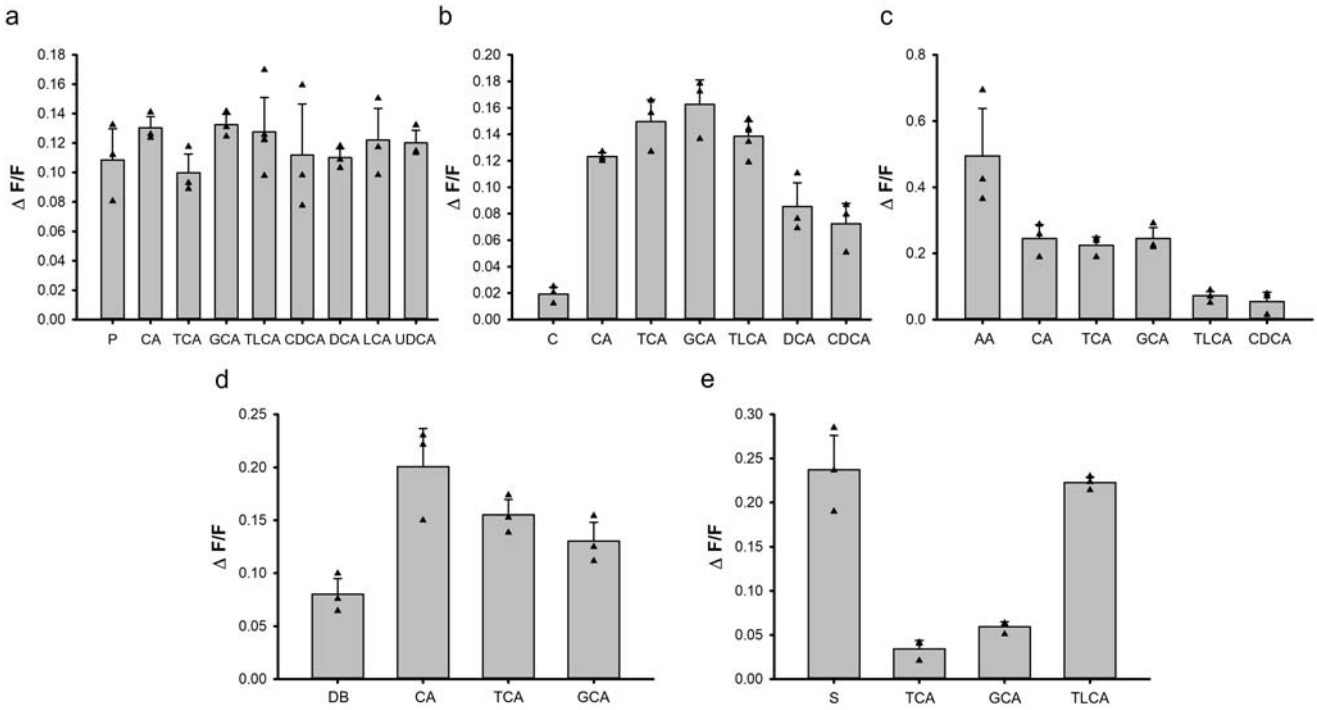

Fig. 5 Comparison of the efficacies of bile acids with prototypical TAS2R agonists. Human bitter taste receptors TAS2R1 **a**), TAS2R4 **b**), TAS2R14 **c**), TAS2R39 **d**), and TAS2R46 **e**) activated by highest applied bile acid concentrations (cholic acid (CA), taurocholic acid (TCA), glycocholic acid (GCA), taurolithocholic acid (TLCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA)) are presented ($n = 3$). For comparison, maximal signal amplitudes (ΔF/F) obtained with control stimuli of the corresponding TAS2Rs were added. The control stimuli were: 1 mM picrotoxinin (P) for TAS2R1, 3 mM colchicine (C) for TAS2R4, 10 μM aristolochic acid (AA) for TAS2R14, 3 mM denatonium benzoate (DB) for TAS2R39 and 10 μM strychnine (S) for TAS2R46. Data are presented as the mean ± standard deviation (STD). Individual data points are depicted by black triangles.

Table 2 Activation of mouse Tas2rs by bile acids.

	Max c (in μM)	Tas2r105	Tas2r108	Tas2r117	Tas2r123	Tas2r126	Tas2r144
Cholic Acid	1000	300	100	10	10	100	100
Chenodeoxycholic Acid	30						
Lithocholic Acid	3	3					
Deoxycholic Acid	30	10	10		10		
Taurocholic Acid	1000		100	30	100		300
Glycocholic Acid	1000	300	100	3	30	100	100
Taurolithocholic Acid	100		1				3
Ursodeoxycholic Acid	30				10		

Presentation of the mouse Tas2rs that were activated by bile acids. Determined threshold concentrations ($p < 0.01$) for receptor activation and maximum applied bile acid concentrations (Max c) are given in μM.

reported by Lossow et al.[13]. has been confirmed, although other, previously not tested bile acids, were able to elicit responses of this receptor.

We further compared the efficacies of bile acids with cognate agonists of the identified human TAS2Rs to evaluate the relative strength of TAS2R activation by bile acids. The results demonstrated that for TAS2R1, bile acid responses are on a similar level with the agonist picrotoxinin indicating that bile acids represent full agonists of this receptor. In contrast, the bile acid agonists of TAS2R14 were not able to trigger a response similar to aristolochic acid, which is one of the most efficient agonists for this receptor. Therefore, the tested bile acids represent only partial agonists of TAS2R14. Responses to control stimuli of TAS2R4 and TAS2R39 were found to be less effective than the activating bile acid agonists, indicating that those may represent full agonists for both receptors. As indicated already by the determined dose-response relationships, the TAS2R46 seems to be a receptor specialized to detect distinct bile acids. Beside the differences in activation threshold concentrations, also the efficacy of TLCA is significantly higher than that of TCA and GCA and comparable to the control stimulus strychnine. Hence, TCLA can be judged as another full agonist of this receptor.

As there are several evidences for the functional conservation of bitter taste receptors between species like the metal ion response of human and vampire bats or the overlapping agonist profiles of coelacanth and zebrafish T2R1, we further investigated mouse bitter taste receptors for their bile acid response[11,41,42]. To compare responses of one-to-one orthologous receptors of both species, we consulted the phylogenetic tree that was generated in a former study[13]. Here, we identified the two receptors TAS2R1

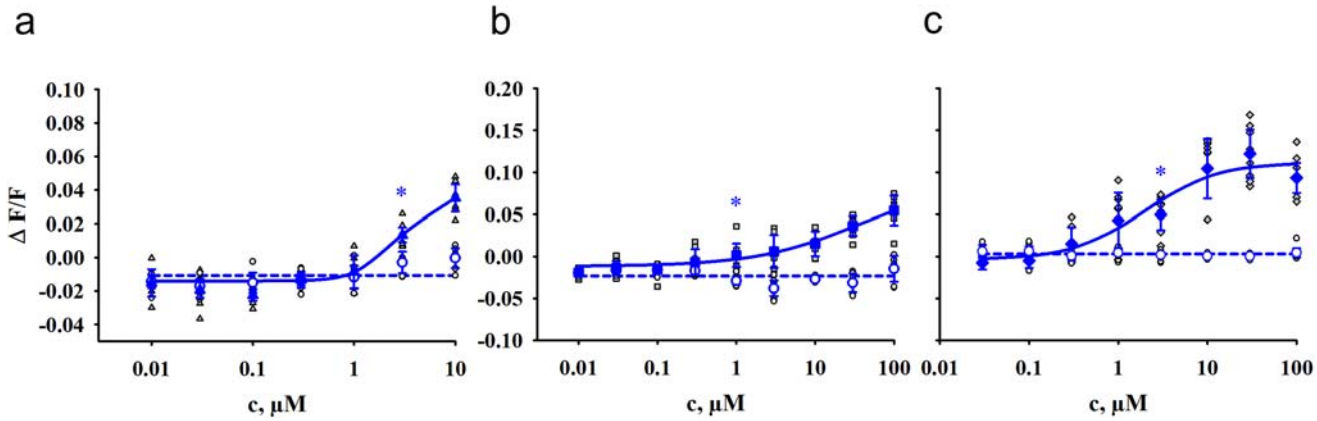

Fig. 6 Concentration-response relationships for the activation of mouse Tas2rs. HEK293T-Gα16gust44 cells were transiently transfected with the murine Tas2r105 (triangle, blue), Tas2r108 (square, blue) or Tas2r144 (diamond, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was recorded by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, increasing concentrations of the bile acids lithocholic acid **a**) and taurolithocholic acid **b**) and **c**) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM ($n = 3$ biologically independent experiments). Data are presented as the mean ± standard deviation (STD). Beginning statistical significance ($p < 0.01$) is indicated by (*).

Table 3 Docking and MM/GBSA scores of analyzed bile acids within the TAS2R1 binding site.

	Activation thresholds [µM]	Docking scores [kcal/mol]	MM/GBSA dG Bind [kcal/mol]
Taurolithocholic Acid	0.30	−5.61	−86.45
Lithocholic Acid	0.30	−5.28	−70.11
Chenodeoxycholic Acid	3.00	−5.08	−69.75
Ursodeoxycholic Acid	3.00	−5.78	−69.60
Deoxycholic Acid	3.00	−5.43	−68.36
Cholic Acid	100.00	−4.57	−65.75
Glycocholic Acid	100.00	−3.36	−65.27
Taurocholic Acid	100.00	−3.33	−48.76

and TAS2R39 responding to bile acids, but their corresponding orthologues Tas2r119 and Tas2r139 do not. In contrast, the orthologue of TAS2R4, called Tas2r108 is activated by bile acids. They share common agonists among the tested bile acids, but there are also significant differences. The human receptor is very sensitive to ursodeoxycholic acid with an activation threshold concentration of 3 µM, whereas the mouse receptor does not respond (Table 1, Table 2). We have analyzed the sequences of the orthologous receptors TAS2R4 and Tas2r108. Among the considerable number of differences between the two receptors (98 positions (~33%) are not identical, see Supplementary Figures 13 – 15), only few minor differences occur in positions which have been demonstrated previously to be important for agonist binding (Supplementary Figures 14 and 15). As ursodeoxycholic acid is the only compound in which the C7 hydroxylgroup is positioned above the plane of ring B, we speculate that sterical hindrance between Tas2r108 residues and the C7 hydroxylgroup could be responsible for the lack of activation.

For the remaining responding receptors, no one-to-one orthologues were identified as they are organized in species-specific gene expansion groups. Therefore, we can confirm that a general conservation of the functionality of orthologous receptors between mice and human does not exist, as it was already proposed elsewhere[13]. We did not obtain any response of mouse receptors to CDCA, whereas human TAS2R1 and TAS2R14 were activated by this bile acid. It is known that mice possess an enzyme called Cyp2c70 in the liver, which is converting CDCA into muricholic acid (MCA)[43]. This keeps CDCA levels low, and hence might be the reason why mouse bitter taste receptors did not exhibit responsiveness to CDCA. Whether mouse Tas2rs instead are more specialized to the mouse-specific MCAs has to be clarified.

Furthermore, we demonstrated that the secondary bile acid lithocholic acid and its taurine conjugated form taurolithocholic acid are the most potent tested bile acids in activating human as well as mouse bitter taste receptors. These bile acids are activating the already known bile acid receptor TGR5 with EC_{50} values of 600 nM for lithocholic and 300 nM for taurolithocholic acid[44]. For the human TAS2R1 we measured EC_{50} values of 900 nM and 1.9 µM, respectively, concluding the TGR5 receptor to be slightly more sensitive to lithocholic acid and taurolithocholic acid. Related to these data, it can be assumed that the activation of bitter taste receptors by bile acids is only of biological relevance in tissues, cells or at subcellular localizations where the TGR5 is not expressed, respectively if bile acid concentration is increasing, the activation of bitter taste receptors might be additive to the TGR5 signal. According to literature, there are some tissues with overlapping TGR5 and TAS2R expression profiles, including the small intestine and the testis[45,46]. In particular in testis, TAS2R1 mRNA was detected in late spermatids at quite high levels, whereas TAS2R4 and TAS2R14 mRNAs were found at lower levels in several cell types of the small (TAS2R4: enteroendocrine cells, Paneth cells, goblet cells, enterocytes; TAS2R14: enterocytes) and large (TAS2R4: undifferentiated cells, enterocytes, goblet cells, enteroendocrine cells; TAS2R14: T-cells, enterocytes, undifferentiated cells, goblet cells, enteroendocrine cells) intestine[47],(https://www.proteinatlas.org/search/TAS2R). It is a matter of future research to elucidate the function of TAS2Rs in these tissues and conclude the interplay between both receptor types. Furthermore, we only tested a subset of the variety of all bile acids. Therefore, we might have missed the best bile acid agonist for human bitter taste receptors, which is more potent to TAS2Rs than to TGR5. However, there are differences in published EC_{50}-values of TGR5 activation by bile acids observed and a recent study reported EC_{50}-concentrations of 20 µM for LCA and 2.3 µM for TLCA[48]. These results suggest, that occasionally TAS2Rs can be more sensitive to bile acids and fulfill their

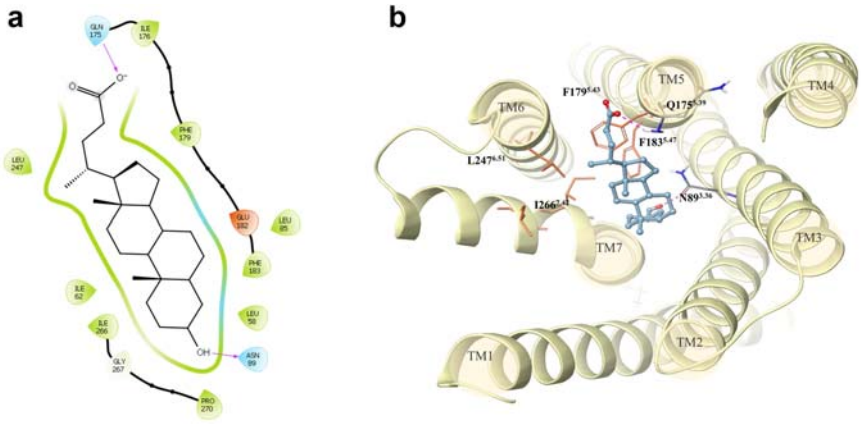

Fig. 7 2D and 3D representations of the putative binding mode of lithocholic acid in the TAS2R1 binding site obtained with MM/GBSA refinement. The 2D plot **a**) was generated using the Ligand Interaction Diagram tool available in Maestro (Schrödinger Release 2022-3) showing residues at 4 Å from the ligand. In the 3D representation **b**), the ligand is shown as blue ball&stick, polar residues in CPK-colored sticks and hydrophobic residues as orange sticks. Hydrogen bonds are shown as dashed magenta lines in both representations.

function independent from TGR5 or that the additive effect is mediated by TGR5.

To answer the question of potential biological relevance, we compared if the quantified concentrations of bile acids in human body fluids listed in the Human Metabolome Database[35] match with our measured data. As already expected, concentrations in bile are in the millimolar range, for which reason a biological relevance of bitter taste receptors in tissues like the gallbladder, the liver or the small intestine is questionable as these concentrations would lead to a permanent activation of the receptors. A previous study, which showed the absence of the known bile acid bitter taste receptors Tas2r117, Tas2r123 and Tas2r144 in mouse small intestine therefore concluded that the absence of the bile acid-sensitive Tas2rs is due to the fact that such receptors are useless if physiological bile acid concentrations exceed threshold concentrations at all times and hence would constantly signal or remain constantly desensitized[49]. As already mentioned, we were able to identify the Tas2r105, the Tas2r108 and the Tas2r126 as further bile acid bitter taste receptors. Published expression data reveals high intestinal expression levels of the Tas2r108 and the Tas2r126[49]. For human TAS2R expression in the small intestine, it is reported that TAS2R4, TAS2R14, TAS2R39 and TAS2R46, which are all responding to bile acids, are expressed in jejunal crypts. In this context, some functions of these receptors in the small intestine are suggested. The activation of TAS2R4 by taurocholic acid was reported to increase the release of molecules that have a positive impact on *E. coli* growth[50]. Therefore, the ingestion of food, which results in the release of bile acids into the small intestine may have positive effects on *E. coli* growth and consequently for the process of digestion. Furthermore, activation of TAS2R14 in a human colorectal cancer cell line is supposed to result in increased GDF15 levels, which is involved in several biological functions like anti-inflammatory and apoptotic pathways[50–52]. To clarify, if these receptors play a role in bile acid detection in the small intestine, further research is necessary.

Beside high concentrations in bile, blood serum bile acid levels increase from 0.2 – 0.7 µM to 4 – 5 µM postprandial[53,54] and for lithocholic acid, which is one of the most potent identified bile acids, a serum concentration of 0.33 µM was measured previously in healthy children subjects[55]. As blood is the main transporting unit in the body, this bile acid will be distributed in concentrations that were shown to be sufficient to activate the human TAS2R1, which is highly expressed in the human brain and testis[46], in particular in late spermatids [47],(https://www.proteinatlas.org/search/TAS2R). It was further shown that the lithocholic acid serum concentration is decreased in children with cystic fibrosis and it is known that men with cystic fibrosis go later through puberty than healthy subjects[55,56]. As brain and testis are important players in puberty a role of bile acids in development from child to adult is conceivable, but further research is required.

Interestingly, the potency of activation of the human TAS2R1 seemed to depend a lot on the presence of hydroxyl groups at positions 7 and 12 of the steroid scaffold structure in our experiments. Docking simulations of bile acids investigated in this paper highlighted the main interactions established with TAS2R1. We found that hydroxyl groups at positions 7 and 12 can affect hydrophobic interactions between the ligands and two hydrophobic patches in the receptor binding site: one made by Leu85$^{3.32}$, Phe183$^{5.47}$, and Phe179$^{5.43}$ and the other one made by Ile266$^{7.42}$ and Leu247$^{6.51}$. We also suggest that the H-bond between the ligands and N89$^{3.36}$ is highly important for the ligand-receptor interaction, and it is supposed to be a key interaction for receptor selectivity. In fact, position 3.36 is highly conserved among the investigated TAS2Rs, but the residue in the close position 3.32 can influence the access to this interaction. In TAS2R1, a leucine occupies this position, but we have bulkier residues (F or W) in TAS2R4, TAS2R14, TAS2R39 and TAS2R46. This difference causes a change of pose in other receptors (we report the predicted binding mode for taurolithocholic acid within the TAS2R46 binding site in Supplementary Figure 16).

In conclusion, we were able to show the activation profile of human and mouse bitter taste receptors by bile acids. We identified five human and six mouse receptors, which are responsive to subsets of tested bile acids. Comparing the determined activation threshold concentrations with physiological bile acid concentrations in the human body, this compound class is very promising as endogenous agonists of bitter taste receptors. The comparative investigation of primary cell lines, intestinal organoids, or mouse models derived from TGR5-knockout[57] and wildtype mice can provide further insights into the activation mechanism and downstream signaling of bitter taste receptors activated by bile acids. It is a future task to experimentally clarify, the exact biological functions of bitter taste receptor activation by bile acids.

Methods

Bile acids. Functional characterization of 25 human[12] and 34 out of 35 mouse bitter taste receptors[13] was performed using a set of 8 different bile acids, including the primary bile acids cholic (Calbiochem, San Diego, United States) and

chenodeoxycholic acid (Sigma-Aldrich, Steinheim, Germany), the secondary bile acids lithocholic (AcrosOrganics, Geel, Belgium) and deoxycholic acid (Sigma-Aldrich, Steinheim, Germany), the conjugated bile acids taurocholic (Biochemika), taurolithocholic (Sigma-Aldrich, Steinheim, Germany), glycocholic acid (Sigma-Aldrich, Steinheim, Germany), as well as the tertiary bile acid ursodeoxycholic acid (Alfa Aesar, Kandel, Germany). This set of bile acids was chosen because of their commercial availability in high purities, their diversity within the class of bile acids and their previous detection and quantitation in human blood. Stock solutions were prepared in DMSO. For the prevention of unspecific cellular responses, the stock solutions are diluted in the assay buffer C1 (130 mM NaCl, 10 mM HEPES pH 7.4, 5 mM KCl, 2 mM $CaCl_2$, 0.18 % glucose) to reduce the DMSO concentration to a maximum of 0.5 % in the final experiments. Maximal applied concentrations are due to solubility problems or receptor-independent artefacts during measurement at high bile acid concentrations (Table 1, Table 2)[12,58].

Cell lines. As basal growth medium for the HEK293T-Gα16gust44 cell line[17,59] served Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific, Darmstadt, Germany) supplemented with 10 % fetal bovine serum (Sigma-Aldrich, Steinheim, Germany), 2 mM L-glutamine (Sigma-Aldrich, Steinheim, Germany), 100 units/ml penicillin (Sigma Aldrich, Steinheim, Germany) and 100 µg/ml streptomycin (Sigma Aldrich, Steinheim, Germany). Growth conditions were 37 °C, 5 % CO_2 and saturated air-humidity[60].

Transient transfection. HEK293T-Gα16gust44 cells were seeded on poly-D-lysine (10 µg/ml) coated 96-well plates to reach a confluence of 40-60 % the next day. For transient transfection 150 ng of plasmid DNA containing the receptor of interest and 0.3 µl lipofectamine 2000 (Thermo Fisher Scientific, Darmstadt, Germany) per well were used and transfection took place according to the manual of lipofectamine 2000. Mouse Tas2r116 was excluded because successful cloning was not possible in a previous work[13]. The empty vector DNA (mock) was transfected as negative control[14,41].

Calcium imaging assay. Cells were loaded using the calcium-sensitive fluorescent dye Fluo-4-AM (Abcam, Cambridge, Great Britain) in the presence of 2.5 mM probenecid (Sigma-Aldrich, Steinheim, Germany) the day after transfection[13,41]. 1 h after loading, cells were washed with C1 buffer using a BioTek Cell Washer, incubated in the dark for half an hour and washed again. For automated agonist application and measurement of fluorescence changes, a $FLIPR^{TETRA}$ device (Molecular Devices, San José, United States) was used. Viability of cells was tested by application of 100 nM Somatostatin 14 (Bachem, Bubendorf, Switzerland)[42].

Data analysis. Measured data were negative control corrected by subtracting the signal of the mock-transfected cells and exported to Microsoft Excel using the FLIPR software ScreenWorks 4.2. In Microsoft Excel software, standardization of maximum fluorescence intensities to the basal fluorescence and normalization to the buffer-only control was done to calculate the relative fluorescence changes (ΔF/F).

Statistics and reproducibility. Initial screening experiments performed in duplicate wells were confirmed by at least one replication and representative traces were selected for display. All dose-response relationships were determined by three independent experiments (biological replicates) performed in duplicates (technical replicates). Threshold concentrations, defined as lowest substance concentrations leading to statistically significant elevated fluorescence changes in receptor-transfected cells compared with empty vector-transfected cells, were determined using SigmaPlot with Student's t-test to evaluate statistical significance ($p < 0.01$).

Molecular modeling. 2D structures of bile acids investigated in this work were downloaded from PubChem. Ligprep (Schrödinger Release 2022-3: LigPrep, Schrödinger, LLC, New York, NY, 2022) was used to generate 3D structures and protonation states of all ligands at pH 7 ± 1.

The currently released receptor structure of TAS2R46 (PDB ID: 7XP6) was used as a template for modeling the structures of TAS2R1, -R4, -R14, and -R39 using Prime (Schrödinger Release 2022-3). The sequence identities between TAS2R1, -R4, -R14, and –R39 and the template are 27%, 24%, 43%, and 25%, respectively. All models are available at https://github.com/dipizio/TAS2R-models.

Glide Standard Precision (Schrödinger Release 2022-3) was used for docking studies on TAS2R1. The receptor binding site was prepared using the "Receptor Grid Generation" tool, the grid box was the centroid of the ligand in the experimental structure of TAS2R46. We saved 30 poses per ligand. The docking pose of lithocholic acid with the lowest Glide score was used as a selection filter for the docking poses of all bile acids. MM/GBSA minimization (Prime, Schrödinger, LLC, New York, NY, USA, 2022) was used to rescore the poses. The same procedure was applied to predict the binding mode of taurolithocholic acid within the TAS2R46 binding site. The 2D and 3D representations of lithocholic acid/TAS2R1 binding mode were generated with Maestro 13.2 (Schrödinger Release 2022-3).

Reporting summary. Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data of this study are provided in the main text and supplementary information. Source data for graphs shown in Figs. 2 to 6 are provided as Supplementary data files (Supplementary data 1 for Figs. 2 and 3; Supplementary data 2 for Figs. 4 and 6; Supplementary data 3 for Fig. 5).

Code availability

Receptor models and docking results are available at https://github.com/dipizio/TAS2R-models.

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Author contributions

M.B. and F.Z. contributed to the study conception and design. F.Z. performed experimental work. M.B. and F.Z. analyzed the data. A.S. and A.D.P. performed and analyzed modeling studies. All authors contributed to the written manuscript. All authors have read and agreed to the published version of the manuscript.

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4. General discussion

The detection of bitter taste receptor expression in several tissues beside the oral cavity indicates their physiological role in addition to taste sensation, which was already summarized in numerous reviews [154,228-231]. Former research demonstrated several functions of extra-oral TAS2Rs. In the gastrointestinal tract, the activation of bitter taste receptors results in hormone secretion. In the case of L-cells, cholecystokinin is released upon TAS2R activation, which triggers a delayed gastric emptying. [232,233] Furthermore, bitter compounds are able to activate TAS2Rs expressed on ASM cells of the human lung [168]. This activation results in relaxation of ASM that can be utilized to develop pharmaceuticals for asthma therapy [234]. These findings show the potential of bitter taste receptor research, which can be promising future targets for drug development or to tailor nutrition for human well-being. Bitter taste receptor responses might be necessary to survive by avoiding bitter tasting toxic substances and also may fulfill essential functions in mammalian physiology.

4.1. Structural requirements for bitter taste receptor activation

The development of drugs that are able to activate bitter taste receptors demands an accurate knowledge about structural requirements that are involved in agonist binding, receptor activation and signal transduction. Therefore, we summarized published data on crucial amino acid positions in bitter taste receptors to give insights into the current state of research [122].

In general, bitter taste receptors are present in almost all mammals, but research results have shown that numbers and agonist profiles vary heavily between species [235]. Humans express around 25 functional TAS2Rs [93,235]. According to their characterized agonist profiles, they are divided into broadly, intermediately, and narrowly tuned receptors [97]. Therefore, research interest was focused on the structural features that are responsible for these tuning properties of bitter taste receptors, especially on broadly tuned receptors as they are able to detect a broad range of structurally very diverse agonists [97]. Due to lacking experimental structures of TAS2Rs and lacking homology to other GPCRs [119,120], the method of choice to accomplish this task was an iterative process of homology modeling, functional experiments with point

mutated bitter taste receptors and refinement of homology and docking models based on experimental data [122,123,236]. Thereby, it turned out, that broadly tuned receptors are recognizing a high number of agonists at the expense of sensitivity [127]. It is assumed that this lowered sensitivity is beneficial for mammals, as too high sensitivities could result in prevention of ingestion of harmless plant derived food products, which are well-accepted by the body. Consequently, the availability of foods would be unnecessarily limited [122]. This work summed up several amino acid positions in human bitter taste receptors that were identified to be involved in agonist binding and receptor activation using experimental methods in combination with molecular modeling [122]. The knowledge of structural requirements for agonist binding gained from experimental data can help to establish molecular modeling techniques to elucidate and design very potent agonists and antagonists for bitter taste receptors. This was further shown by a recent study that identified 200 agonists and 10 antagonists for the broadly tuned TAS2R14. They used an iterative approach to refine their model and proved their results with experimental data. [236] A further important step was the elucidation of the first experimental structure of a bitter taste receptor. This study identified position W88$^{3.32}$, E265$^{7.39}$ and Y241$^{6.51}$ as critical for strychnine response of TAS2R46. [143] Whereas W88$^{3.32}$ and E265$^{7.39}$ are necessary for strychnine binding and stabilization of the strychnine-bound form, Y241$^{6.51}$ fulfills a large conformational change upon strychnine binding and stabilizes the active state by forming interactions with amino acid residues in transmembrane domain 5 and 7 [143]. Interestingly, these three positions were already known to be critical for TAS2R46 activation [122,123,125,237]. The structure elucidation was thus able to confirm former experimental data. This study further showed that there are motifs in other GPCRs, which can be transferred to TAS2Rs even if sequence identities are not very high. The corresponding position of Y241$^{6.51}$ in the class A GPCR CXCR2 is W$^{6.48}$ and both fulfill the function as a toggle switch in receptor activation. [143] These results indicate that experimental data of receptor function can give first insights on critical amino acid residues, but to fully understand agonist binding and receptor activation structure elucidation is essential [122]. Nevertheless, sequence homologies of bitter taste receptors among each other as well as to other GPCR families are low [238]. Therefore, the structure of TAS2R46 is not

suitable for proper homology modeling of all other bitter taste receptors and research effort to elucidate further experimental structures of TAS2Rs is still necessary.

In contrast, one-to-one orthologous receptors that descend from a common ancestor, mostly share high sequence homologies, which result in common structural features and functional conservation [239]. Therefore, often model organisms like mice are used to study physiological functions and transfer the observations to humans. Nevertheless, some results that are generated using model organisms are only partially transferable to humans as physiological processes may differ between both species. In the case of bitter taste receptor agonists and function, it is still a matter of debate to which extent inter-species conservation exists.

4.2. Conservation of bitter taste receptor responses

In general, conservation of biological processes in species that divided millions of years ago, hint to the importance of this function. Therefore, the elucidation of conserved agonist profiles for bitter taste receptors can help to concentrate research on essential physiological responses of TAS2R activation. Indeed, we showed the conservation of bitter taste receptor response to di- and trivalent metal ions, which was recently published for the human TAS2R7, in the common vampire bat species *Desmodus rotundus* [130,183,240]. In contrast to this, the loss of agonist sensitivity of one receptor or the genetic loss of some receptors in mammals indicate the lack of importance of these receptor responses for this species. In the case of bitter taste receptors, this may be traced back to different dietary habits or habitats of mammals. [99] Most natural bitter compounds are found in plant material. Therefore, a higher need of bitter taste is conceivable for herbivorous species compared to carnivorous ones. [96,99]

As already mentioned, mice are often used as model organisms in research. Just like humans, mice are also omnivorous species [241]. Therefore, it may be assumed that taste receptors respond to similar compounds and TAS2Rs might be functionally conserved. Compared to humans, mice express even 35 functional Tas2rs. Nevertheless, their receptors can also be categorized according to their tuning breadth and 11 of them have one-to-one orthologous receptors in human. However, previous studies showed that agonist profiles of one-to-one orthologous receptors of human and

mouse, which share a common ancestor, are not overlapping well. [225] This can be explained, as only minor changes in amino acid sequence result in failure of agonist detection. For the human TAS2R46, it is known that the exchange of only some amino acid positions results in a loss of activation by its known agonists. Interestingly, the response of TAS2R46 to the most potent agonist strychnine can be transferred to TAS2R31 by simply mutating only 2 critical amino acids. [123] These findings point out that bitter taste receptor specificity to its agonist is dependent on minor changes in the amino acids constituting the agonist binding pocket. Therefore, it is not too surprising that agonist spectra of one-to-one orthologous receptors of mouse and human do not overlap as amino acid sequence homology is only up to 68.7 %. [242]

Because of these findings for human and mouse TAS2Rs, a functional conservation of one-to-one orthologous bitter taste receptors in general was questionable [225]. However, more and more studies in recent years showed that the functional conservation of one-to-one orthologous bitter taste receptors is more common than initially thought. An agonist overlap of almost 70 % for the orthologous receptors Tas2r3/Tas2r7, respectively 80 % for Tas2r4/Tas2r2 of turkey and chicken, which genetically divided around 30 million years ago was the first evidence for an existing functional conservation of bitter taste receptor responses [94]. Even more distantly related, with their last common ancestor more than 400 million years ago, are the bony fish species *Latimeria chalumnae* and *Danio rerio*. Nevertheless, responses of the one-to-one orthologous receptors lcT2R01 and drT2R1 to their common agonists are quantitatively and qualitatively well conserved. [243]

In line with this, is the shown conserved response of the drTAS2R7 to di- and trivalent metal ions [240]. Some of these, like Fe^{2+} or Mn^{2+}, were present long before more evolved life developed [244]. Therefore, it can be assumed that evolution was driven, among other things, by the influence of metal ions. Thus, the question arises whether animals with the ability to avoid the ingestion of high concentrations of metal ions by its bitter taste had an advantage to survive compared to others, as endogenous metal ion concentrations have to be balanced at a distinct level and excess, as well as deficiencies are connected to disease development [245]. The detection of high concentrations of metal ions can protect the animal from ingestion of toxic doses [183].

In nature, there are some sources with high metal salt concentrations like the mineral springs in Epsom, which possess high levels of bitter tasting magnesium sulfate [184]. To avoid accidental drinking of these high doses, the detection of metal ions by the TAS2R7 may be conserved in further species like *Desmodus rotundus*, which developed from a common ancestor over 90 million years ago [240,246]. Indeed, the habitat of vampire bats also includes areas in Columbia, where volcanic springs with high bitter salt concentrations were found [247]. These observations suggest the detection of metal ions as bitter for protection mechanisms to avoid the ingestion of high concentrations. As already mentioned before, bitter taste receptors including TAS2R7 are found to be expressed in several non-gustatory tissues and endogenous metal ions are essential for human well-being [210,245]. Therefore, a further role of extra-oral TAS2R7 activation by metal ions in physiology can be considered.

4.3. The role of endogenous compounds as agonists of non-gustatory bitter taste receptors

Bitter taste receptors in general are rather insensitive and consequently, local endogenous agonist concentrations have to reach levels up to mM ranges, depending on their activation thresholds [248]. Therefore, it is questionable whether bitter substances that are taken up by ingestion will reach distant non-gustatory tissues expressing TAS2Rs in concentrations that suffice to activate these receptors as high concentrations of bitter substances will be eliminated by avoiding ingestion, because of the unpleasant taste of bitterness. Nevertheless, there are various approaches, how activation threshold concentrations of bitter taste receptor agonists can be reached endogenously. First, the bitter taste of some compounds may be masked by other substances. This was already shown for the bitter off-taste of the non-caloric sweetener saccharin. By combination with cyclamate, which is assumed to block the access of saccharin to the orthosteric binding site, the bitter taste is decreased. [221] This observation can be beneficial in food industry for improved use of saccharin as a sweetener. Furthermore, bitter compounds that are ingested in doses not sufficient for TAS2R activation, may be enriched endogenously in different tissues. It is known for several substances especially pollutants that they are not metabolized in the human body and therefore bioaccumulate endogenously. [249] This process is also possible for

some bitter taste receptor agonists that will then reach activation threshold concentrations. Finally, the body may synthesize the bitter substances by itself, either by metabolization of ingested non-bitter compounds or by de-novo synthesis. These endogenously available compounds or metabolites can then serve as agonists for non-gustatory TAS2Rs. [122]

The bitter taste of the body fluid bile has long been known [185] and former studies already hint to the group of bile acids as agonists for bitter taste receptors [225,243]. Therefore, it was an important task during this work to further characterize the bitter taste receptor response of humans to bile acids and transfer the gained results to a more physiological context. With the activation threshold concentrations of 300 nM of lithocholic acid (LCA) for TAS2R1 and TLCA for TAS2R1, TAS2R14 and TAS2R46, they turned out to be the most potent ones identified during this study. [226] Comparison of these data with published endogenous bile acid concentrations, summarized in the human metabolome database, revealed levels of lithocholic acid in the blood that are matching our measured activation threshold concentration [250,251]. To assume a putative biological function, we had a look at published expression data, which detected expression of TAS2R1 in the brain, the kidney, as well as in the reproductive tissues - ovary and testis [210]. In literature, there are already some hints that TAS2R1 and the mouse Tas2r105, which was identified as bile acid receptor during this study as well [226], fulfill important physiological functions upon bile acid activation in these tissues. In the kidney, the Tas2r105 is assumed to be involved in glomerulus and renal tubule structure maintenance. [252] As circulating bile acids are filtered by the kidney, local concentrations may reach threshold concentrations [253]. Therefore, bile acids might participate in reproduction and maintaining kidney structure by activation of Tas2rs. In the case of humans, children with cystic fibrosis were shown to have decreased serum levels of LCA, which compared to healthy subjects range below the measured activation threshold concentration [250]. In combination with the knowledge that male children with cystic fibrosis go through puberty later than healthy subjects, an involvement of TAS2R1, activated by bile acids in brain and testis, in the development of male adolescents to adults can be assumed. [254]

Finally, only a set of eight different bile acids was investigated, but due to modifications by the gut microbiome hundreds of structurally diverse bile acids are present in the human body [194,195]. Therefore, there might be more potent bile acid derived bitter taste receptor agonists missed [226]. It is further possible that some bile acids bind to bitter taste receptors without activation instead acting as inhibitors by competitive antagonism. Both possibilities are topics for further investigation for the elucidation of bile acid's role in human physiology by TAS2R activation or inhibition.

Similar to previous research on the functional characterization of mouse Tas2rs [225], overlapping agonist profiles of one-to-one orthologous receptors of both species are very scarce also in the case of bile acid detection [226]. It has to be taken into account, that molecular mechanisms between species that are only distantly related differ significantly. In the case of bile acids, mice express an enzyme called Cyp2c70 that converts the primary bile acid CDCA into muricholic acids (MCA), which are major bile acid forms in mice, but are barely present in human [255]. Therefore, mice bitter taste receptors may have adapted more to MCAs, whereas they are not detecting CDCA. Nevertheless, the response of Tas2rs to bile acids in general is still present in mice, indicating the importance of bile acid detection by bitter taste receptors to avoid ingestion or for endogenous processes [226].

Beside bile acids, metal ions are known to be important endogenous compounds especially known for their roles as second messengers or co-factors of enzymes [172,175]. The elucidation of their ability to activate the TAS2R7 opens the possibility of further unknown physiological functions [130,183]. To elucidate these functions, extra-oral tissue expression of the metal ion bitter taste receptor TAS2R7 and endogenous metal ion concentrations that are in the range of activation threshold for the TAS2R7 should be considered. Former RNA-sequencing data detected the human TAS2R7 in the brain, the heart, and the ovary [210]. Interestingly, all these tissues were already associated with influences of metal ions. In recent years, more and more studies highlight the importance of metal ions in the development of Alzheimer's disease [256]. The accumulation of metal ions resulting in high concentrations in different brain regions, as well as the expression of TAS2R7 in the brain hint to a correlation between disease development and bitter taste receptor activation. The activation of the human TAS2R7

by Zn^{2+} was previously published with a threshold concentration of 300 µM. [130,183,240] Whereas normal concentrations of Zn^{2+} in the human brain are reported to be 150 µM [257], concentrations can increase up to 3-fold at distinct locations during Alzheimer's disease [258]. Thus, compared to healthy individuals, concentrations would exceed the activation threshold concentration of TAS2R7 in brains of Alzheimer patients. In the hearts of mice, the activation of Tas2rs is correlated to negative inotropic effects [259]. Furthermore, it is known that magnesium has an important effect on heart function by influencing cardiac conduction and contraction [260]. Very high concentrations of magnesium are even associated with cardiac arrest [261]. Finally, the expression of TAS2R7 in ovaries and the use of magnesium sulfate as tocolytic to avoid preterm birth is indicating the putative role of this bitter taste receptor in uterine muscle relaxation [210,262]. To clarify these hypotheses further research is necessary. This includes questions like are local metal ion concentrations sufficient to activate the TAS2R7 and is there a direct correlation between receptor activation by metal ions and the mentioned physiological effects.

5. Conclusion and future perspectives

Nowadays, it is known that bitter taste receptors are expressed in several non-gustatory tissues [154]. By activation they might be involved in physiological processes like immune responses, heart functions or fertility [157,263,264], indicating their importance apart from taste perception in human health and well-being. Therefore, it is an important task to elucidate their distinct functions and agonists. This information can be beneficial for the food and pharma industries to develop food additives or pharma products that target bitter taste receptors as agonists or antagonists at different locations to contribute to human health.

By presenting a comprehensive overview of the potential of bile acids to activate non-gustatory bitter taste receptors and elucidating the conservation of metal ion responses of TAS2R7 in species that divided millions of years ago, this work took a first step in this direction. By comparison of measured activation threshold concentrations with endogenously available bile acid and metal ion concentrations, we were further able to give insights into physiological functions of these compound classes by activation of TAS2Rs. [226,240]

Indeed, it is also important to mention that there are living mammals like the bottlenose dolphin, which lack functional bitter taste receptors [96]. Therefore, one may note that bitter taste sensation in the mouth and physiological functions of non-gustatory bitter taste receptors are not essential for survival. Hence, a complete elucidation of the functions of extra-oral bitter taste receptors is important. It is further conceivable that the bottlenose dolphin developed TAS2R independent biological pathways, that make bitter taste receptors unnecessary for this species in taste perception or physiology.

It is a future task to prove non-gustatory functions of bitter taste receptors by endogenous substances like metal ions and bile acids. Bitter salts for example are present in high concentrations in springs in Epsom [184]. Besides, mammals are feeding on their prey and humans consume innards like the liver of calf or pigs, which bitter taste is well known. As this organ is the site of bile acid production, it may contain high amounts of bitter tasting bile acids [186]. Therefore, it is further possible that bitter taste

of both compound classes is simply important to avoid ingestion of high concentrations that might be harmful for the organism.

Beside metal ions and bile acids, there are numerous other compounds present in the human body with the potential to activate bitter taste receptors. Even the group of bile acids is composed of hundreds of structurally diverse members [194,195]. Therefore, it is likely that there are more potent compounds than those identified in this work as activators for bitter taste receptors. Beside agonists, the impact of antagonists of bitter taste receptors on physiology is also worth studying. However, the experimental workload to investigate these amounts of compounds for their ability to activate or inhibit bitter taste receptors is immense. For this reason, in silico docking experiments will have an enormous impact in future. For reliable results, the major task will be the elucidation of experimental structures of bitter taste receptor.

Nevertheless, the examples of bile acids and metal ions show the high potential of endogenous compounds as agonists for non-gustatory bitter taste receptors and hint to their involvement in physiological functions and disease development. This, in combination with already known bitter substances that are highly efficient for asthma therapy [234], shows the future role of bitter taste receptors as drug targets for agonists, as well as antagonists. The endogenous occurrence of bile acids and metal ions in concentrations that are sufficient to activate TAS2Rs and their conservation in distantly related species in combination with former studies that showed the influence of bitter taste receptor activation on different tissues prove this assumption. Nevertheless, it will take many years to completely understand the role of extra-oral bitter taste receptors, but their future importance in pharmacology and food science is already obvious.

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7. Supplementary Information

Supplementary information for “Bitter taste receptors of the common vampire bat are functional and show conserved responses to metal ions in vitro”

Appendix S1: Sequences of Desmodus rotundus bitter taste receptors

Sequence of the Desmodus rotundus T2R1 (NCBI accession number: XM_024578389)

ATGCTAGAGTCTCTCGTTATTACCCATCTTATTTTTGCAACAATAGAGTGTCTCATGGGGGTTTTA
GCAAATGGCATCATTGTGGATGTGACCAGCACAGGCTCGATCAAGCAGAGAAAGATGCTGCCCT
TCGACCTCCTCCTTTGCTGCCTGGCGACTTCCAGGATTTGTCTCCAGGTAGTCATGTTCTACTTTA
ATCTGGCTCTTCTTTCCCTGATTGAAATCTCTCTTTCTGCTGGGTGTTTTATAATTTTCATGTTTATA
TACAAATCGGAACTTTGGCTTGCCACGTGGCTCAGCGTTTTCTATTGCACCAAGGTCTCCACCAT
CGCTCACCCGCTCTTCTTTTGGTTGAAGTTGAGAATCTCCAAGTTGGTGCCATGGCTGATTTGGG
GGACCTTGCTGTGTACATCTCTCACTTCTGTTTTCCACAGAAAATGTGCATGGATTCTCTTCCAAA
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TCTCTTGATATTTTCCCTGGGGAGACACACCCGGCAGATGATAAGCACAGCCACGGGCAGCAGG
CCCCCTAGCGTGAGTGTCCACATCAGCGCTCTCCTGTCCTTCCTGTCCTTCCTGGTCCTCTACGT
GTCCCAGTACATGATGGCTACTTTAGTCCTTTCTCATATTTTCAAGATCAGAAACTTCATCTTTCTC
TTCTGCCTGTTGCTAGTAGGTTTATACCCCTCTGTACACTCCATTGTCTTAATTTTAGGAAATCCTA
AACTGAAACAAAATGCAAAGAAGTTCCTCCTCCACAGTAAGTGCTGTCCG

Sequence of the Desmodus rotundus T2R4 (NCBI accession number: XM_024555089)

ATGCTGGAAATACTCTTTGCTGTTATTGTCTCAGCAACTTTGAATTTTGTAGGACTCATTGTGAATC
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TCCTGTTCAGCTTGGGCATCAGCAGATGCCTTATGCTGGCGCTGTCTCTCCTGGCCGTCATCTAC
CCCTTCGTCTCTCCGCGTGTCGGGAGGCCAGTCAGCCTGTTTGCTGTCCTGCTGTGTTGGGTGT
TTTTGGACTCCAGCAGTCTCTGGTTTGTAACCTTGCTCAGTGTCCTGTACTGTGTGGAGATTACTA
ACTTGCAGCACTCAGTTTTTCTCCTGCTGAAACAAAATCTCTCCTCAAAGACCCTGAGGCTGCTG
CTGGCCTGTGTGCTGATTTCCACCCTCACCACGCTTCTGTACGCTGTGCTCAGGCAGACGCTACA
GTTTCCTGAATCTGTGACCGGGAGGAATGGCACAGAATTCAGCGTCAGTGAGCGCATCTTGTCTT
CGGTGATCTCTTTGTTCTCGAGCTCATTTCTCCAGTTCATCATTAACGTGACTTCTGCTTCCTTGTT
AATAAACAAGTTGAGGAGACACATACAGATGATGCAGAGAAACGCCAGTGGCTTCTGGAATCCCC
AGACTGAAGCTCACGTGGGCGCTATGAAGCTGATGGTCTATTCCCTCCTCCTGTACATCCCCTAC
TCTGTAGCTGCCCTGTCTCTCCACCTCCCGTCTTCTGTAGGGATGGATCTGAGAACCAGATCCGT
GTGTGTGGTTCTGTCCGCTGTTTACCACCCAGGACATTCTGTTCTCATTATTGTCACACATCCTAA
ACTGAGAACAAAAGCAAAGAAGATTCTGTGCTTCAACAAATGGTGGAATATCAGTAGGGGA

Sequence of the Desmodus rotundus T2R7 (NCBI accession number: XM_024575901)

ATGTCAGGTGAAGTAAACAGCACCTTAATGCTCATAGCAGTTGGAGAGTTTTCAGTGGGGATTTT
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TGATTTCAGGCTTTGTGTCAAGACAAAGGTGAGAAGAAACTTAACTTTGAGATGCTGGGAAAATAA
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CCTCCTTTTCATTGTCTACTCTCTGTCCTTTCTCGTAGCCACCTCCAGTTACTTCATTCCAGAGAG
TGAGTTAGCTGTGATGATTGGTGAGTTGGTAGCTCTAATCTATCCCTCAAGCCATTCATTTATCTT
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Appendix S2 of "Bitter taste receptors of the common vampire bat are functional and show conserved responses to metal ions in vitro"

Description: Method and presentation of the immunocytochemical staining of the Desmodus rotundus bitter taste receptors expressed in HEK 293T cells

Method:

Immunocytochemistry

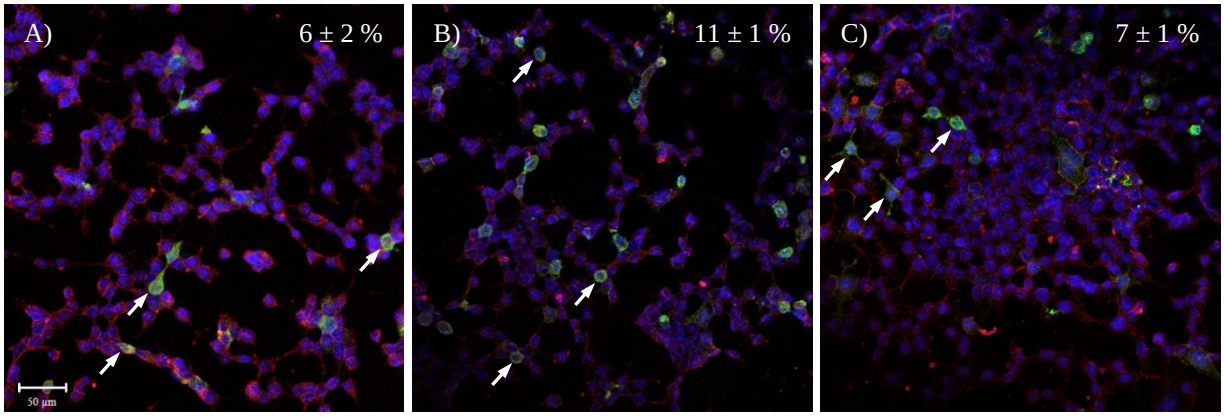
Immunocytochemical staining was done as described previously (Behrens et al., 2017). Briefly, HEK293T-Gα16gust44 cells were grown on round glass cover slips coated with 10 µg/mL poly-D-lysine in 24-well plates and transiently transfected as described in the main text. Per well 0.6 µg of plasmid DNA and 1.2 µl of lipofectamine 2000 were used. The next day, cells were washed twice with warm (37 °C) PBS for 1 min. After incubation on ice for half an hour, cells were treated with concanavalin A diluted in ice-cold PBS (1:2000) for 1 h on ice. Cells were then washed 3 times with ice-cold PBS for 1 min and fixed with a 1:1 mixture of methanol and acetone for 2 min on ice. Further experimental procedures were performed at room temperature. Three washing steps with PBS for 5 min each were followed by a blocking step using blocking buffer 1 (5 % normal horse serum and 0.5 % Triton X – 100 in PBS) for 45 min. Subsequently, mouse anti-HSV, diluted 1:15000 in blocking buffer 2 (5 % horse serum and 0.2 % Triton X – 100 in PBS), was applied and incubated for 1 h. After 4 washing steps with PBS for 5 min each, anti-mouse Alexa Fluor 488 and Streptavidin Alexa Fluor 633, diluted 1:2000 and 1:1000 in blocking buffer 2, respectively, were applied and cells were further incubated in the dark for 1 h. Before and after a 15 min staining step with 4′,6-Diamidin-2-phenylindol (DAPI), cells were washed 3 times with PBS for 5 min each. Finally, cells were washed with deionized H_2O and placed on a glass slide using Dako mounting medium. Until confocal microscopy, cells were stored at 4 °C in the dark.

Confocal microscopy

For imaging of the stained cells, a Zeiss LSM 780 confocal microscopy system was used. The fluorophores DAPI, Alexa 488 and Alexa 633 were excited by laser lights at wavelengths of

405 nm, 488 nm and 633 nm, respectively. For detection, emission spectra filters with wavelength ranges of 410 – 508 nm, 490 – 633 nm and 638 – 747 nm, respectively, were used. For optimal results, sequential scanning option was chosen. Three representative images for each receptor were used to count cells.

Figure and caption:



Immunocytochemical staining of cells transfected with Desmodus rotundus T2R1, T2R4 and T2R7. HEK293T-Gα16gust44 cells were transfected with *D. rotundus* bitter taste receptors T2R1, T2R4 and T2R7. 24 h post transfection, cell membranes were stained with concanavalin A (red), nuclei with DAPI (blue), and the receptors with an anti-HSV antibody (green). Confocal laser scanning microscopy was done with a Zeiss LSM 780. Representative images of the expression of *D. rotundus* bitter taste receptors T2R1 (A), T2R4 (B) and T2R7 (C) are presented. Arrows point to exemplarily chosen T2R expressing cells. Expression efficiencies and the corresponding standard deviation are given in the upper right corners of the images. No major differences between the three receptors were detected. Beside cell surface expression, also intracellular immunoreactivity was evident. Scale bar, 50µm.

Appendix S3 of "Bitter taste receptors of the common vampire bat are functional and show conserved responses to metal ions in vitro"

Description: Amino acid sequences of human TAS2R7 and *Desmodus rotundus* T2R7. Conserved amino acids are printed in red on a yellow background. Supposed metal ion binding sites are highlighted in bold and are underscored.

H. sapiens TAS2R7 (NCBI Reference Sequence: NP_076408.1)

D. rotundus T2R7 (NCBI Reference Sequence: XP_024431669.1)

```
                                1                                                50
H. sapiens  TAS2R7     (1) MADKVQTTLLFLAVGEFSVGILGNAFIGLVNCMDWVKKRKIASIDLILTS
D. rotundus   T2R7     (1) MSGEVNSTLMLIAVGEFSVGILGNAFIGLVNCVDWIKNKKIASIGLILTS
          Consensus    (1) M   V  TL   AVGEFSVGILGNAFIGLVNC DW K  KIASI LILTS

                               51                                               100
H. sapiens  TAS2R7    (51) LAISRICLLCVILLDCFILVLYPDVYATGKEMRIIDFFWTLTNHLSIWFA
D. rotundus   T2R7    (51) LAISRICLLCVILLDYVMLVLYPDIYATGKQMRIIDFFWTLTNHVSVWFA
          Consensus   (51) LAISRICLLCVILLD   LVLYPD YATGK MRIIDFFWTLTN H SVWFA

                              101                                              150
H. sapiens  TAS2R7   (101) TCLSIYYFFKIGNFFHPLFLWMKWRIDRVISWILLGCVVLSVFISLPATE
D. rotundus   T2R7   (101) TCLSIFYFFKIANFFHPLFLWIKWRIDRVIPGILLVCFALSVFISLPVTE
          Consensus  (101) TCLSI YFFKI NFFHPLFLW KWRIDRVI  ILL C  LSVFISLP TE

                              151                                              200
H. sapiens  TAS2R7   (151) NLNADFRFCVKAKRKTNLTWSCRVNKTQHASTKLFLNLATLLPFCVCLMS
D. rotundus   T2R7   (151) NLNDDFRLCVKTKVRRNLTLRCWENKAQYASIKVYLNLLTLFPFSVSLIS
          Consensus  (151) NLN DFR CVK K   NLT  C  NK Q AS K  LNL TL PF V L S

                              201                                              250
H. sapiens  TAS2R7   (201) FFLLILSLRRHIRRMQLSATGCRDPSTEAHVRALKAVISFLLLFIAYYLS
D. rotundus   T2R7   (201) FLLLILSLCGHVRQMQLNATGCRDPSTDAHVGAMKAVVSFLLLFIVYSLS
          Consensus  (201) F LLILSL  H R MQL ATGCRDPST AHV A KAV SFLLLFI Y LS

                              251                                              300
H. sapiens  TAS2R7   (251) FLIATSSYFMPETELAVIFGESIALIYPSSHSFILILGNNKLRHASLKVI
D. rotundus   T2R7   (251) FLVATSSYFIPESELAVMIGELVALIYPSSHSFILILGNNKLRQASQRVL
          Consensus  (251) FL ATSSYF PE ELAV  GE  ALIYPSSHSFILILGNNKLR AS  V

                              301           318
H. sapiens  TAS2R7   (301) WKVMSILKGRKFQQHKQI
D. rotundus   T2R7   (301) CKVAHALKGRHF------
          Consensus  (301)  KV   LKGR F
```

Figures S1 and S2 of "Bitter taste receptors of the common vampire bat are functional and show conserved responses to metal ions in vitro"

Description: Presentation of dose-response relationships for the activation of human TAS2R7 and Desmodus rotundus T2R7 by the bitter salts $MgCl_2$, $MnCl_2$, $FeCl_2$ and $ZnSO_4$.

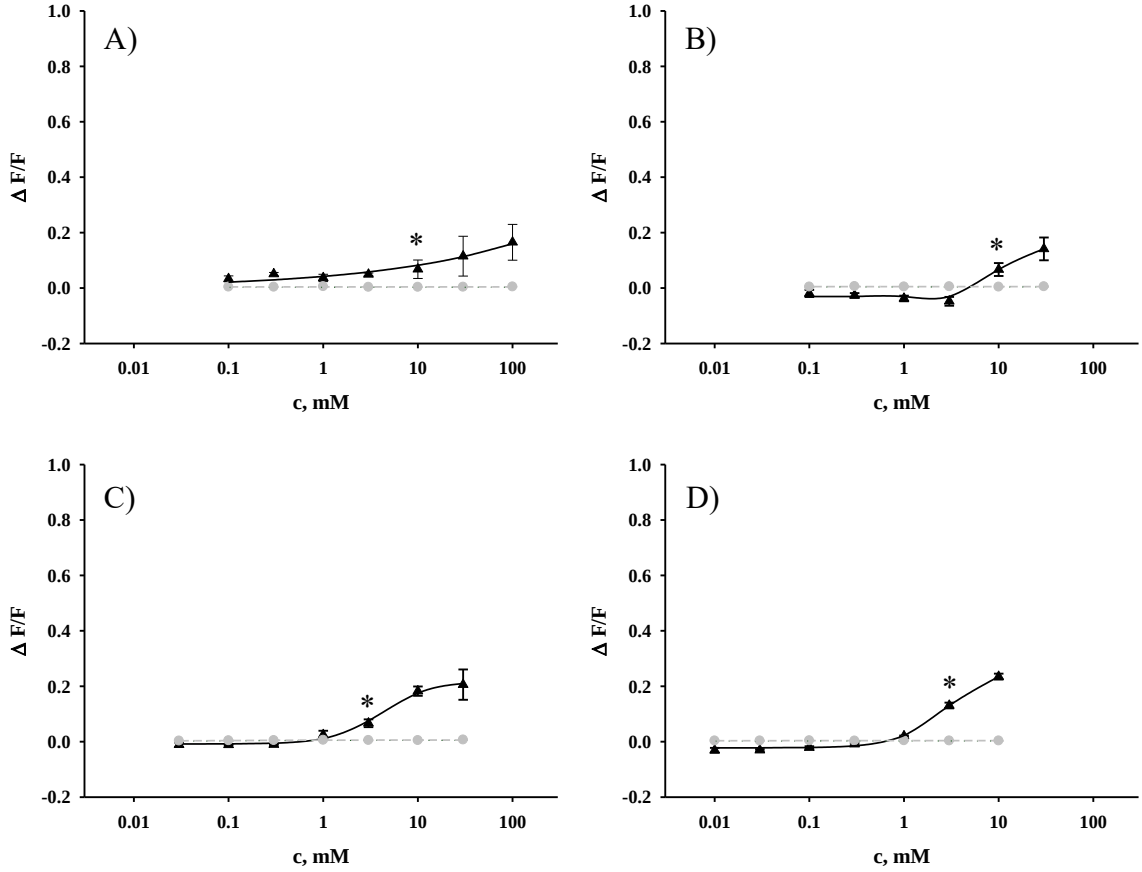


Figure S1: Concentration – response relationships of the *Desmodus rotundus* bitter taste receptor T2R7 challenged with metal ions. HEK293T – Gα16gust44 cells were transiently transfected with *Desmodus rotundus* T2R7 and the empty vector control (mock). Fluorescence intensities, according to the Ca^{2+} - release upon receptor activation, were measured with an automated fluorometric imaging plate reader ($FLIPR^{Tetra}$). Different concentrations of $MgCl_2$ (A), $MnCl_2$ (B), $FeCl_2$ (C) and $ZnSO_4$ (D) were used. The relative fluorescence intensities (black) were mock (grey) subtracted and plotted against the ligand concentration in mM (n = 3). Statistical significance ($P < 0.01$) is presented by (*).

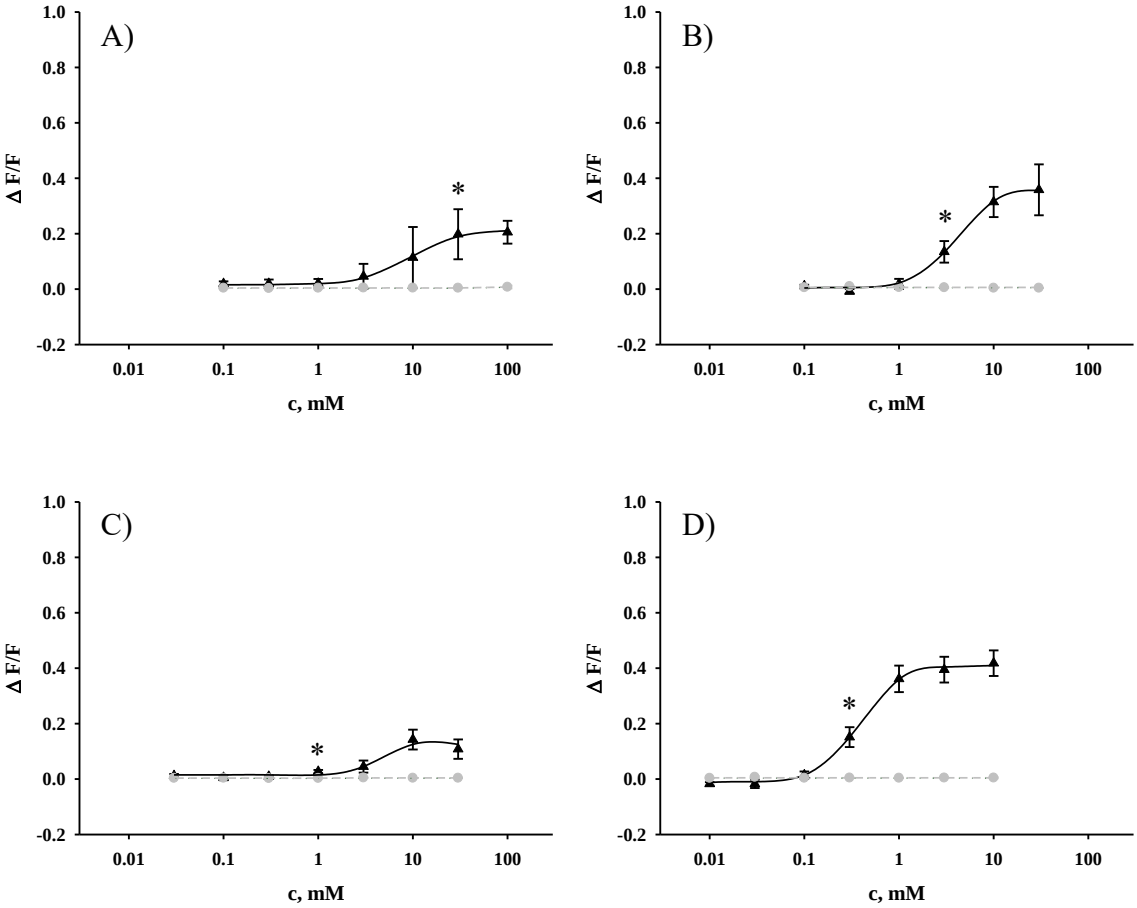


Figure S2: Concentration – response relationships of the human bitter taste receptor TAS2R7 challenged with metal ions. HEK293T – Gα16gust44 cells were transiently transfected with human TAS2R7 and the empty vector control (mock). Fluorescence intensities, according to the Ca^{2+} - release upon receptor activation, were measured with an automated fluorometric imaging plate reader ($FLIPR^{Tetra}$). Different concentrations of $MgCl_2$ (A), $MnCl_2$ (B), $FeCl_2$ (C) and $ZnSO_4$ (D) were used. The relative fluorescence intensities (black) were mock (grey) subtracted and plotted against the ligand concentration in mM (n = 3). Statistical significance ($P < 0.01$) is presented by (*).

Table S1 of "Bitter taste receptors of the common vampire bat are functional and show conserved responses to metal ions in vitro"

Description: Bitter test substances presented with their maximal applied concentration (C_{max}) in Ca^{2+} - imaging experiments. Furthermore, the suppliers and the CAS numbers of the compounds are given. Agonists of *Desmodus rotundus* bitter taste receptors T2R1, T2R4 and T2R7 are shown in italics.

	C_{max}	Supplier	CAS number
Acetaminophen	3 mM	Sigma-Aldrich	103-90-2
Aloin	10 µM	Fluka	1415-73-2
Amarogentin	1 mM	ChromaDex	21018-84-8
Arbutin	30 mM	Sigma-Aldrich	497-76-7
Aristolochic acid	10 µM	Sigma-Aldrich	10190-99-5
Artemisinin	30 µM	ChromaDex	63968-64-9
Azathioprin	0.3 mM	Sigma-Aldrich	446-86-6
Brucin	0.1 mM	Sigma-Aldrich	357-57-3
$CaCl_2$	30 mM	VWR Chemicals	10035-04-8
Caffeine	0.3 mM	Sigma-Aldrich	58-08-2
Carisoprodol	0.1 mM	Sigma-Aldrich	78-44-4
Camphor	1 mM	Fluka	464-49-3
Chloramphenicol	1 mM	Sigma-Aldrich	56-75-7
Chloroquine	10 mM	Sigma-Aldrich	50-63-5
Chlorpheniramin	0.3 mM	Sigma-Aldrich	113-92-8
Colchicin	3 mM	Sigma-Aldrich	64-86-8
Costunolide	0.1 mM	ChromaDex	553-21-9
Coumarin	1 mM	Sigma-Aldrich	91-64-5
Coumestrol	1 mM	Sigma-Aldrich	479-13-0
Cromolyn	10 mM	Sigma-Aldrich	15826-37-6
$CuCl_2$	30 mM	Sigma-Aldrich	7447-39-4
Curcumin	1 µM	Sigma-Aldrich	458-37-7
Cycloheximid	1 mM	Sigma-Aldrich	66-81-9
Denatonium benzoate	10 mM	Sigma-Aldrich	3734-33-6
Dextrometorphan	30 µM	Sigma-Aldrich	125-71-3
Diphenhydramin	0.3 mM	Sigma-Aldrich	58-73-1
Diphenidol	0.3 mM	Sigma-Aldrich	3254-89-5
Diphenylthiourea	1 mM	Fluka	102-08-9
Ethylhydrocupreine	30 µM	Sigma-Aldrich	522-60-1
Emetin	30 µM	Sigma-Aldrich	7083-71-8
Erythromycin	0.3 mM	Sigma-Aldrich	114-07-8
$FeCl_2$	100 mM	Alfa Aesar	13478-10-9
$FeCl_3$	100 mM	Sigma-Aldrich	7705-08-0
KCl	100 mM	VWR Chemicals	7447-40-7
Limonin	0.3 mM	Sigma-Aldrich	1180-71-8
$MgCl_2$	100 mM	Merck	7791-18-6
$MgSO_4$	100 mM	Alfa Aesar	10034-99-8
$MnCl_2$	100 mM	Sigma-Aldrich	13446-34-9
Naringin	10 µM	Sigma-Aldrich	10236-47-2
NaSCN	10 mM	Fluka	540-72-7
Nicotine	0.1 mM	Sigma-Aldrich	54-11-5
Noscapin	10 µM	Sigma-Aldrich	912-60-7
Ouabain	3 mM	Sigma-Aldrich	11018-89-6
Papaverin	10 µM	Sigma-Aldrich	61-25-6
Phenylthiocarbamid	10 µM	Sigma-Aldrich	103-85-5
Picrotin	3 mM	Santa Cruz Biotechnology	21416-53-5
Picrotoxinin	0.1 mM	Sigma-Aldrich	17617-45-7
Pyrocatechol	1 mM	Sigma-Aldrich	120-80-9
Quassin	1 mM	CPS	76-78-8
Quinidin	0.3 mM	Sigma-Aldrich	56-54-2

Quinine sulfate	30 µM	Fluka	130-95-0
Resveratrol	3 mM	Apin Chemicals Ltd.	501-36-0
Saccharin	10 mM	Sigma-Aldrich	82385-42-0
Salicin	10 mM	Sigma-Aldrich	138-52-3
Sinigrin	1 mM	Sigma-Aldrich	3952-98-5
Sodium cyclamate	30 mM	Sigma-Aldrich	139-05-9
Solanin, alpha	10 µM	Sigma-Aldrich	20562-02-1
Strychnine	10 µM	Sigma-Aldrich	57-24-9
Sucralose	30 mM	Sigma-Aldrich	56038-13-2
Thiamin	1 mM	Sigma-Aldrich	67-03-8
α-Thujon	0.3 mM	Sigma-Aldrich	546-80-5
Warfarin	1 mM	Sigma-Aldrich	81-81-2
Xanthohumol	10 µM	Carl Roth	6754-58-1
Xanthotoxin	0.1 mM	Fluka	298-81-7
Yohimbine	0.3 mM	Sigma-Aldrich	65-19-0
$ZnSO_4$	30 mM	Sigma-Aldrich	7446-20-0

Supplementary information for "Physiological activation of human and mouse bitter taste receptors by bile acids"

Supplementary Information

Physiological activation of human and mouse bitter taste receptors by bile acids

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Supplementary Figures

Supplementary Figure 1: Concentration-response relationships of the activation of the human TAS2R4 by bile acids

Supplementary Figure 2: Concentration-response relationships of the activation of the human TAS2R14 by bile acids

Supplementary Figure 3: Concentration-response relationships of the activation of the human TAS2R39 by bile acids

Supplementary Figure 4: Concentration-response relationships of the activation of the human TAS2R46 by bile acids

Supplementary Figure 5: Concentration-response relationships of the activation of the murine Tas2r105 by bile acids

Supplementary Figure 6: Concentration-response relationships of the activation of the murine Tas2r108 by bile acids

Supplementary Figure 7: Concentration-response relationships of the activation of the murine Tas2r117 by bile acids

Supplementary Figure 8: Concentration-response relationships of the activation of the murine Tas2r123 by bile acids

Supplementary Figure 9: Concentration-response relationships of the activation of the murine Tas2r126 by bile acids

Supplementary Figure 10: Concentration-response relationships of the activation of the murine Tas2r144 by bile acids

Supplementary Figure 11: 3D representations of the putative binding modes of bile acids in the TAS2R1 binding site obtained with MM/GBSA refinement.

Supplementary Figure 12: Correlation plots for all investigated ligands.

Supplementary Figure 13: Amino acid sequence identities of human and mouse bile acid-sensitive receptors.

Supplementary Figure 14: Amino acid sequence alignment of human and mouse bile acid-sensitive receptors.

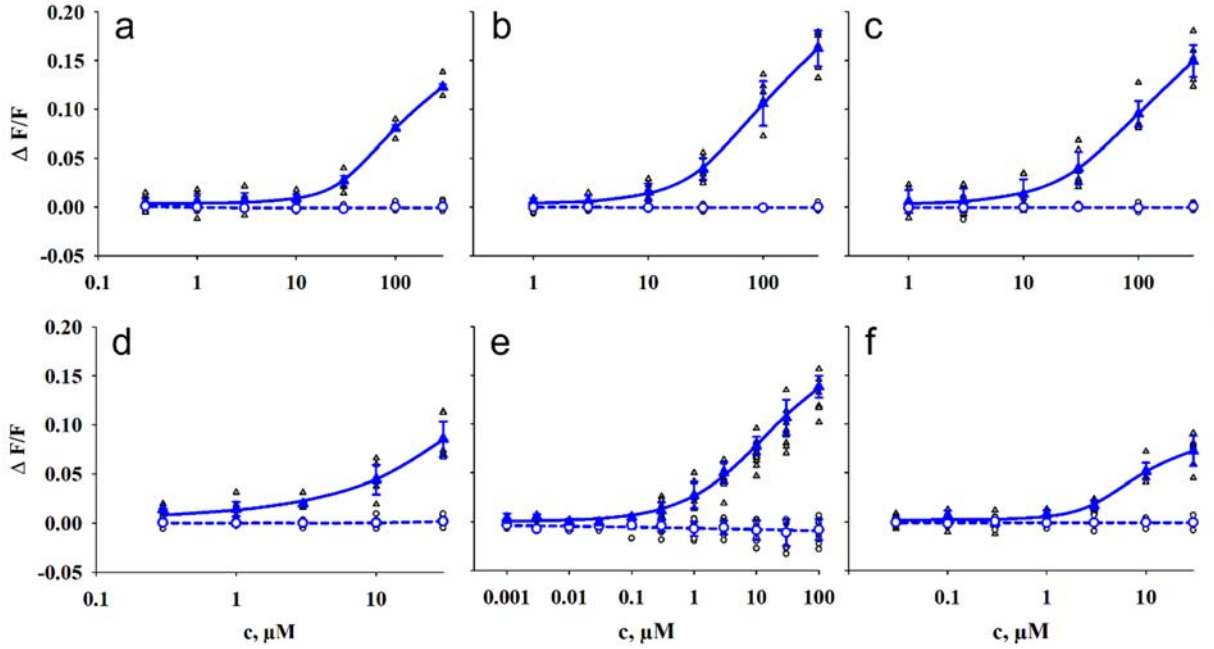
Supplementary Figure 15: WebLogo depiction of the amino acid sequence alignment of bile acid-sensitive human and mouse bitter taste receptors.

Supplementary Figure 16: 3D representation of the putative taurolithocholic acid binding mode in the TAS2R46 binding site obtained with MM/GBSA refinement.

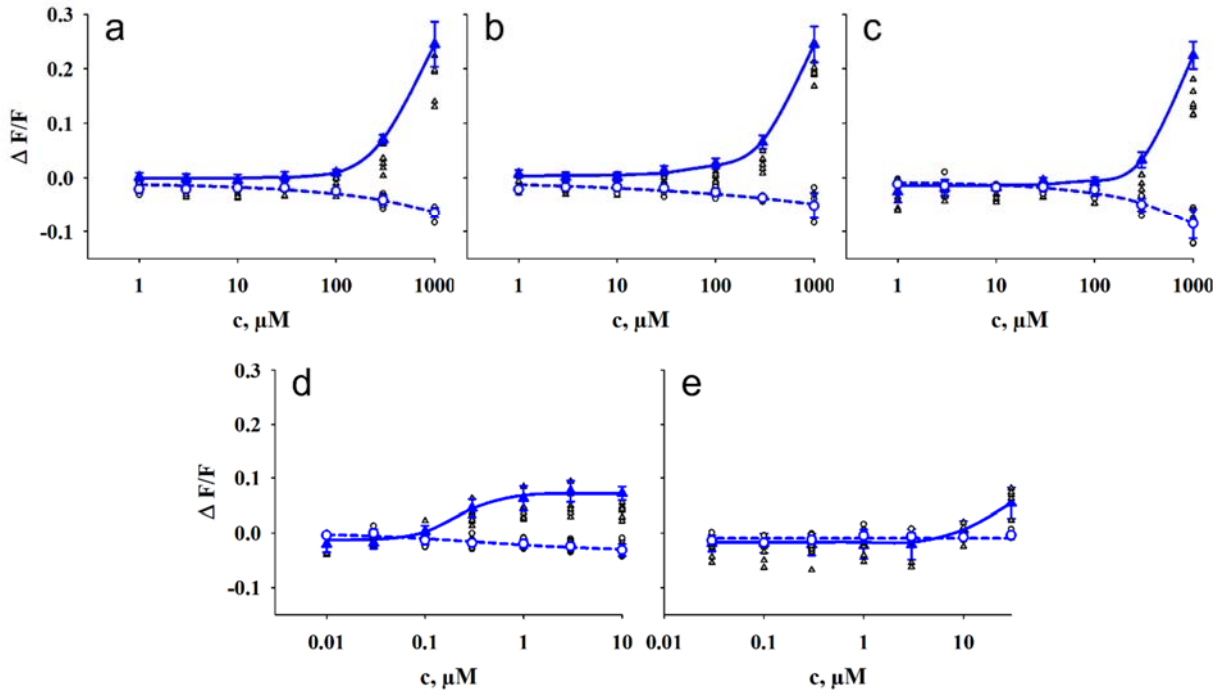
Supplementary Table

Supplementary Table 1: Calculated EC_{50}-values of human and murine bitter taste receptors activated by bile acids

Supplementary Figures

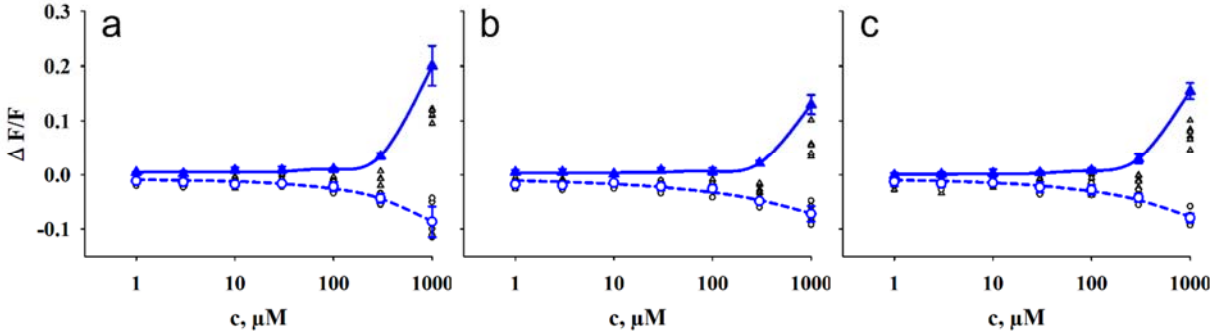


Supplementary Figure 1: Concentration-response relationships of the activation of the human TAS2R4 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human TAS2R14 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids cholic acid a), glycocholic acid b), taurocholic acid c), deoxycholic acid d), taurolithocholic acid e) and ursodeoxycholic acid f) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).

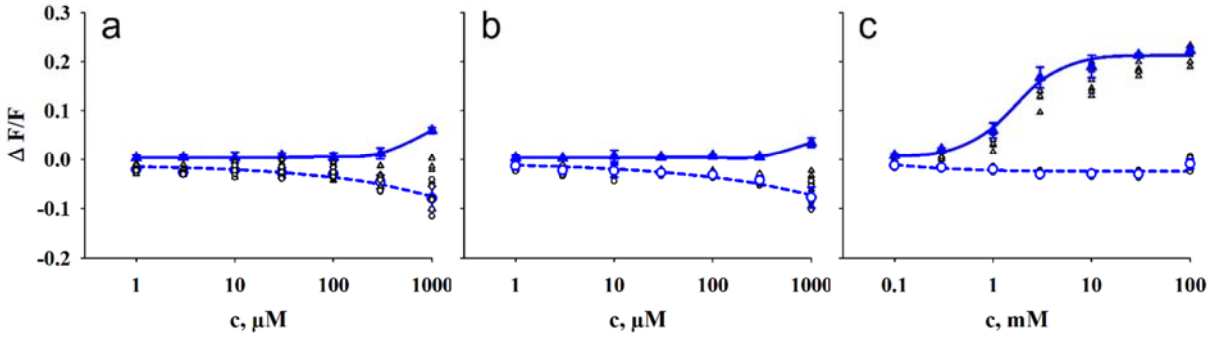


Supplementary Figure 2: Concentration-response relationships of the activation of the human TAS2R14 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human TAS2R14 (triangle, blue) and

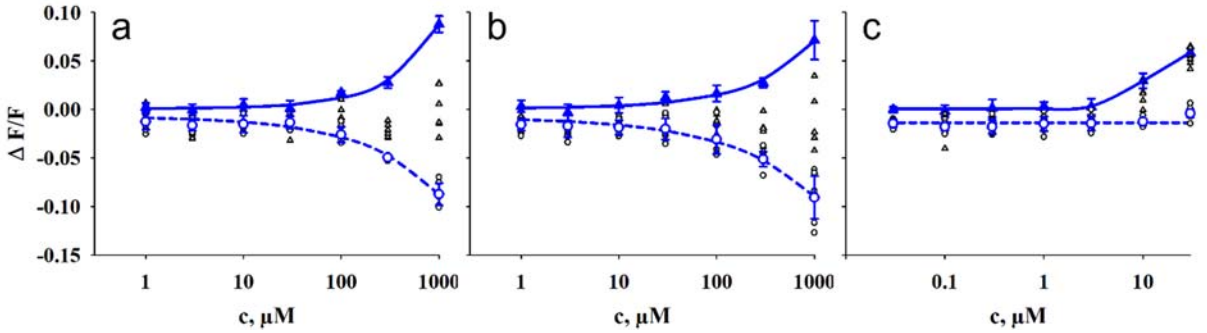
an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids cholic acid a), glycocholic acid b), taurocholic acid c), taurolithocholic acid d) and chenodeoxycholic acid e) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).



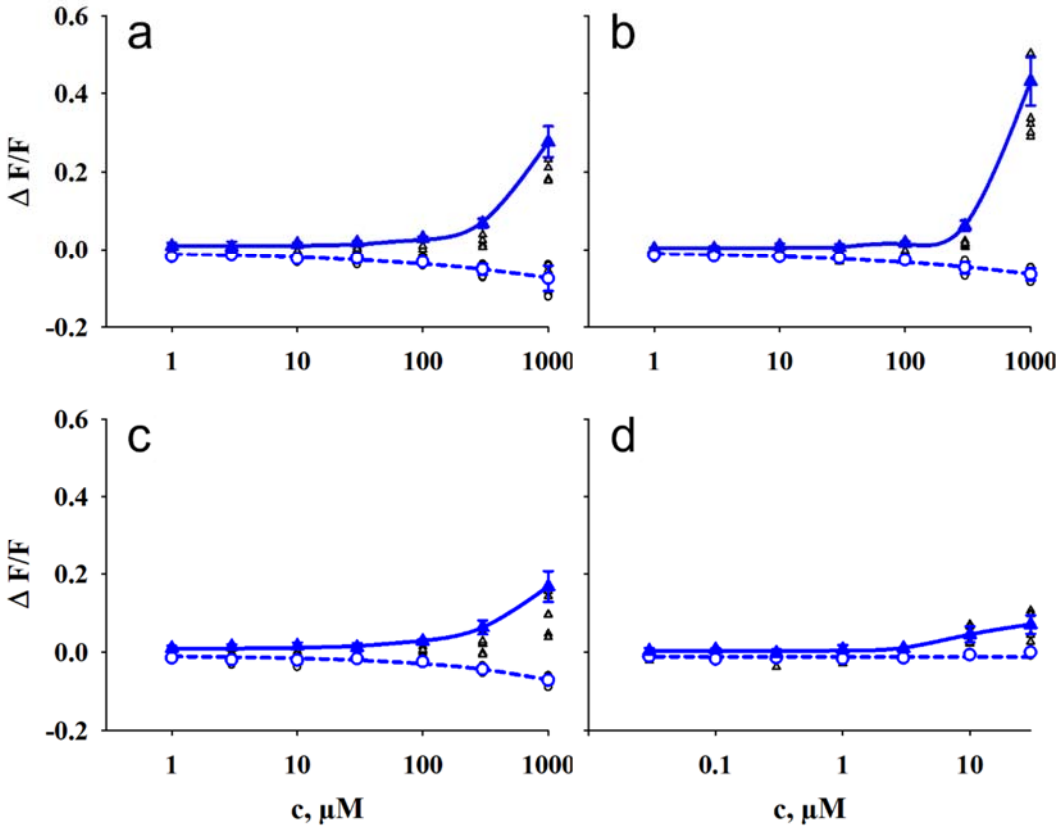
Supplementary Figure 3: Concentration-response relationships of the activation of the human TAS2R39 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human TAS2R39 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids cholic acid a), glycocholic acid b) and taurocholic acid c) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).



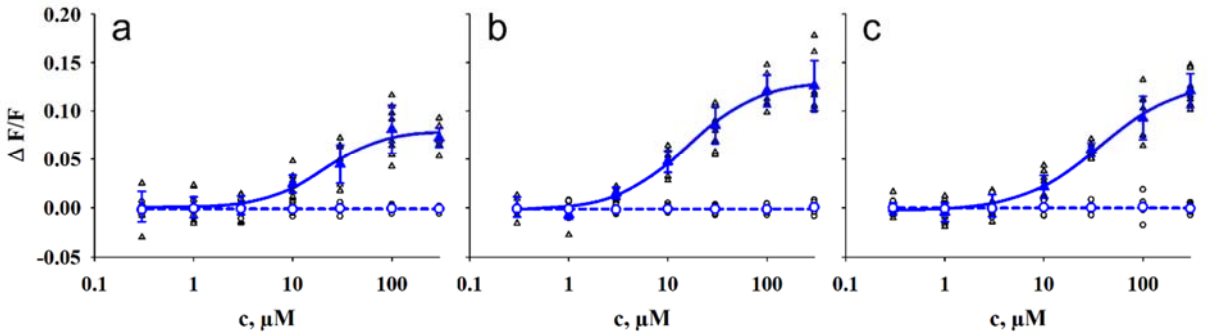
Supplementary Figure 4: Concentration-response relationships of the activation of the human TAS2R46 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human TAS2R46 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids glycocholic acid a), taurocholic acid b) and taurolithocholic acid c) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).



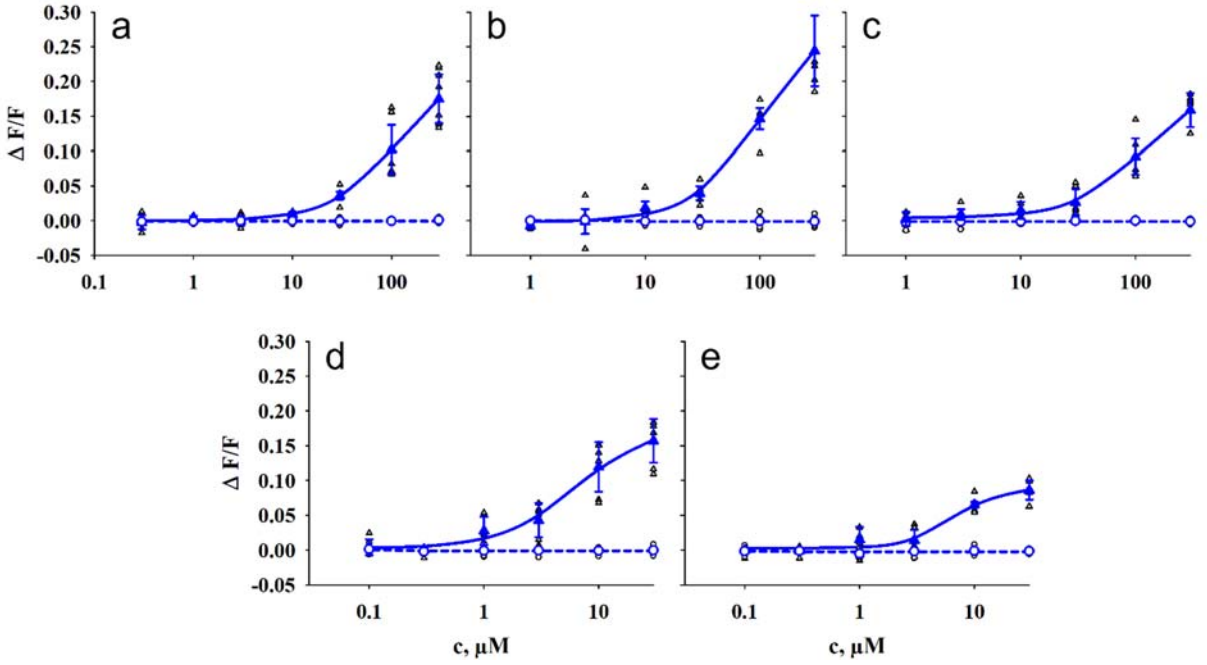
Supplementary Figure 5: Concentration-response relationships of the activation of the murine Tas2r105 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human Tas2r105 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids cholic acid a), glycocholic acid b) and deoxycholic acid c) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).



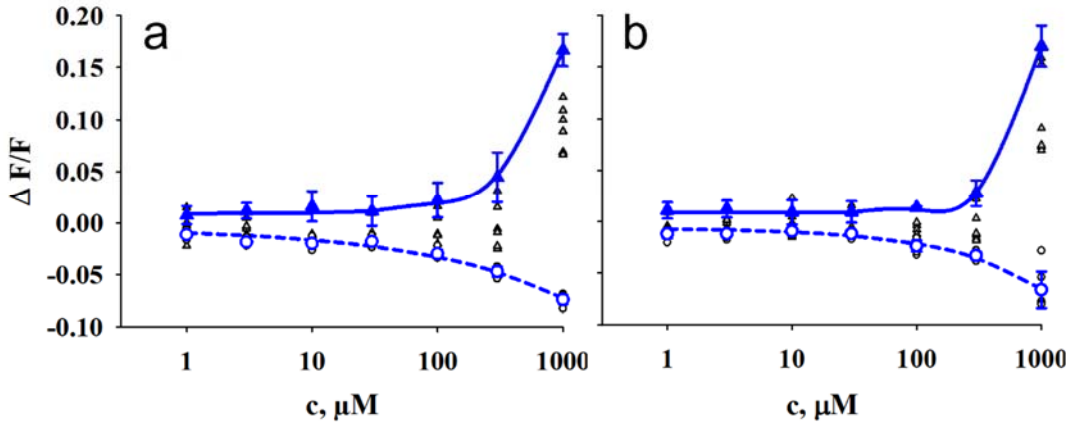
Supplementary Figure 6: Concentration-response relationships of the activation of the murine Tas2r108 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human Tas2r108 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids cholic acid a), glycocholic acid b), taurocholic acid c) and deoxycholic acid d) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).



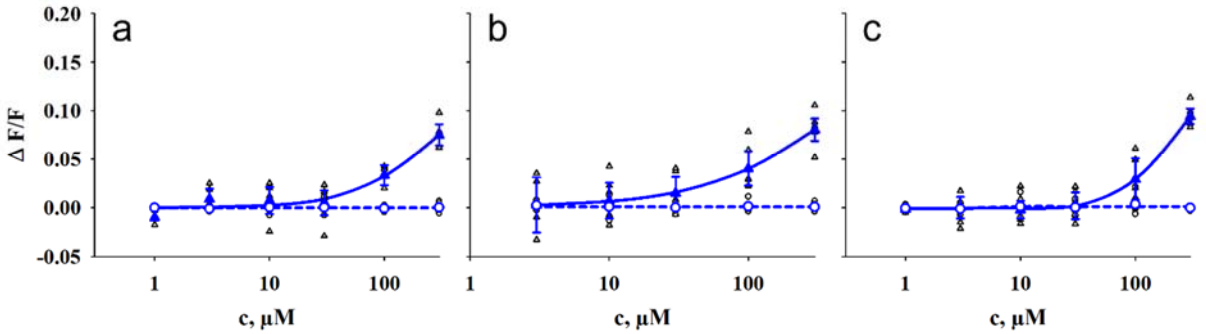
Supplementary Figure 7: Concentration-response relationships of the activation of the murine Tas2r117 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human Tas2r117 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids cholic acid a), glycocholic acid b) and taurocholic acid c) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).



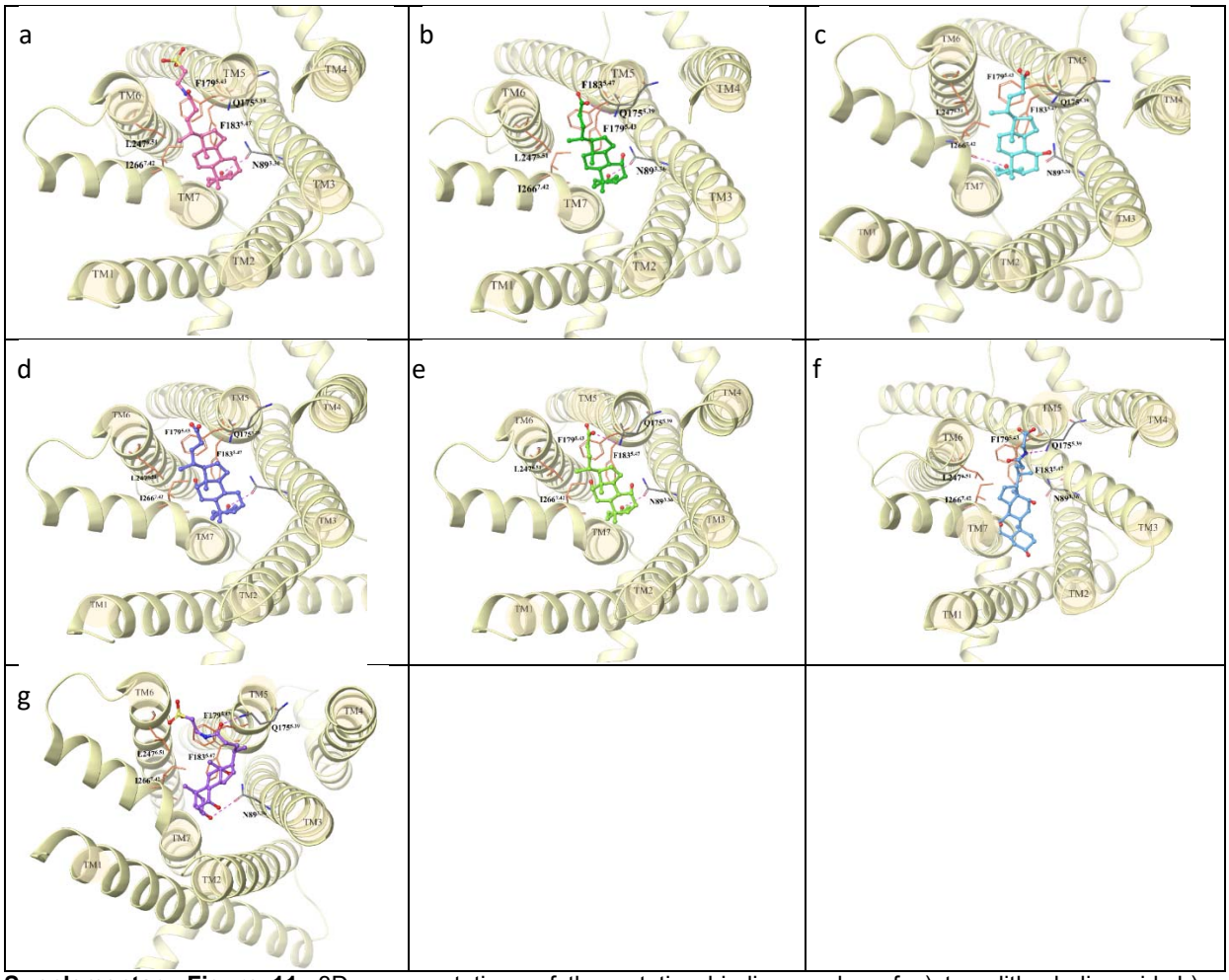
Supplementary Figure 8: Concentration-response relationships of the activation of the murine Tas2r123 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human Tas2r123 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids cholic acid a), glycocholic acid b), taurocholic acid c), deoxycholic acid d) and ursodeoxycholic acid e) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).



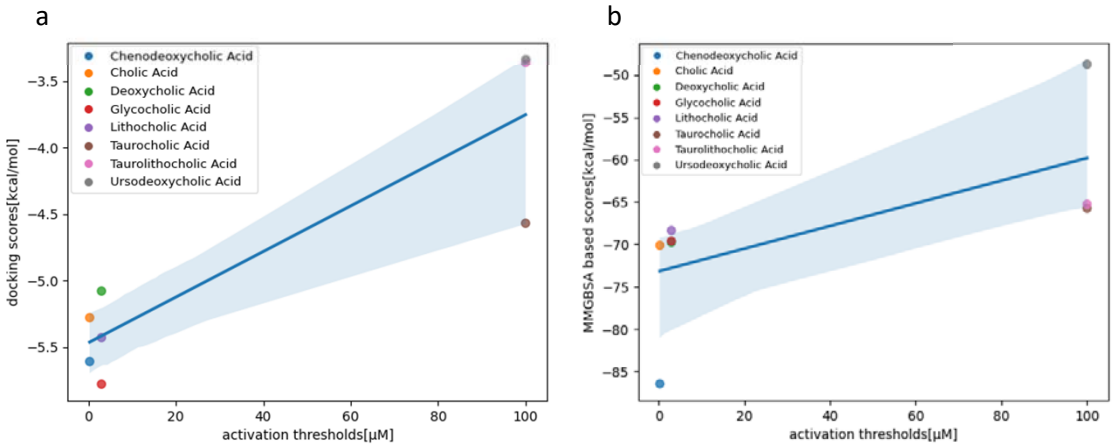
Supplementary Figure 9: Concentration-response relationships of the activation of the murine Tas2r126 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human Tas2r126 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids cholic acid a) and glycocholic acid b) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).



Supplementary Figure 10: Concentration-response relationships of the activation of the murine Tas2r144 by bile acids. HEK293T-Gα16gust44 cells were transiently transfected with the human Tas2r144 (triangle, blue) and an empty vector control (circle, blue). Individual data points are depicted accordingly by black symbols. Receptor activation was measured by increasing fluorescence intensities upon Ca^{2+} - release using an automated fluorometric imaging plate reader ($FLIPR^{TETRA}$). For dose-response relationships, different concentrations of the bile acids cholic acid (a), glycocholic acid (b) and taurocholic acid (c) were applied. The relative fluorescence intensities were mock subtracted and plotted against the bile acid concentration in µM (n ≥ 3 biologically independent experiments). Data are presented as the mean ± standard deviation (STD).



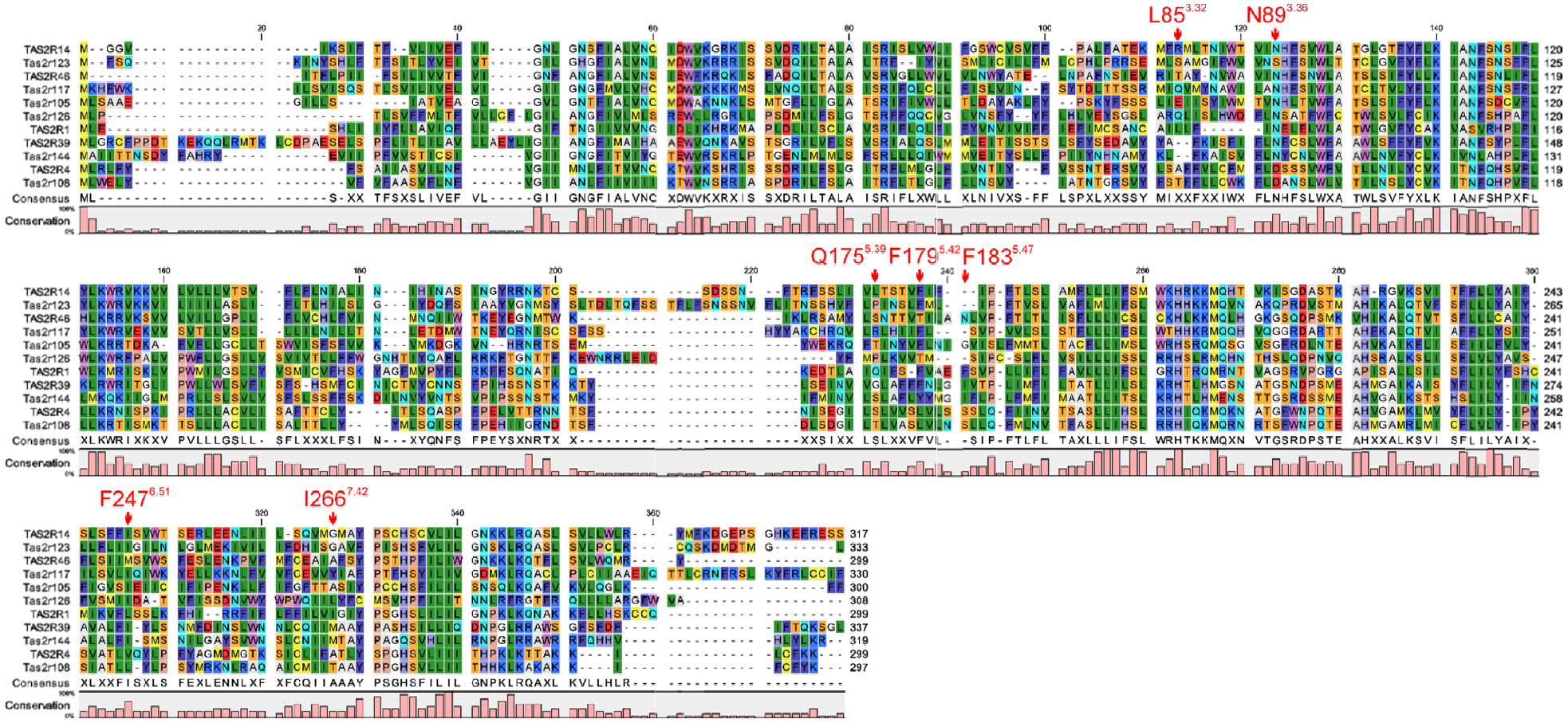
Supplementary Figure 11: 3D representations of the putative binding modes of a) taurolithocholic acid, b) chenoxydecholic acid, c) ursodeoxycholic acid, d) deoxycholic acid, e) cholic acid, f) glycocholic acid, g) taurocholic acid in the TAS2R1 binding site obtained with MM/GBSA refinement.



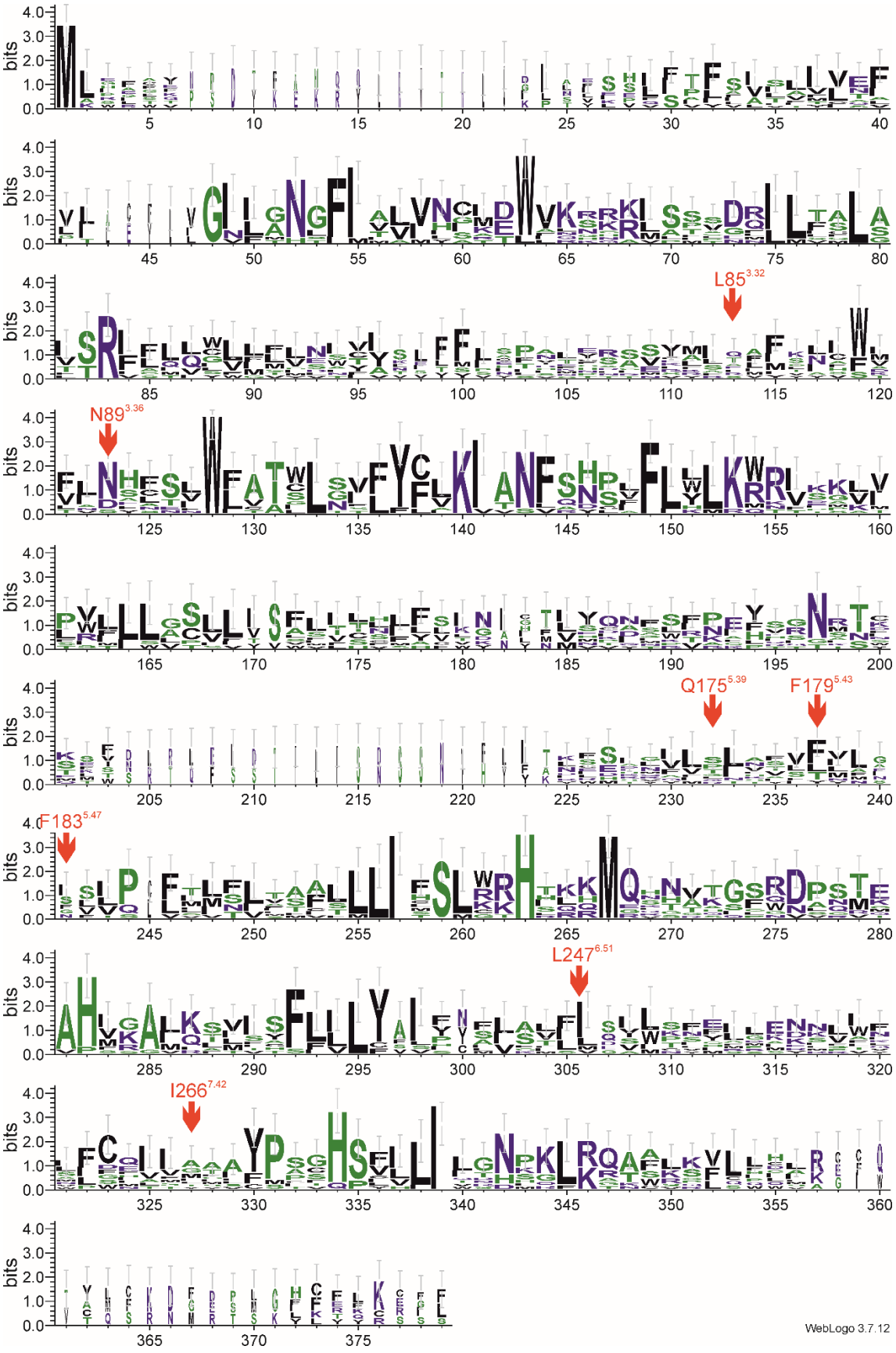
Supplementary Figure 12: Correlation plots of a) docking scores vs. activation thresholds and b) MM/GBSA scores vs. activation thresholds for all investigated ligands.

	TAS2R14	Tas2r123	TAS2R46	Tas2r117	Tas2r105	Tas2r126	TAS2R1	TAS2R39	Tas2r144	TAS2R4	Tas2r108
TAS2R14		44,15	42,38	41,12	30,82	22,64	24,93	23,33	20,52	20,54	21,43
Tas2r123	44,15		38,46	37,54	28,49	24,72	23,21	22,46	19,61	20,51	21,37
TAS2R46	42,38	38,46		33,83	33,12	29,88	28,43	23,12	19,28	22,81	24,45
Tas2r117	41,12	37,54	33,83		29,29	24,21	24,05	22,65	19,37	20,94	20,94
Tas2r105	30,82	28,49	33,12	29,29		26,14	28,21	23,26	20,72	22,74	21,50
Tas2r126	22,64	24,72	29,88	24,21	26,14		25,93	24,44	23,10	25,91	24,39
TAS2R1	24,93	23,21	28,43	24,05	28,21	25,93		23,70	25,23	20,69	21,32
TAS2R39	23,33	22,46	23,12	22,65	23,26	24,44	23,70		47,93	28,45	26,76
Tas2r144	20,52	19,61	19,28	19,37	20,72	23,10	25,23	47,93		27,95	25,23
TAS2R4	20,54	20,51	22,81	20,94	22,74	25,91	20,69	28,45	27,95		66,56
Tas2r108	21,43	21,37	24,45	20,94	21,50	24,39	21,32	26,76	25,23	66,56	

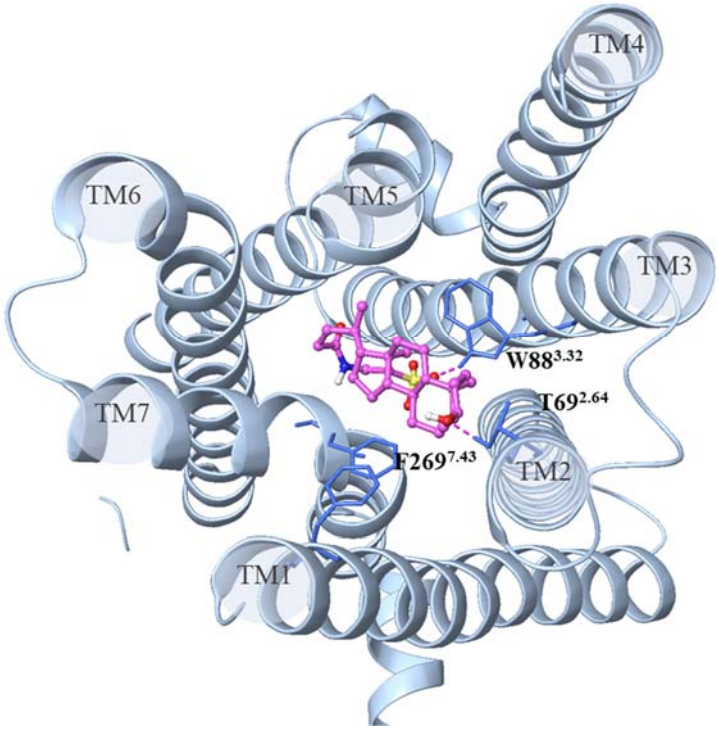
Supplementary Figure 13: Amino acid sequence identities of human and mouse bile acid-sensitive receptors. The pairwise amino acid sequence identities of the indicated bitter taste receptors in % were determined with CLC Main Workbench 22.0.2.



Supplementary Figure 14: Amino acid sequence alignment of human and mouse bile acid-sensitive receptors. The alignment was done using CLC Main Workbench 22.0.2. The color code is provided according to ClustalW conservation code. Positions indicated by red arrows refer to bile acid interacting positions in TAS2R1.



Supplementary Figure 15: WebLogo depiction of the amino acid sequence alignment (done with CLC Main Workbench 22.0.2) of bile acid-sensitive human and mouse bitter taste receptors. Positions indicated by red arrows refer to bile acid interacting positions in TAS2R1. WebLogo was created with the Web Logo 3 tool (https://weblogo.threeplusone.com).



Supplementary Figure 16: 3D representation of the putative binding mode of taurolithocholic acid in the TAS2R46 (PDB ID: 7XP5) binding site obtained with MM/GBSA refinement (score: -70.21 kcal/mol).

Supplementary Table

Supplementary Table 1: Calculated EC_{50}-values of human and murine bitter taste receptors activated by bile acids.

	TAS2R1	TAS2R46	Tas2r108	Tas2r117
Cholic Acid	-	-	-	20.6 ± 5.1 µM
Chenodeoxycholic Acid	-	-	-	-
Lithocholic Acid	0.9 ± 0.1 µM	-	-	-
Deoxycholic Acid	6.1 ± 0.6 µM	-	-	-
Taurocholic Acid	-	-	-	37.4 ± 7.3 µM
Glycocholic Acid	-	-	-	16.0 ± 2.5 µM
Taurolithocholic Acid	1.9 ± 0.9 µM	1.7 ± 0.2 µM	2.3 ± 1.8 µM	-
Ursodeoxycholic Acid	-	-	-	-

Eidesstattliche Erklärung

Ich, Florian Ziegler, erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung

Leibniz-Institut für Lebensmittel-Systembiologie

der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Identification of endogenous agonists of non-gustatory bitter taste receptors

unter der Anleitung und Betreuung durch: PD Dr. Maik Behrens

ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 7 Abs. 6 und 7 angegebenen Hilfsmittel benutzt habe.

☒ Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuer*innen für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

☒ Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

☒ Teile der Dissertation wurden in Molecules, Proceedings of the Royal Society B und Communications Biology veröffentlicht.

☒ Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

☐ Ich habe bereits am _____ bei der promotionsführenden Einrichtung _____ der Hochschule _____ unter Vorlage einer Dissertation mit dem Thema _____ die Zulassung zur Promotion beantragt mit dem Ergebnis:

_____

☒ Ich habe keine Kenntnis über ein strafrechtliches Ermittlungsverfahren in Bezug auf wissenschaftsbezogene Straftaten gegen mich oder eine rechtskräftige strafrechtliche Verurteilung mit Wissenschaftsbezug.

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Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich

☒ einverstanden, ☐ nicht einverstanden.

Töging, 24.03.2024

Ort, Datum, Unterschrift