Ancient coexistence of norepinephrine, tyramine, and octopamine signaling in bilaterians

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Abstract
Norepinephrine/noradrenaline is a neurotransmitter implicated in arousal and other aspects of vertebrate behavior and physiology. In invertebrates, adrenergic signaling is considered absent and analogous functions are attributed to the biogenic amine octopamine. Here we describe the coexistence of signaling by norepinephrine, octopamine, and its precursor tyramine in representatives of the two major clades of Bilateria, the protostomes and the deuterostomes. Using phylogenetic analysis and receptor pharmacology we show that six receptors coexisted in the protostome-deuterostome last common ancestor, two each for the three ligands. All receptors were retained in the genomes of the deuterostome *Saccoglossus kowalewskii* (a hemichordate) and the protostomes *Platynereis dumerilii* (an annelid) and *Priapulus caudatus* (a priapulid). Adrenergic receptors were lost from most insects and nematodes and tyramine and octopamine receptors were lost from most deuterostomes. These results clarify the history of monoamine signaling in animals and highlight the importance of studying slowly evolving marine taxa.

Introduction

Norepinephrine is a major neurotransmitter in vertebrates with a variety of functions including roles in promoting wakefulness and arousal (Singh et al., 2015), regulating aggression (Marino et al., 2005), and autonomic functions such a heart beat (Kim et al., 2002). Signaling by the monoamine octopamine in protostome invertebrates is often considered equivalent to vertebrate adrenergic signaling (Roeder, 2005) with analogous roles in promoting aggression and wakefulness in flies (Crocker and Sehgal, 2008; Zhou et al., 2008), or the regulation of heart rate in annelids and arthropods (Crisp et al., 2010; Florey and Rathmayer, 1978). Octopamine is synthesized from tyramine (Figure 1A) which itself also acts as a neurotransmitter or neuromodulator in arthropods and nematodes (Jin et al., 2016; Kutsukake et al., 2000; Nagaya et al., 2002; Rex and Komuniecki, 2002; Roeder, 2005; Saudou et al., 1990; Selcho et al., 2012). Octopamine and norepinephrine are chemically similar, are synthesized by homologous enzymes (Monastirioti et al., 1996; Wallace, 1976), and signal by similar G-protein coupled receptors (GPCRs) (Evans and Maqueira, 2005; Roeder, 2005), further arguing for their equivalence. However, the precise evolutionary relationship of these transmitter systems is unclear.

The evolution of neurotransmitter systems has been analyzed by studying the distribution of monoamines or biosynthetic enzymes in different organisms (Gallo et al., 2016). This approach has limitations, however, because some of the biosynthetic enzymes are not specific to one substrate (Wallace, 1976) and because trace amounts of several monoamines are found across many organisms, even if specific receptors are often absent. For example, even if invertebrates can synthesize trace amounts of norepinephrine and vertebrates can synthesize tyramine and octopamine, these are not considered to be active neuronal signaling molecules, since the respective receptors are lacking. Consequently, the presence of specific neuronally expressed monoamine receptors is the best indicator that a particular monoamine is used in neuronal signaling (Arakawa et al., 1990; Saudou et al., 1990). We thus decided to analyze the evolution of adrenergic, octopamine, and tyramine receptors in bilaterians.

Results

Using database searches, sequence-similarity-based clustering, and phylogenetic analysis, we identified a few invertebrate GPCR sequences that were similar to vertebrate adrenergic α1 and α2 receptors (Figure 1B). An adrenergic α1 receptor ortholog is present in the sea urchin *Strongylocentrotus purpuratus*. Adrenergic α1 and α2 receptors were both present in *Saccoglossus kowalewskii*, a deuterostome hemichordate (Figure 1B and Supplementary figures 1-2), as previously reported (Krishnan et al., 2013). We also identified adrenergic α1 and α2 receptor orthologs in annelids and mollusks (members of the Lophotrochozoa), including *Aplysia californica*, and in the priapulid worm *Priapulus caudatus* (member of the Ecdysozoa)(Figure 1B). Adrenergic α receptors are also present in a few arthropods, including the crustacean *Daphnia pulex* and the moth *Chilo suppressalis* (the *Chilo* receptor was first described as an octopamine receptor (Wu et al., 2012)), but are absent from most other insects (Supplementary figures 1-2). We also identified α2 receptors in some nematodes. The identification of adrenergic α1 and α2 receptor orthologs in ambulacrarians, lophotrochozoans, and ecdysozoans indicates that both families were present in the protostome-deuterostome last common ancestor. We could not identify adrenergic β receptors outside chordates (Supplementary figure 3).

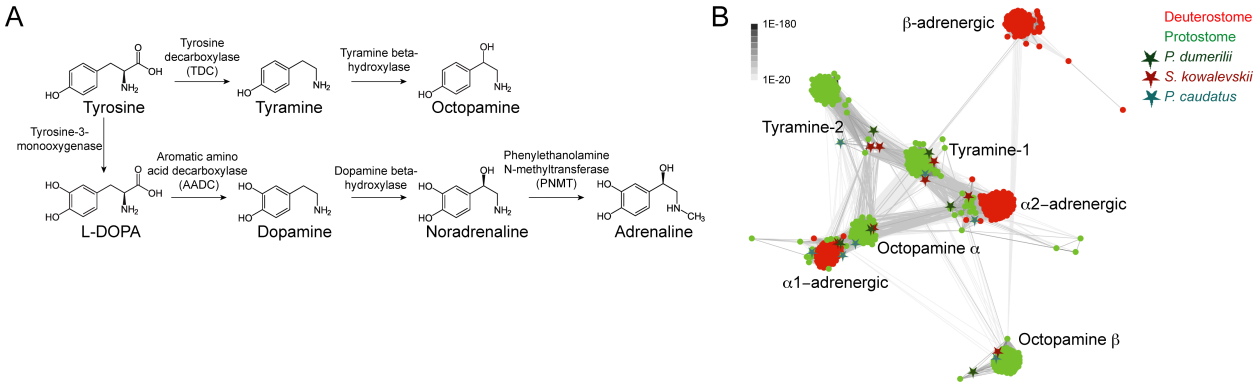

Figure 1. Biosynthesis of monoamines and cluster map of GPCR sequences.
(A) Biosynthesis of tyramine, octopamine, norepinephrine and epinephrine from tyrosine. The enzymes catalyzing the reaction steps are indicated. (B) Sequence-similarity-based cluster map of bilaterian octopamine, tyramine, and adrenergic GPCRs. Nodes correspond to individual GPCRs and are colored based on taxonomy. Edges correspond to BLAST connections of P value >1e-20.

To characterize the ligand specificities of these putative invertebrate adrenergic receptors we cloned them from *S. kowalewskii*, *P. caudatus*, and the marine annelid *Platynereis dumerilii*. We performed *in vitro* GPCR activation experiments using a Ca^{2+}-mobilization assay (Bauknecht and Jékely, 2015; Tunaru et al., 2005). We found that norepinephrine activated both the adrenergic α1 and α2 receptors from all three species with EC_{50} values in the nanomolar range. In contrast, tyramine, octopamine, and dopamine were either inactive or only activated the receptors at higher concentrations (Figure 2, Table 1, and Supplementary figures 4-5). These phylogenetic and pharmacological results collectively establish these invertebrate receptors as bona fide adrenergic α receptors.

To investigate if adrenergic signaling coexists with octopamine and tyramine signaling in protostomes we searched for octopamine and tyramine receptors in *P. dumerilii* and *P. caudatus*. In phylogenetic and clustering analyses, we identified orthologs for tyramine type 1 and type 2 and octopamine α and β receptors in both species (Figure 1B and Supplementary figures 6-9). We performed activation assays with the *P. dumerilii* receptors. The tyramine type 1 and type 2 receptors orthologs were preferentially activated by tyramine with EC_{50} values in the nanomolar range (Figure 2, Table 1, and Supplementary figures 10-11). The *P. dumerilii* octopamine α receptor was activated by octopamine at a lower concentration than by tyramine and dopamine. The *P. dumerilii* octopamine β receptor was not active in our assay. These results show that specific receptor systems for norepinephrine, octopamine, and tyramine coexist in *P. dumerilii* and very likely also *P. caudatus*.

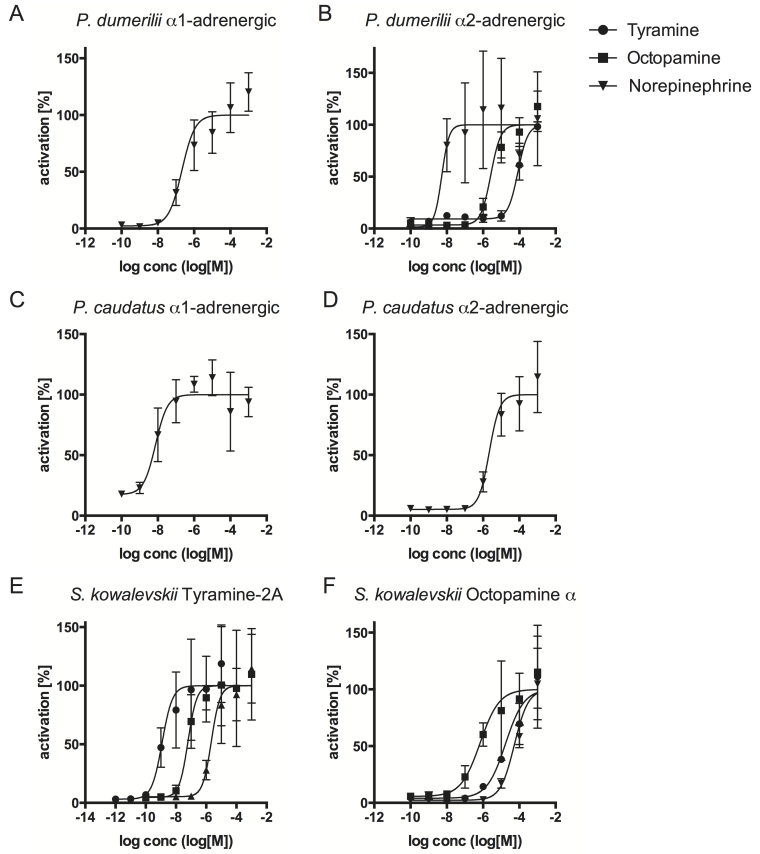

Figure 2. Dose-Response curves of selected monoamine GPCRs from *P. dumerilii*, *P. caudatus*, and *S. kowalevskii* treated with varying concentrations of ligand.
Data, representing luminescence units relative to the maximum of the fitted dose-response curves, are shown as mean ± SEM (n = 3) for selected monoamine GPCRs. All dose-response curves with diverse agonists and antagonists are shown in the Supplementary material. EC_{50} values are listed in Table 1.

When did tyramine and octopamine signaling originate? To answer this, we surveyed available genome sequences for tyramine and octopamine receptors. As expected, we identified several receptors across the protostomes, including ecdysozoans and lophotrochozoans (Supplementary figures 6-9). However, chordate genomes lacked clear orthologs of these receptors. Strikingly, we identified tyramine type 1 and 2 and octopamine α and β receptor orthologs in the genome of the hemichordate *S. kowalewskii* (Figure 1B). In phylogenetic analyses, we recovered at least one *S. kowalewskii* sequence in each of the four receptor clades (one octopamine α, one octopamine β, two tyramine type 1, and two tyramine type 2 receptors), establishing these sequences as deuterostome orthologs of these predominantly protostome GPCR families (Supplementary figures 6-9).

We cloned the candidate *S. kowalewskii* tyramine and octopamine receptors and performed ligand activation experiments. The *S. kowalewskii* type 2 receptors were preferentially activated by tyramine in the nanomolar range. The type 1 receptor was only activated at very high ligand concentrations. The octopamine α and β receptors were preferentially activated by octopamine in the nanomolar, and low micromolar range, respectively (Figure 2, table 1, and Supplementary figures 10-11). These data show that octopamine and tyramine signaling also

coexists with adrenergic signaling in this deuterostome, as in *P. dumerilii* and *P. caudatus*. The presence of tyramine signaling in *S. kowalewskii* is also supported by the phyletic distribution of tyrosine decarboxylase, a specific enzyme for tyramine synthesis (Alkema et al., 2005). Tyrosine decarboxylase is present in protostomes and *S. kowalewskii* but is absent from other deuterostomes (Supplementary figure 12).

We also tested the α adrenergic agonist clonidine and the GPCR antagonists mianserin and yohimbine on several receptors from all three species. These chemicals did not show specificity for any of the receptor types, suggesting these chemicals may not be useful for studying invertebrate neurotransmission (Table 1, and Supplementary figures 4-5, Supplementary figures 9-10).

Discussion

Our results established the coexistence of adrenergic, octopaminergic, and tyraminergic signaling in the deuterostome *S. kowalewskii* and the protostomes *P. dumerilii* and *P. caudatus.* The discovery of adrenergic signaling in some protostomes and octopamine and tyramine signaling in a deuterostome changes our view on the evolution of monoamine signaling in bilaterians (Figure 3). It is clear from the phyletic distribution of orthologous receptor systems that two families of receptors per each ligand were present in the protostome-deuterostome last common ancestor. These include the adrenergic α1 and α2 receptors, the tyramine type 1 and type 2 receptors, and the octopamine α and β receptors. From the six ancestral families, the octopamine and tyramine receptors were lost from most deuterostomes, and the adrenergic receptors were lost from most ecdysozoans.

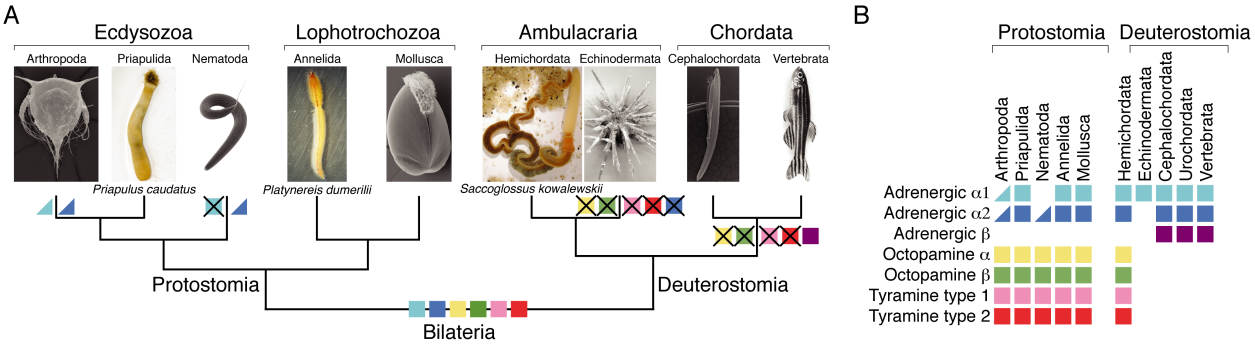

Figure 3. Evolution of adrenergic, octopamine, and tyramine signaling in bilaterians.
(A) Phylogenetic tree of major clades of bilaterian animals with the loss of specific GPCR families indicated. (B) Phyletic distribution of adrenergic, octopamine, and tyramine GPCR families across major bilaterian clades.

We consider the measured *in vitro* ligand preferences for these receptors as indicative of their physiological ligands. In general, there is a two orders of magnitude difference in the EC_{50} values between the best ligand and other related ligands, indicating the high specificity of the receptors. The preferred ligand of all six orthologous receptor families is the same across protostomes and deuterostomes, indicating the evolutionary stability of ligand-receptor pairs, similar to the long-term stability of neuropeptide GPCR ligand-receptor pairs (Jékely, 2013; Mirabeau and Joly, 2013).

Understanding the ancestral role of these signaling systems and why they may have been lost differentially in different animal groups will require functional studies in organisms where all three neurotransmitter systems coexist. Signaling by norepinephrine in vertebrates has often been considered as equivalent to signaling by octopamine in invertebrates. Our results change this view and show that these signaling systems coexisted ancestrally and still coexist in some bilaterians, and thus cannot be equivalent. This has important implications for our interpretation of comparative studies of neural circuits and nervous system evolution.

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Materials and Methods

Gene identification and receptor cloning

Platynereis protein sequences were collected from a *Platynereis* mixed stages transcriptome assembly (Conzelmann et al., 2013). The accession numbers of all newly identified GPCRs tested here are GenBank: KX372342, KX372343, KP293998, KU715093, KU530199, KU886229). GPCR sequences from other species were downloaded from NCBI. GPCRs were cloned into pcDNA3.1(+) (Thermo Fisher Scientific, Waltham, USA) as described before (Bauknecht and Jékely, 2015). Forward primers consisted of a spacer (ACAATA) followed by a

BamHI or EcoRI restriction site, the Kozak consensus sequence (CGCCACC), the start codon (ATG) and a sequence corresponding to the target sequence. Reverse primers consisted of a spacer (ACAATA), a NotI restriction site, a STOP codon, and reverse complementary sequence to the target sequence. Primers were designed to end with a C or G with 72°C melting temperature. PCR was performed using Phusion polymerase (New England Biolabs GmbH, Frankfurt, Germany). The sequences of all *Platynereis* GPCRs tested here were deposited in GenBank (accession numbers: α1-adrenergic receptor, KX372342; α2-adrenergic receptor, KX372343 Tyramine-1 receptor, KP293998; Tyramine-2 receptor, KU715093; Octopamine α receptor, KU530199; Octopamine β receptor, KU886229). Tyramine receptor 1 has been previously published (Bauknecht and Jékely, 2015) as Pdu orphan GPCR 48.

Cell culture and receptor deorphanization

Cell culture assays were done as described before (Bauknecht and Jékely, 2015). Briefly, CHO-K1 cells were kept in Ham's F12 Nut Mix medium (Thermo Fisher Scientific, Waltham, USA) with 10 % fetal bovine serum and penicillin-streptomycin (PenStrep, Invitrogen). Cells were seeded in 96-well plates (Thermo Fisher Scientific, Waltham, USA) at approximately 10,000 cells/well. After 1 day, cells were transfected with plasmids encoding a GPCR, the promiscuous Gα-16 protein (Offermanns and Simon, 1995), and a reporter construct GFP-apoaequorin (Baubet et al., 2000) (60 ng each) using 0.375 µl of the transfection reagent TurboFect (Thermo Fisher Scientific, Waltham, USA). After two days of expression, the medium was removed and replaced with Hank's Balanced Salt Solution (HBSS) supplemented with 1.8 mM Ca^{2+}, 10 mM glucose and 1 mM coelenterazine h (Promega, Madison, USA). After incubation at 37°C for 2 hours, cells were tested by adding synthetic monoamines (Sigma, St. Louis, USA) in HBSS supplemented with 1.8 mM Ca^{2+} and 10 mM glucose. Solutions containing norepinephrine, epinephrine or dopamine were supplemented with 100 µM ascorbic acid to prevent oxidation. Luminescence was recorded for 45 seconds in a plate reader (BioTek Synergy Mx or Synergy H4, BioTek, Winooski, USA). For inhibitor testing, the cells were incubated with yohimbine or mianserin (Sigma, St. Louis, USA) for 1 hour. Then, synthetic monoamines were added to yield in each case the smallest final concentration expected to elicit the maximal response in the absence of inhibitor and luminescence was recorded for 45 seconds. Data were integrated over the 45-second measurement period. Data for dose-response curves were recorded in triplicate for each concentration. Dose-response curves were fitted with a four-parameter curve using Prism 6 (GraphPad, La Jolla, USA). The curves were normalized to the calculated upper plateau values (100% activation).

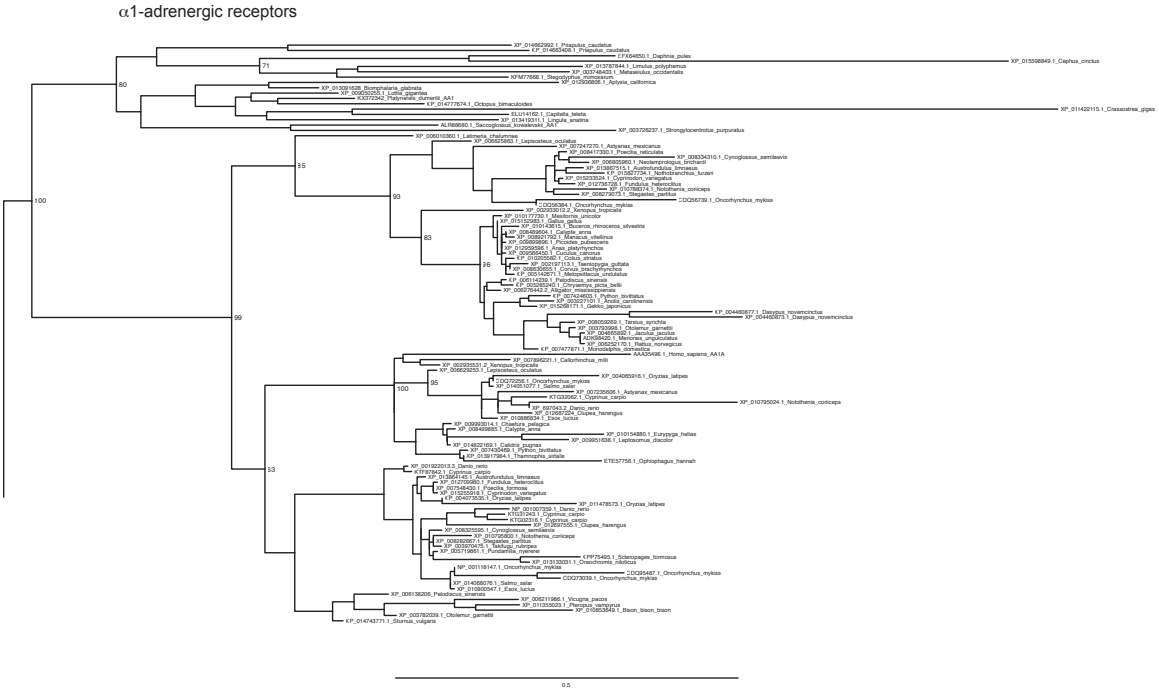
Bioinformatics

Protein sequences were downloaded from the NCBI. Redundant sequences were removed from the collection using CD-HIT (Li and Godzik, 2006) with an identity cutoff of 95%. Sequence cluster maps were created with CLANS2 (Frickey and Lupas, 2004) using the BLOSUM62 matrix and a P-value cutoff of 1E-70. For phylogenetic trees, protein sequences were aligned with MUSCLE (Edgar, 2004). Alignments were trimmed with TrimAI (Capella-Gutiérrez et al., 2009) in "Automated 1" mode. The best amino acid substitution model was selected using ProtTest 3 (Darriba et al., 2011). Maximum likelihood trees were calculated with RAxML (Stamatakis, 2014) using the CIPRES Science Gateway (Miller et al., 2010). Bootstrap analysis was done and automatically stopped (Pattengale et al., 2010) when the Majority Rule Criterion (autoMRE) was met. The resulting trees were visualized with FigTree

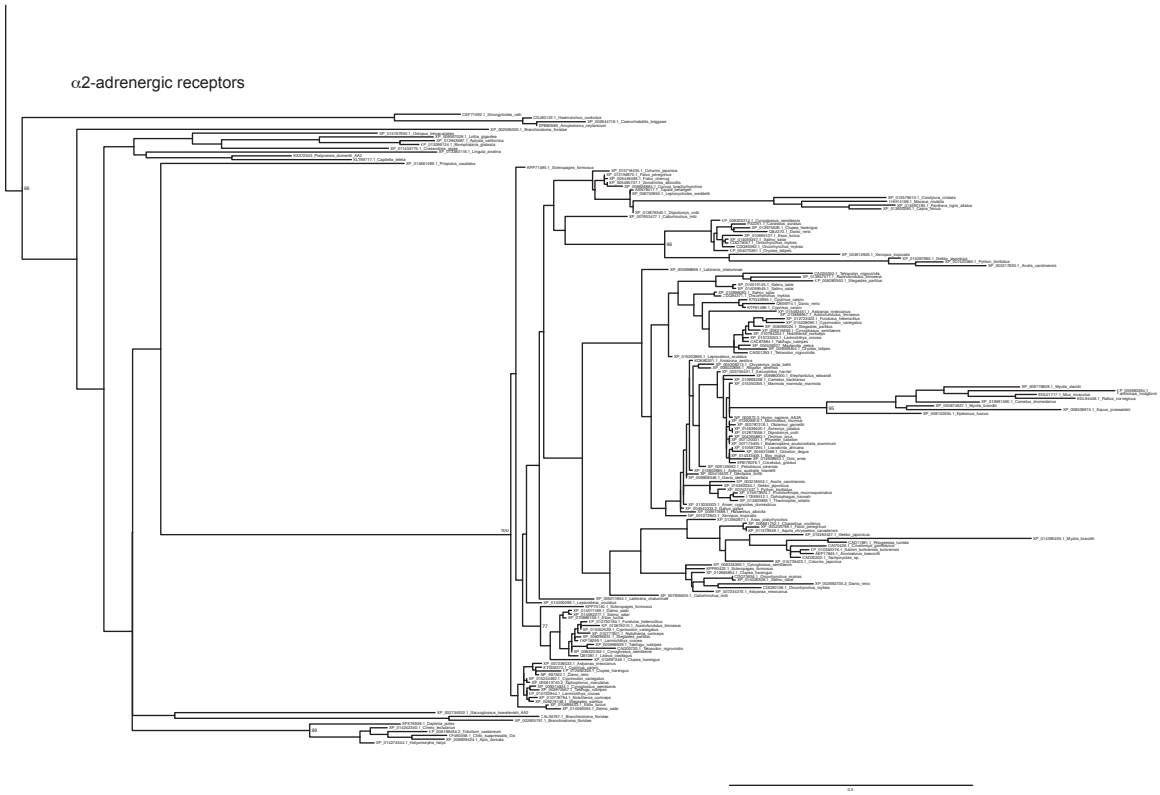
(http://tree.bio.ed.ac.uk/software/figtree/). The identifiers of deorphanized octopamine and tyramine receptors (Balfanz et al., 2014; Blais et al., 2010; Chang et al., 2000; Chen et al., 2010; Gerhardt et al., 1997; Gross et al., 2015; Huang et al., 2012; Jezzini et al., 2014; Kastner et al., 2014; Lind et al., 2010; Rex and Komuniecki, 2002; Verlinden et al., 2010; Wu et al., 2012; Wu et al., 2014; Wu et al., 2015) were tagged with _Oa, _Ob, _T1, or _T2.

EC50 (M)/IC50 (M)	Tyramine	Octopamine	Norepinephrine	Epinephrine	Dopamine	Clonidine	Yohimbine	Mianserin
P. dumerilii α1-adrenergic KX372342	inactive	inactive	**2.12E-07**	3.79E-07	1.25E-04	inactive	4.41E-06	3.79E-06
P. dumerilii α2-adrenergic KX372343	8.36E-05	2.67E-06	**5.25E-09**	1.06E-08	1.63E-06	2.57E-06	5.71E-06	2.53E-05
S. kowalevskii α1-adrenergic ALR88680	inactive	n/a	**1.67E-08**	1.94E-08	3.79E-06	inactive	1.32E-05	4.46E-06
S. kovalewskii α2-adrenergic XP_002734932	3.75E-06	1.94E-06	**1.16E-13**	2.31E-09	5.60E-09	3.61E-08	7.90E-05	n/a
P. caudatus α1-adrenergic XP_014662992	inactive	inactive	**7.49E-09**	inactive	inactive	inactive	n/a	n/a
P. caudatus α2-adrenergic XP_014681069	inactive	inactive	2.20E-06	**4.52E-07**	inactive	1.02E-07	n/a	9.85E-07
P. dumerilii Tyramine-1 KP293998	**1.12E-08**	2.70E-06	n/a	n/a	7.79E-06	n/a	2.06E-06	1.05E-05
P. dumerilii Tyramine-2 KU715093	**7.02E-09**	7.82E-07	n/a	n/a	3.89E-06	n/a	5.38E-05	6.36E-06
S. kovalewskii Tyramine-1 XP_002742354	**8.40E-05**	n/a	inactive	inactive	n/a	2.86E-04	1.68E-06	1.70E-05
S. kovalewskii Tyramine-2A XP_002734062	**1.22E-09**	6.24E-08	inactive	inactive	7.18E-08	1.39E-06	n/a	1.61E-04
S. kovalewskii Tyramine-2B XP_006812999	**3.71E-09**	1.57E-06	1.15E-04	2.85E-05	1.44E-06	1.60E-05	2.06E-05	1.88E-05
P. dumerilii Octopamine α KU530199	1.34E-05	**2.56E-07**	n/a	n/a	inactive	n/a	9.02E-09	1.62E-06
S. kowalevskii Octopamine α XP_006823182	1.72E-05	**6.89E-07**	5.30E-05	1.85E-05	2.65E-04	1.64E-07	7.78E-06	2.16E-05
S. kowalevskii Octopamine β XP_002733926	inactive	**6.37E-08**	3.49E-06	inactive	inactive	inactive	1.57E-04	6.45E-06

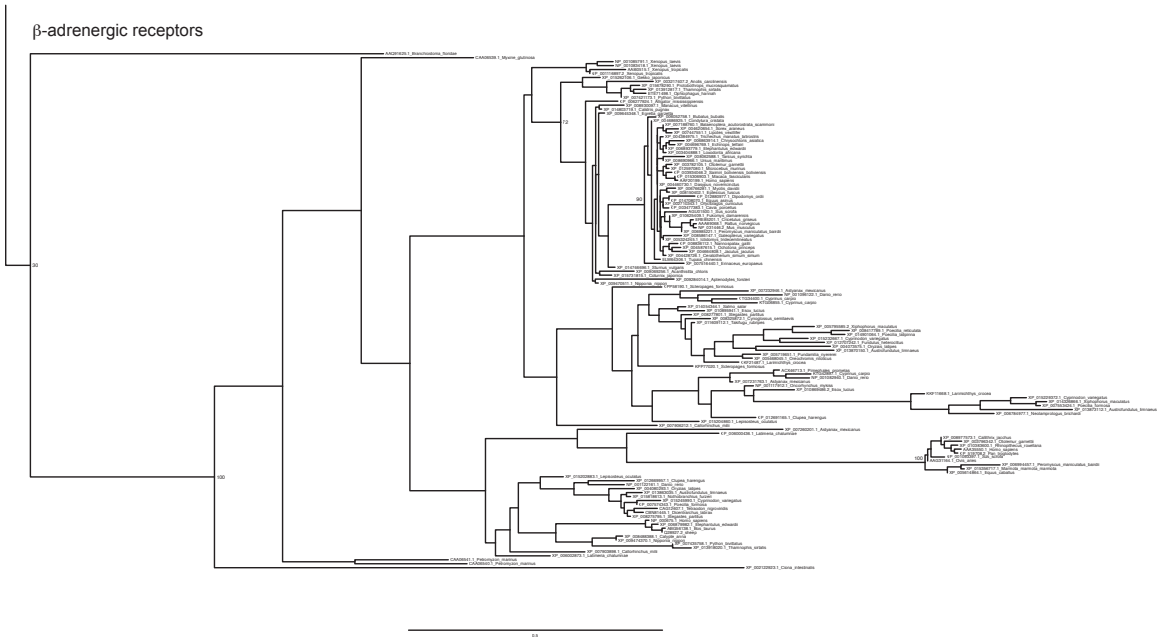
Table 1. EC_{50} (M)/IC_{50} (M) values of all tested GPCRs with the indicated ligands or inhibitors. The most effective ligand for each receptor is shown in bold.



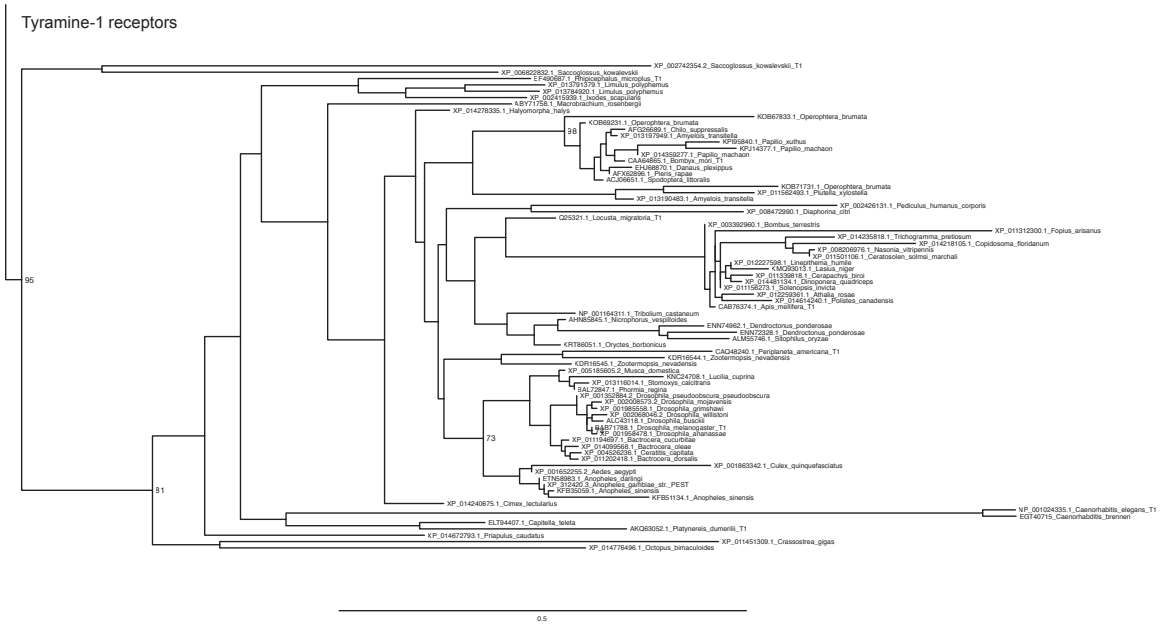
Supplementary figure 1. Maximum likelihood tree of α1-adrenergic receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs.



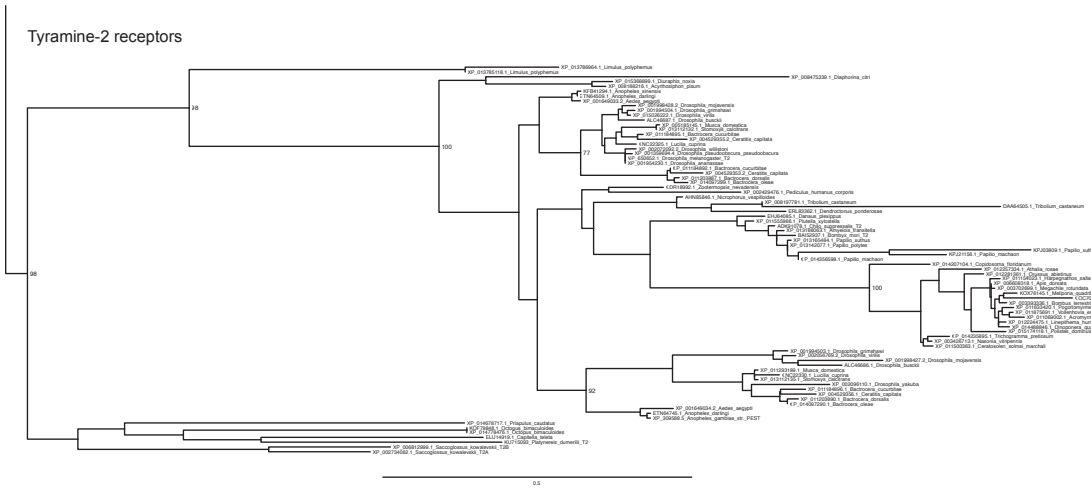
Supplementary figure 2. Maximum likelihood tree of α2-adrenergic receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs.



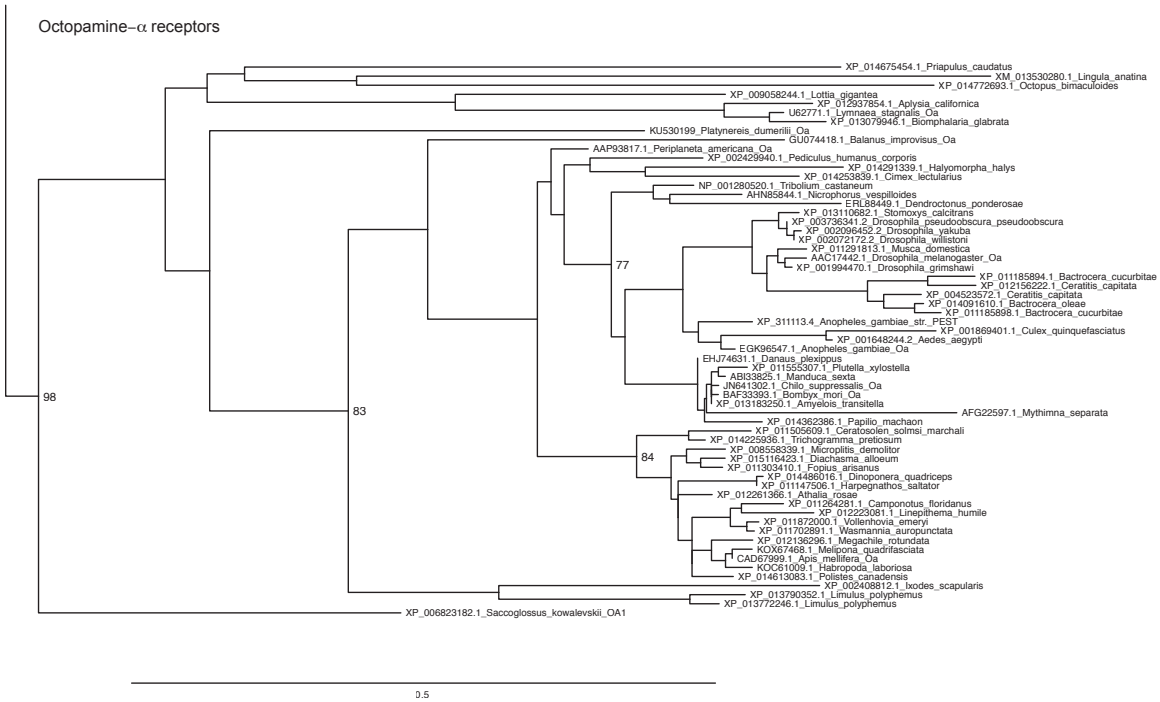
Supplementary figure 3. Maximum likelihood tree of β-adrenergic receptors. Bootstrap support values are shown for some nodes of interest. This tree is part of a larger tree containing all investigated GPCRs.



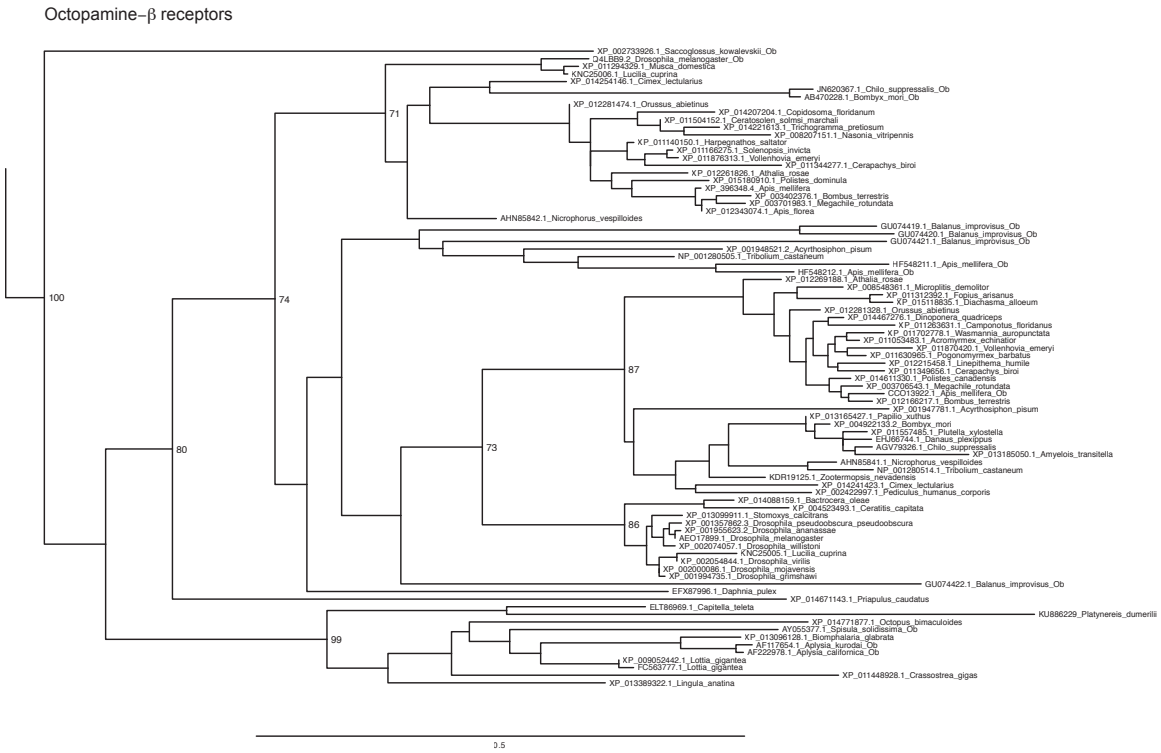
Supplementary figure 4. Maximum likelihood tree of Tyramine-1 receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. The identifiers of deorphanized tyramine receptors were tagged with _T1.



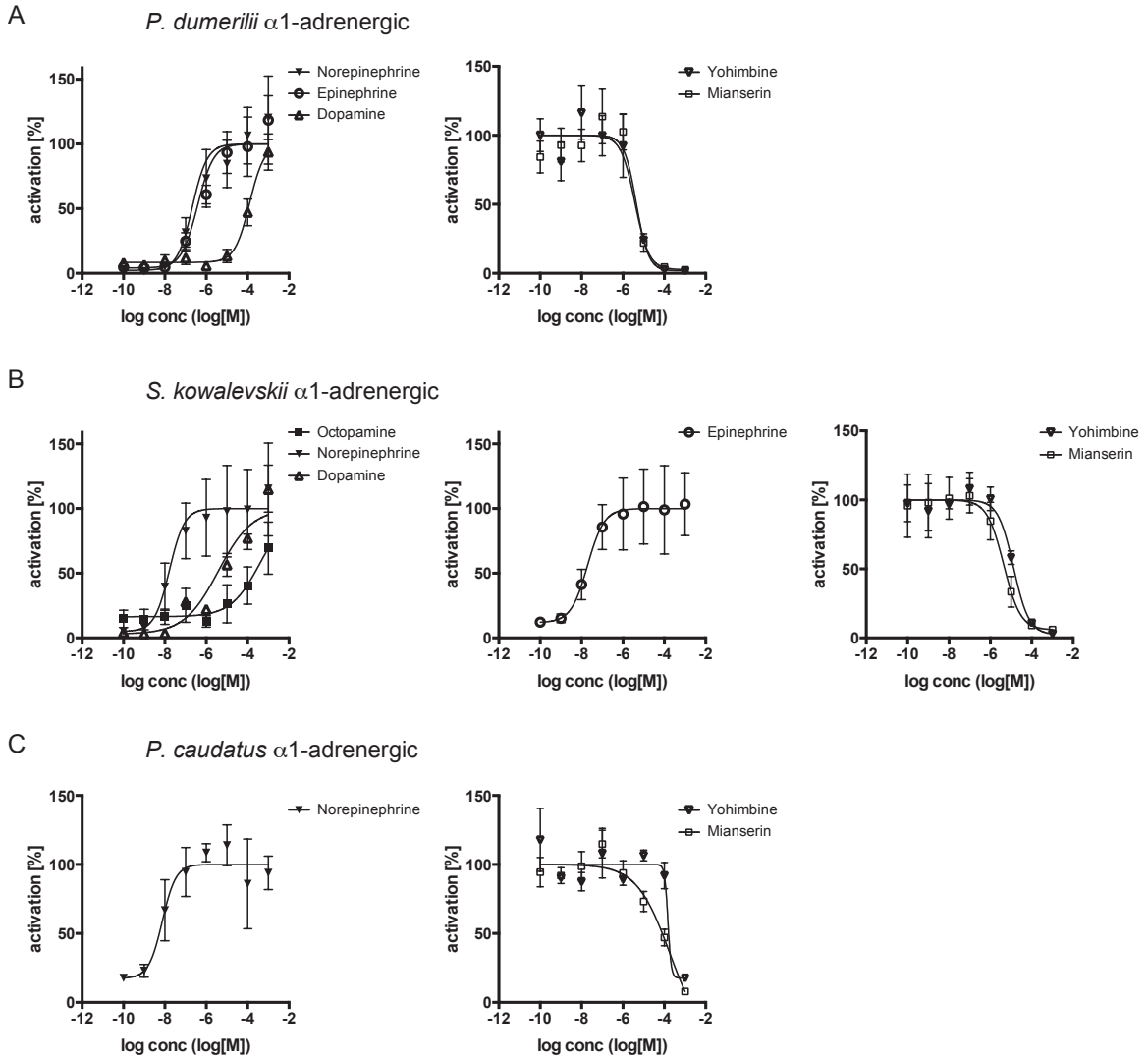
Supplementary figure 5. Maximum likelihood tree of Tyramine-2 receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. The identifiers of deorphanized tyramine receptors were tagged with _T2.



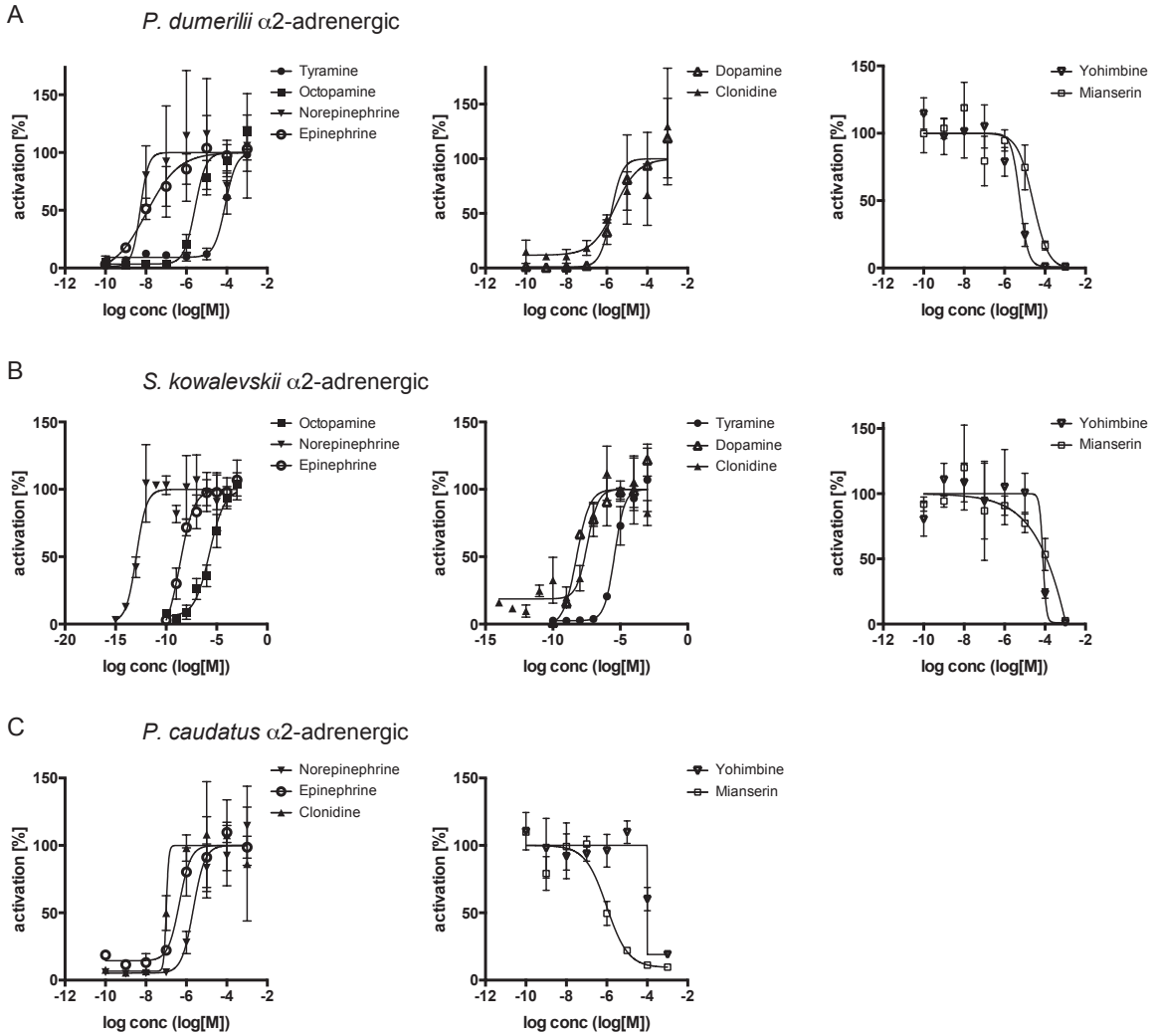
Supplementary figure 6. Maximum likelihood tree of Octopamine-α receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. The identifiers of deorphanized octopamine receptors were tagged with _Oa.



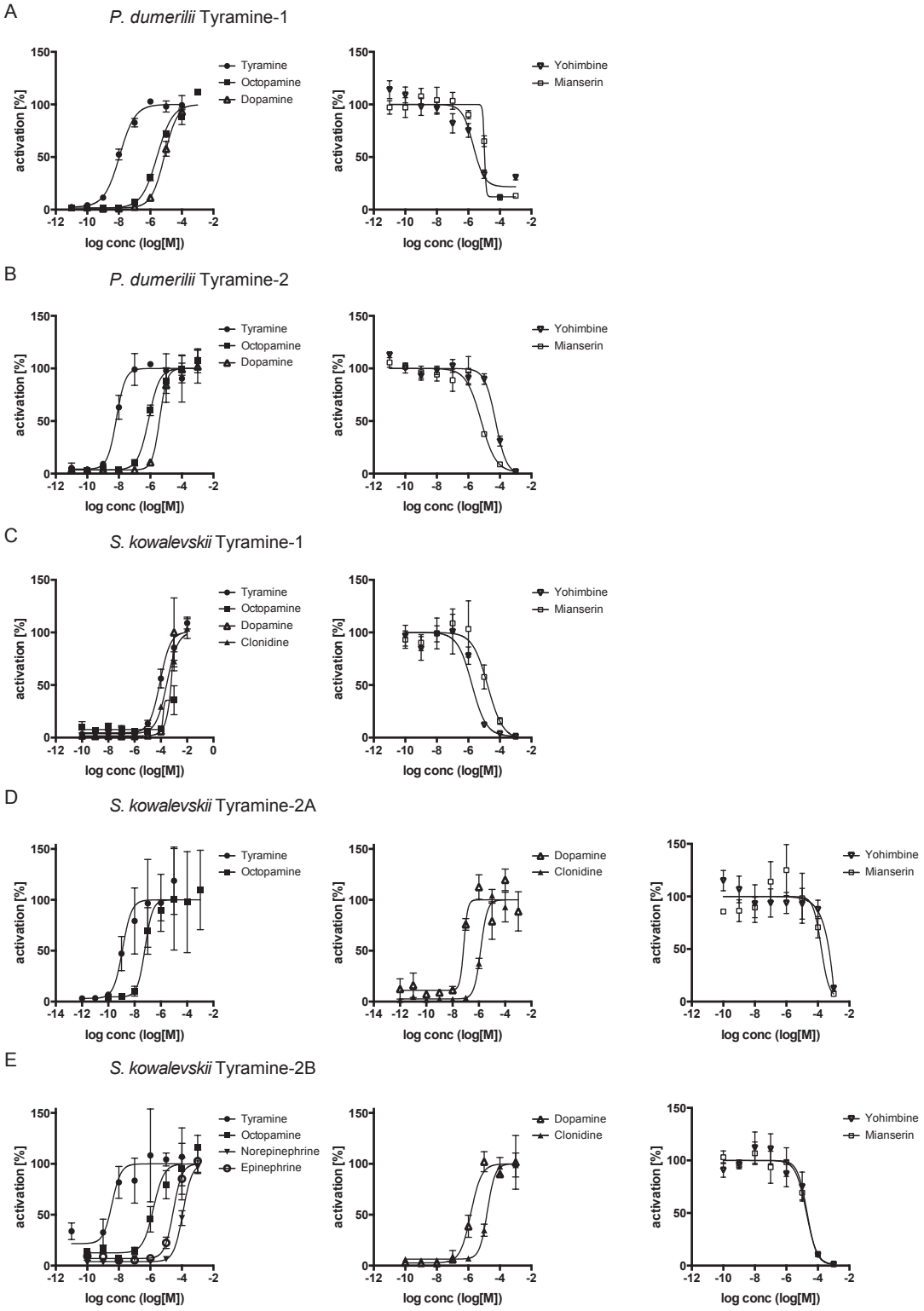
Supplementary figure 7. Maximum likelihood tree of Octopamine-β receptors. Bootstrap support values are shown for selected nodes. This tree is part of a larger tree containing all investigated GPCRs. The identifiers of deorphanized octopamine receptors were tagged with _Ob.



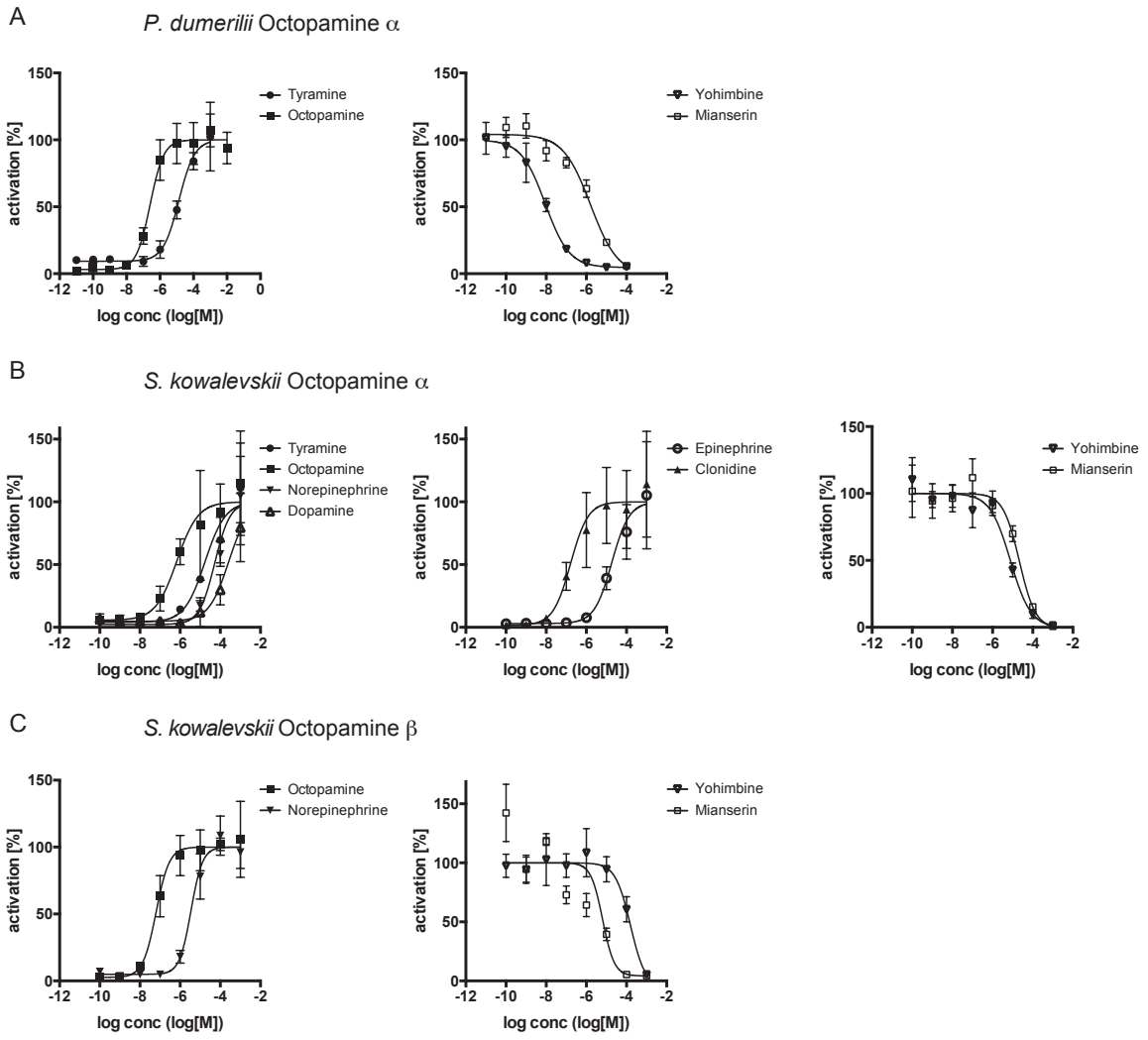
Supplementary figure 8. Dose-response curves of α1-adrenergic receptors from *P. dumerilii*, *P. caudatus*, and *S. kowalevskii* treated with varying concentrations of ligand or inhibitor. Data, representing luminescence units relative to the maximum of the fitted dose-response curves, are shown as mean ± SEM (n = 3). EC_{50} and IC_{50} values are listed in Table 1.



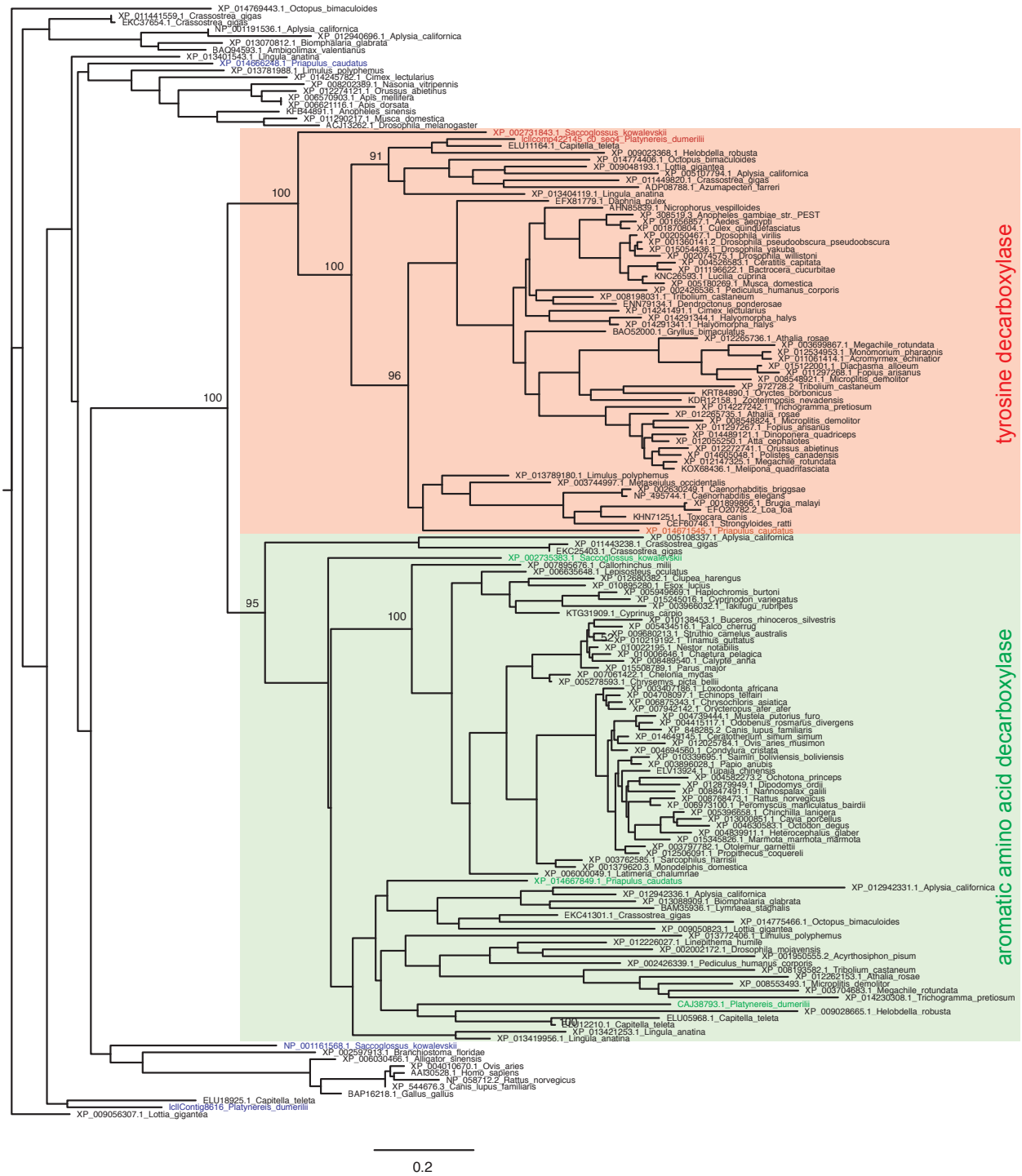
Supplementary figure 9. Dose-response curves of α2-adrenergic receptors from *P. dumerilii*, *P. caudatus*, and *S. kowalevskii* treated with varying concentrations of ligand or inhibitor. Data, representing luminescence units relative to the maximum of the fitted dose-response curves, are shown as mean ± SEM (n = 3). EC_{50} and IC_{50} values are listed in Table 1.



Supplementary figure 10. Dose-response curves of tyramine receptors from *P. dumerilii* and *S. kowalevskii* treated with varying concentrations of ligand or inhibitor. Data, representing luminescence units relative to the maximum of the fitted dose-response curves, are shown as mean ± SEM (n = 3). EC_{50} and IC_{50} values are listed in Table 1.



Supplementary figure 11. Dose-response curves of octopamine receptors from *P. dumerilii* and *S. kowalevskii* treated with varying concentrations of ligand or inhibitor. Data, representing luminescence units relative to the maximum of the fitted dose-response curves, are shown as mean ± SEM (n = 3). EC_{50} and IC_{50} values are listed in Table 1.



Supplementary figure 12. Maximum likelihood tree of tyrosine decarboxylase and aromatic amino acid decarboxylase enzymes. Bootstrap support values are shown for selected nodes. *P. dumerilii*, *P. caudatus*, and *S. kowalevskii* sequences are highlighted in color. The *Caenorhabditis elegans* tyrosine decarboxylase was experimentally shown to be required for tyramine biosynthesis (Alkema et al., 2005).