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SUPPLEMENTAL DATA

MATERIALS AND METHODS

Rearing of barnacle cypris larvae

Cypris larvae of *B. improvisus* were produced and reared in a laboratory culture of adult individuals as earlier described with some modifications (Berntsson *et al.* 2000). Cyprids were allowed to settle on plexiglass panels placed in the sea off the west coast of Sweden, in the vicinity of Tjärnö Biological Laboratory (58° 53'N, 11° 08'E). The panels were brought to the laboratory and placed in buckets/aquaria with running seawater and used as brood stock. Adult *B. improvisus* spawn throughout the year when regularly fed with nauplii of *Artemia* sp. When kept at 26°C, the development to cypris larvae takes 6-7 days from the first nauplius stage. The newly moulted cypris larvae were filtered to separate the cyprids from the nauplius larvae using three filters with 340, 200 and 160 μm pore size. Cypris larvae were washed to remove algae and detritus.

Cyprid motility assay

Cyprids were made immobile by using agarose (A-2576 from Sigma-Aldrich, St. Louis, MO, USA). Agarose was dissolved in filtered seawater (FSW) to a final concentration of 2 %, heated and melted and thereafter allowed to cool to just above the gelling temperature. Cyprids were added to a Petri dish that had been marked at the bottom into 8 different area zones. Excessive sea water was thereafter removed and the melted agarose was gently poured into the Petri dish. The cyprid larvae were then incorporated and distributed randomly into the agarose by shaking the dish gently. When the agarose was no longer fluid, 5 ml of FSW was added atop of the agarose layer and thereafter the dishes were left for one hour to allow a steady state to form between cyprid, agarose and FSW. Medetomidine (10 nM), atipamezole (100 nM) or a combination of these ligands were then added and individual cyprid movements were counted as kicks per minute under a stereo microscope. The added concentration of medetomidine was approximately ten-fold above its efficacy in preventing settling as noted in earlier studies (Dahlstrom & Elwing 2006). Approximately 20-30 animals were individually identified by the help of the zones mapped on the bottom of the Petri dishes. In the case of adding medetomidine or atipamezole alone, the numbers of kicks were counted before and after one hour incubation with the ligands. When adding atipamezole and medetomidine in combination, the cyprids were first incubated for 3 hours with atipamezole, whereafter the numbers of kicks were registered. Medetomidine was then added and the cyprids were incubated for another hour, whereafter the kicks were counted again. In a control sample, kicks were counted before and after one hour incubation with water.

Collection of adult barnacles for expression studies

Cyprids were allowed to settle during the summer on plexiglass panels placed in the sea off the west coast of Sweden, in the vicinity of Kristineberg Marine Research Station (58° 15'N, 11° 27'E). Adult animals were collected in October.

RNA preparation and cDNA synthesis

For each RNA extraction one adult animal, or 20-30 mg of cyprids (wet weight) corresponding to 600-1000 animals were used. The adult animal was removed from its shell by opening the operculum plates and pulling it out with forceps. The adult tissue or cypris larvae were sonicated in micro-centrifuge tubes on ice at 10 micron amplitude with a Soniprep 150® device (MSE, London, UK), in buffer RLT (QIAgen RNeasy mini kit, Qiagen, Valencia, CA, USA) for 2 x 5 seconds with a 2 min rest in between. To get rid of tissue remnants, the sample was then spun at 13000 rpm for 2 min in a micro-centrifuge. Total RNA was extracted from the supernatant with the QIAgen RNeasy mini kit according to the manufacturer's instructions. The integrity and purity of RNA was measured both spectrophotometrically and by gel electrophoresis. The RNA was used as template for cDNA synthesis using either the iScript kit (BioRad, Hercules, CA, USA) or SuperscriptIII first strand kit (Invitrogen, Invitrogen Life Technologies Inc., Rockville, MD, USA) according to the manufacturers' protocols.

Preparation of genomic DNA from cyprids

A batch of 20-30 mg cyprids (wet weight) was sonicated in Elution buffer (E.Z.N.A Blood DNA Kit, Omega Bio-Tek, Norcross, GA, USA) for 2 x 5 seconds and then centrifuged to remove tissue remnants. Genomic DNA was thereafter prepared according to the E.Z.N.A Blood DNA Kit protocol. About 20 µg DNA was obtained per batch.

Strategy for cloning of the B. improvisus octopamine receptors

Fragments of *B. improvisus* octopamine receptor genes were obtained using different approaches (see below). To identify the sequences of the 5´ and 3´ends, rapid amplification of cDNA ends (RACE) using the GeneRacer™ kit (Invitrogen) was performed. The obtained 5´ and 3´ sequences were used to design primers for cloning of the full-length receptors, from sequences just upstream of the predicted start methionine codon to just downstream of the stop codon. For amplification of each of the 5´ or 3´ends, one initial PCR was run followed by a nested PCR using 1 µl of the first reaction. All PCR reactions contained 5 % DMSO. For PCR with degenerate PCR primers, Truestart™Taq DNA Polymerase (Fermentas Life Sciences, Burlington, Canada) was used. In all other PCR reactions, PfuUltra High-Fidelity DNA polymerase® (Stratagene, La Jolla, CA, USA) was used. Fragments obtained with degenerate PCR primers were cloned into pCR4®-TOPO® (Invitrogen) using the TOPO® TA Cloning® Kit and sequenced. All other fragments were cloned into pCR®4Blunt-TOPO® using the Zero Blunt® TOPO® PCR Cloning Kit for Sequencing (Invitrogen) and sequenced. For PCR primer

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sequences and PCR programs, see tables S1 and S2, respectively. The GeneRacer primers are supplied with the kit and their sequences can be found in the GeneRacer^{TM} kit manual.

Cloning of the α-like octopamine receptor R0

To clone the *B. improvisus* α-like octopamine receptor, PCR primers R0-TM-I and R0-TM-VII, corresponding to regions in TM-I and TM-VII of the known *B. amphitrite* octopamine receptor, were used to amplify a fragment of a putative *B. improvisus* homolog. PCR was run on both genomic DNA (1 µg) and cDNA (50 ng) obtained from a pool of approximately 1000 cyprids, using PCR program P1. The resulting fragment was about 1100 bp in size.

The 5´ end was amplified by an initial PCR using primer R0-5´ together with primer GeneRacer 5´ and running PCR program P2. The subsequent nested PCR was run using primer R0-5´_n together with the GeneRacer 5´ nested primer and applying PCR program P3. The 3´ end was amplified by an initial PCR using primer R0-3´ together with the GeneRacer 3´ primer and applying program P4. The subsequent nested PCR was run using primer R0-3´_n together with the GeneRacer 3´ nested primer and applying program P5. Fragments of approximately 1800 bp and 1100 bp were obtained for the 5´ and 3´ ends, respectively.

To clone the full-length receptor, PCR program P6 was run on both genomic DNA and cDNA using primers R0-fl_fw and R0-fl_rev. Fragments of approximately 1500 bp were obtained. Sequence analysis showed that the cloned fragments contained an open reading frame for a receptor of 1470 bp with high similarity to the previously cloned α-like octopamine receptor from *B. amphitrite*.

Cloning of the β-like octopamine receptors R1 and R2

Molaei *et al*. previously used degenerate primers based on sequences conserved in TM-III and TM-VI of biogenic amine receptors to clone an Oct/Tyr receptor from the grasshopper *Locusta migratoria* (Molaei *et al.* 2005). We used the same primers for running PCR on 1 µg genomic DNA from *B. improvisus*. The PCR was run according to the temperature protocol of Molaei *et al*., except that the denaturing temperature during cycling was 95° C and the annealing temperature was 57° C. Fragments of approximately 800 bp were obtained. Twenty-nine clones were sequenced and 3 different receptor sequences were found. One was identical to the already cloned α-like receptor of *B. improvisus* and a BLAST search showed that the other two were most similar to β-like octopamine receptors and were named R1 and R2.

The 5´ ends of R1 and R2 were amplified by initial PCR reactions using the primer GeneRacer 5´ together with primers R1-5´ or R2-5´. The PCR program P4 was applied for both reactions. The subsequent nested PCR reactions were run using the GeneRacer 5´ nested primer together with primer R1-5´_n or primer R2-5´_n. PCR programs P7 and P8 were used for R1 and R2, respectively. Fragments of approximately 1200 bp for R1 and 1100 bp for R2 were obtained. The 3´ ends were amplified by initial PCR reactions using primer GeneRacer 3´ together with R1-3´ or R2-3´, applying

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PCR program P4. The subsequent nested PCR reactions were run using primer R1-3´_n or primer R2-3´_n, together with the GeneRacer 3´ nested primer. PCR programs P8 and P7 were used for R1 and R2, respectively. Fragments of about 800 bp for R1 and about 1100 bp for R2 were obtained.

To clone the full-length receptors, PCR was run on both genomic DNA and cDNA using PCR program P9. The primers used were R1-fl_fw and R1-fl_rev for R1 and primers R2-fl_fw and R2-fl_rev for R2. Fragments of approximately 1500 bp were obtained. Sequence analysis showed that the cloned fragments contained open reading frames for R1 or R2 of 1500 and 1494 bp, respectively.

Cloning of the β-like octopamine receptors R3 and R4

PCR was performed on 1 µg genomic DNA with degenerate primers annealing to conserved sequences within TM-VI and TM-VII of biogenic amine receptors. These primers were previously used by Smith *et al*. to clone a serotonin receptor from the nematode *Haemonchus contortus* (Smith *et al.* 2003). PCR was run using PCR program P10. A fragment of approximately 100 bp was obtained. Sequencing of 12 clones revealed three receptor gene fragments. A BLAST search showed that one was most similar to a dopamine receptor whereas the other two were most similar to β-like octopamine receptors. To obtain the sequences of the 5´ and 3´ ends of the two receptors most similar to β-like octopamine receptors, called R3 and R4, RACE was performed.

The 5´ ends of R3 and R4 were amplified by initial PCR reactions using the primer GeneRacer 5´ together with primers R3-5´ or R4-5´. For both reactions, PCR program P11 was applied. The subsequent nested PCR reactions were run using primer R3-5´_n or R4-5´_n together with the GeneRacer 5´ nested primer, applying PCR program P12. Fragments of approximately 1400 bp for R3 1200 bp for R4 were obtained.

The 3´ ends were amplified by initial PCR reactions using the primer GeneRacer 3´ together with primers R3-3´ and R4-3´, applying PCR program P11. The subsequent nested PCR reactions were run using primer R3-3´_n for R3 and primer R4-3´_n for R4 together with the GeneRacer 3´ nested primer. PCR program P12 was used. Fragments of about 1200 bp for R3 and 750 bp for R4 were obtained.

To clone the full-length receptors, PCR was run on cDNA using PCR program P13. The primers used were R3-fl_fw and R3-fl_rev for R3 and primers R4-fl_fw and R4-fl_rev for R4. Sequence analysis showed that the cloned fragments contained open reading frames for R3 or R4 of 1071 and 1638 bp, respectively.

Prediction of phosphorylation and glycosylation sites

Prediction of potential phosphorylation and glycosylation sites in the cloned *B. improvisus* octopamine receptors were made using the NetPhosK and NetNGlyc prediction programs on the CBS prediction servers (http://www.cbs.dtu.dk/services/)(Blom *et al.* 2004).

Construction of the 3D octopamine receptor/ligand structure model

A homology model of the R4 receptor was built based on the turkey β1-adrenoceptor structure ((Warne *et al.* 2008), pdb id 2vt4) using ICM (www.molsoft.com). The pairwise sequence alignment was extracted from a manually corrected multiple sequence alignment including the *B. improvisus* receptors and the sequences of several functionally characterized invertebrate and human biogenic amine receptors (Supplementary Figure S1 and Table S4). An octopamine model was introduced and repeatedly relaxed with selected receptor binding pocket side-chains flexible by means of Monte Carlo geometry optimizations in torsion space, using soft van der Waals potentials (4.0 kcal/mole cutoff) and other parameters at default values. Several solutions were used for automatic docking of a small library of compounds (including stereoisomers of octopamine and medetomidine) using ICM (www.molsoft.com, (Abagyan & Totrov 1994)). Residues in the TMs are referred to by residue number and the nomenclature of Ballesteros and Weinstein (Ballesteros & Weinstein 1995).

Octopamine is smaller than cyanopindolol that is present in the β1-adrenoceptor crystal structure, and the position of the docked octopamine differs from cyanopindolol mainly by leaving a region near TM-VII unoccupied. This region is blocked in the octopamine receptor model by F7.39, corresponding to N7.39 in the β1-adrenoceptor. In the crystallized β1- and β2-adrenoceptor structures (Rosenbaum *et al.* 2007, Rasmussen *et al.* 2007, Cherezov *et al.* 2007, Warne *et al.* 2008), the β-hydroxyl groups of cyanopindolol or carazolol form hydrogen bonds with asparagine N7.39, but corresponding hydrogen bonds cannot form with F7.39 of the octopamine receptor. Instead, two other residues are sufficiently close to potentially form direct hydrogen bonds in two different docking orientations: N3.29 and S3.36. Both positions are conserved within the β-like octopamine receptor group. We chose the model with the ligand hydrogen bonded to the N3.29 position for further analysis since the corresponding position has aliphatic, hydrophobic residues in tyramine receptors (tyramine lacks a hydroxyl group in the β-position).

Phylogenetic analysis of identified B. improvisus octopamine receptor clones

Translated sequences from the CDS of the *B. improvisus* clones R0-R4 were analyzed together with biogenic amine receptors from three insects *D. melanogaster*, *T. castaneum* and *A. mellifera*, the barnacle *B. amphitrite*, the molluscs *A. californica, A. kurodai* and *Spisula solidissima* and the crustacean *Daphnia pulex*. For *D. pulex*, the three biogenic amine receptors most similar to octopamine receptors were collected from the pre-release protein predictions available from JGI (Dappu1) (GNO_656044, GNO_630044, GNO_324094). These sequence data were produced by the US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/ in collaboration with the Daphnia Genomics Consortium http://daphnia.cgb.indiana.edu and annotated by Dr. Giuseppe Cazzamali (Department of Biology, University of Copenhagen). No other putative crustacean octopamine receptors have been reported to our knowledge.

In phylogenetic comparisons, two rhodopsin receptors from *D. melanogaster* (Dm_Rh4 and Dm_Rh6) were included to act as an outgroup. As the *A. mellifera* OctB3 seemed to be truncated at the C-terminus, thus missing the last TMs, an alternative sequence (ENSAPMP00000022772=Am_OctB-b), which had a complete C-terminus but a different N-terminus, was also added to the analysis.

Alignments were created with CLUSTALW (1.83) and manually corrected when needed, so that the TMs were aligned correctly. Several alignments were made: an alignment based on full-length sequences, an alignment without N- and C-termini, and alignments in which highly variable regions that were hard to align properly were removed, leaving mainly the TMs.

Phylogenetic trees were built using the maximum likelihood method. Trees were calculated using PhyML with both WAG and PAM options, bootstrap 100, employing the PhyML server 2.4 at http://atgc.lirmm.fr/phyml/. For comparison, trees were also generated with neighbour-joining (PHYLIP package v. 3.57) or using Bayesian inference of phylogeny (MrBayes, v. 3.1.2), with similar results

To make a phylogenetic analysis based on ligand-interacting residues only, all protein side-chain atoms within 5 Å from octopamine in the R4 receptor homology model were identified using ICM (Molsoft LLC, www.molsoft.com). Twenty-one binding site residues were selected. The corresponding twenty-one amino acids in functionally characterized biogenic amine receptors were extracted from the multiple sequence alignment created when constructing the octopamine receptor 3D model. Three of these residues, belonging to the EC loop 2, were hard to align properly and were therefore removed. The selection radius was generously set to 5 Å to minimize the risk that binding site residues were excluded. A phylogenetic tree based on the predicted ligand binding pocket of eighteen amino acids was constructed using PhyML with the WAG option. Bovine rhodopsin was used as an outgroup.

Expression of cloned receptors in CHO cells

The *B. improvisus* receptors were subcloned from pCR®4Blunt-TOPO® into the XbaI and HindIII sites of the pcDNA3.1(-) expression vector (Invitrogen), and subsequently linearized utilizing the SspI restriction site present in the plasmid. The resulting fragments were purified using the NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany).

Stable transfections were performed using the commercial FuGENE® HD transfection kit (Roche Applied Science, Indianapolis, IN, USA) with slight modifications to the manufacturer's instructions. Briefly, 2 µg of the linearized plasmid were added onto 30-40 % confluent CHO cell cultures in 6-well plates together with 5 µl of FuGENE® HD transfection reagent. Water (10 µl) was used instead of plasmid DNA in control reactions. The cells were at this stage cultured in serum- and antibiotic-free α-MEM medium (α-Minimum Essential Medium, GIBCO™, Invitrogen). Cells were maintained at 37° C in a humidified atmosphere containing 5 % CO_2. After 24 h recovery in an incubator, the

transfected cell cultures were washed with phosphate-buffered saline (PBS), and fresh α-MEM supplemented with 26 mM $NaHCO_3$, 50 IU/ml penicillin, 50 µg/ml streptomycin and 5 % heat-inactivated fetal bovine serum (FBS) was added. Selection of transfected cells was started 48 h after the transfection by adding 800 µg/ml of the neomycin analogue G418 (Geneticin®) (Sigma-Aldrich).

Once G418-induced cell death was evident in the mock-transfected control cell culture wells, the transfected cell cultures were transferred from the 6-well plates into 75 cm^2 plastic culture flasks containing 20 ml of α-MEM supplemented with 600 µg/ml G418. Cells were grown until 90 % confluent, detached from the culture flasks with 0.25 % trypsin/0.02 % EDTA, centrifuged at 1500 rpm for 5 min at 4° C and suspended into 2 ml of FBS supplemented with 7.5 % DMSO. The resulting cell ampoules were stored frozen in liquid nitrogen until used in experiments. For subsequent experiments, transfected CHO cells were revived from liquid nitrogen and cultured in α-MEM supplemented with 600 µg/ml G418.

Ligands

Octopamine, tyramine, dopamine and histamine were purchased from Sigma-Aldrich. Medetomidine was purchased from Fermion (Finland) and atipamezole was kindly given as a gift from Fermion. [^{3}H]RS79948-197 was from Amersham (Buckinghamshire, UK).

cAMP assay

The cAMP assay was run according to the LANCE kit instructions (PerkinElmer, Life and analytical sciences, Waltham, MA, USA). Briefly, cells that had grown until confluence or near confluence in 75 cm^2 flasks were detached by adding 6 ml Versene solution (Invitrogen). Thereafter 5 ml PBS was added, cells were spun at 1500 rpm for 3-5 min, washed with 5 ml HBSS and suspended in 1-2 ml stimulation buffer to a density of 600000 cells/ml. Finally, the Lance cAMP antibody was diluted 1/100 in the cell suspension. Stimulation of cAMP production was performed by adding the cell/antibody suspension to different concentrations of ligand in a 96-well plate and incubating for 45 minutes. Then 20 µl of detection mix was added and the plate was incubated for 2 h before reading the Lance counts at 615 nm in a Victor 2 instrument (PerkinElmer). In each assay, two concentrations of forskolin alone, 100 nM and 100 µM, were used as positive control for adenylyl cyclase stimulation. When dose-response curves with R1, R3 and R4 were generated, the amount of stimulation was normalized against the stimulation obtained with 100 nM forskolin alone. For ligand-induced stimulation of R0, 100 nM forskolin was included in addition to the ligand and the stimulation was calculated as % of the stimulation by 100 nM forskolin alone.

Ca^{2+} measurements

CHO cells expressing R0 receptors and non-transfected control cells were plated on black 96-well polystyrene plates (PerkinElmer). 48 h later, the FLIPR Calcium 4 Assay Kit (Molecular Devices,

Sunnyvale, CA, USA) was reconstituted in Hepes-buffered medium (137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose, 2.5 mM probenecid and 20 mM Hepes, pH 7.4. Cell culture medium was removed so that 50 µl was left in each well, 50 µl of the FLIPR medium was added, and the cells were incubated for 60 min at 37 °C. The plate was then placed inside a FlexStation automated fluorescence plate reader (Molecular Devices) and fluorescence was monitored at 37 °C at two wavelengths, 485 nm (excitation) and 525 nm (emission). Each well was measured for 230 s. Agonists were added at 30 s. The experiments were conducted in quadruplicate and repeated 3-5 times. Microsoft Excel was used for data analysis.

[^{35}S]GTPγS binding assay

Experiments were carried out using a MultiScreen Vacuum Manifold system (Millipore, Billerica, MA, USA) with Millipore MultiScreen MSFBN 96-well glass fiber filtration plates. Agonist-induced stimulation of [^{35}S]GTPγS binding was measured described previously (Peltonen *et al.* 1998): Membrane suspensions prepared by differential centrifugation of disrupted CHO cells were thawed and 5 µg of membrane protein per sample were added to the reaction buffer (final concentrations: 30 µM ascorbic acid, 25 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 20 mM NaCl, 1 µM GDP and 0.1 nM [^{35}S]GTPγS (pH 7.4) at room temperature (RT) in a total volume of 300 µl. The samples were incubated for 30 min at RT with 7-8 dilutions of the agonists. Reactions were terminated by rapid vacuum filtration, filters were rinsed (with ice-cold 20 mM Tris-HCl, 1 mM EDTA, 5 mM MgCl2, pH 7.4) air dried and impregnated with 50 µl of Super Mix scintillation cocktail (PerkinElmer Wallac). The bound radioactivity was determined with a Wallac 1450 Betaplate liquid scintillation counter.

Specific binding of [^{3}H]RS79948-197 to R0-expressing CHO cell membranes

Saturation binding experiments with the α_2-adrenoceptor antagonist radioligand [^{3}H]RS79948-197 were carried out for the octopamine receptor-transfected CHO cells (Fagerholm *et al.* 2004). Briefly, 20 µg of membrane protein were incubated with final concentrations of [^{3}H]RS79948-197 ranging from 0.125 to 16 nM in 50 mM K+-phosphate buffer (pH 7.4). After 30 min at RT, reactions were terminated using rapid filtration through glass fiber filters (Whatman GF/B). The filters were washed (50 mM Tris-HCl, 10 mM EDTA, pH 7.4), placed into scintillation vials with Optiphase “HiSafe” III (Perkin-Elmer Wallac), and the bound radioactivity was measured in a Wallac 1410 scintillation counter. Non-specific binding was determined with 10 µM phentolamine.

The B_{max} and K_d for R0 were approximately 2.4 pmol/mg membrane protein and 7.3 nM, respectively. The B_{max} and K_d of recombinant human α_{2A}-adrenoceptors expressed in CHO cells (Pohjanoksa *et al.* 1997), used as positive control, was 5.5 pmol/mg protein and 0.2 nM, respectively. No detectable binding of [^{3}H]RS79948-197 was observed for the R1-R4 receptors.

Quantitative real-time PCR analysis of differential gene expression

Q-PCR primers were preferably placed in regions with little or no sequence variation between the clones of a specific receptor, and when possible, in regions that are not conserved between the different receptor subtypes. For primer information see Table S3. To optimize the Q-PCR, the annealing temperature was initially chosen according to the primer melting temperature. Each Q-PCR reaction contained 50 ng cDNA, 12.5 µl iQ SYBR Green supermix (Bio-Rad), and 0.2, 0.5 or 0.8 µM primer in a total volume of 25 µl. The reaction was run on an iQ5 iCycler (Bio-Rad), using an initial denaturing temperature of 95° C for 30 s, followed by 40 cycles of 95° C for 20 s, an annealing temperature specific for each primer for 20 s and elongation at 72° C for 30 s. After 40 cycles, a melting curve was created to ensure that only one PCR product was obtained and product size was checked by gel electrophoresis. If the sample containing no cDNA (negative control) did not give any product and the melting curves indicated one pure product for the different primer concentrations, this temperature was used. If not, the procedure was repeated at other temperatures until the desired result was obtained (see Table S3 for the used optimized temperatures for each primer pair). The reaction efficiencies (*E*) were determined for each primer and calculated according to the equation: $E = 10^{(-1/slope)}$. Efficiencies of 100±5 % were accepted. The mRNA expression was normalized to actin mRNA levels for each sample and calculated using threshold cycle values (C_t) and the equation: $Ratio\ (reference/target) = 2^{CT\ (reference) - CT\ (target)}$.

Since the now identified five GPCRs from *B. improvisus* have large sequence similarities, experiments were performed to ensure that the primer pairs only amplified the receptor it was designed for. Each primer pair was combined with each of the five receptors' cDNA as template (cloned into a plasmid) and Q-PCR was run. Plasmid concentrations from 0.008 to 25 ng per reaction were used. There was a difference of 10-15 cycles when comparing the specific binding of a primer pair to the receptor cDNA it was designed for, with the binding to the other receptor cDNAs. Thus, all primer pairs were shown to be specific for their target cDNA.

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Figure S1

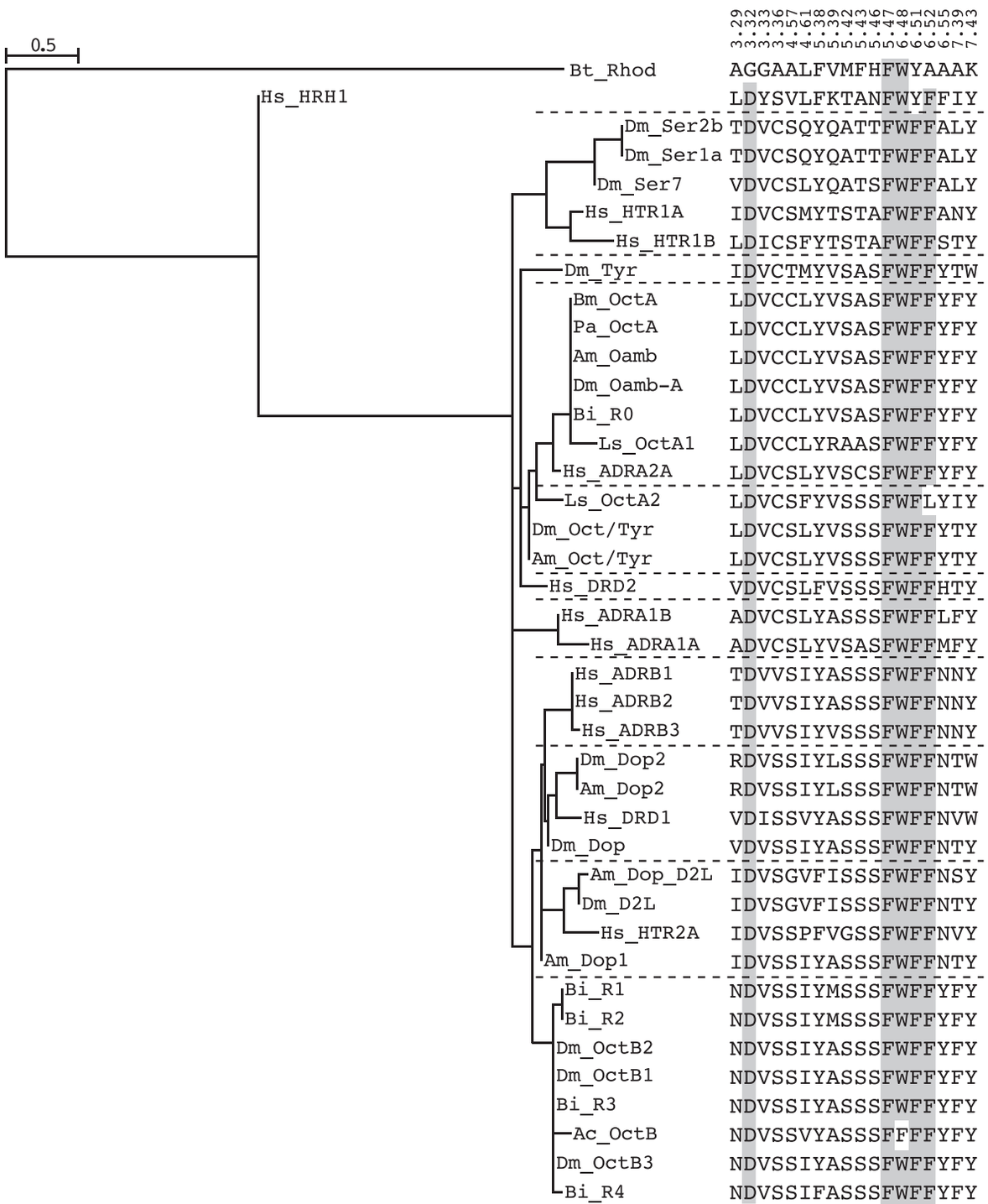

Figure S1. Phylogenetic tree based on sequence alignments of putative ligand-binding residues in functionally characterized biogenic amine receptors. A phylogenetic maximum likelihood tree, using PhyML, was built based on 18 putative ligand-binding residues, identified from the 3D model, in selected functionally characterized biogenic amine receptors from the species B. improvisus (Bi), D. melanogaster (Dm), A. mellifera (Am), A. californica (Ac), A. kurodai (Ak), L. stagnialis (Ls), Periplaneta americana (Pa) and Homo sapiens (Hs). Rhodopsin from Bos taurus (Bt) was used as an outgroup. For accession numbers to the used sequences see Table S4. Horizontal lines in the alignment are shown to aid interpretation, and roughly coincide with the sub-family groups. Positions with more than 90 % identity are shaded.

Lind, U., Rosenblad M.A., Hasselberg-Frank, L., Falkbring, S., Brive, L., Laurila, J.M., Pohjanoksa, K., Vuorenpää, A., Kukkonen, J.P., Gunnarsson, L., Scheinin, M., Mårtensson Lindblad, L.G.E., Blomberg, A., Octopamine receptors from the barnacle Balanus improvisus are activated by the α2-adrenoceptor agonist medetomidine, Molecular Pharmacology, Mol#63594

Table S1. Primers for cloning of the *B. improvisus* octopamine receptors. Name and sequence for the primers used in cloning of the *B. improvisus* octopamine receptors are shown. All primers are written in the 5´ to 3´direction.

Primer name	Primer sequence
R0-TM-I	AATTCCGGCAACCTGCTGGTCGTGAT
R0-TM-VII	CTAGCGAGTTGCAGTAGCCCAACCAG
R0-5´	CAGCCAGACGCTGCACCACACGT
R0-5´_n	GCACGCCAGCGACACGATGAACA
R0-3´	GGCTGCCGTTCTTCGTGTGCTAC
R0-3´_n	GCGGCTTCTGCGCCGACTGCGT
R0-fl_fw	GCTGTGTAGAGCTGTGACTGAC
R0-fl_rev	GCCGGACTGGACTCCTGCTC
R1-5´	CCCAGAAAGATGGGCACGAACGAT
R1-5´_n	GGCCACGGTGCGCTTGTTCATGT
R1-3´	CTCCCGCAAGGCGTCGACCAACT
R1-3´_n	CGCTACTCGACGAGCCAGATGCT
R1-fl_fw	GGCACTTCTGACTGGAGCTGAT
R1-fl_rev	CACGTGGGATGAGGTCAGCTC
R2-5´	CCAGGAAGATCGGCACGAAGGAGAT
R2-5´_n	CGCCACGGTGCGCTTGTTCATGT
R2-3´	GACCTGCGGAAGTCGTCCAACAC
R2-3´_n	CGCTTCTCCACCAGTCACATGCT
R2-fl_fw	TCACGTGTCCGGCGGACCAT
R2-fl_rev	CAGCAGCAGCACGAGGTCCTA
R3-5´	CCACCACGTCTGGGCAGGGACA
R3-5´_n	CGGCACAGGCTGACCGTGATGTA
R3-3´	CAGCCTGTGCCGCGAGGCCTGT
R3-3´_n	GATCGGCTACCTCAACTCGTGTCT
R3-fl_fw	GTGAAGATGTCTCTCGAGGAACT
R3-fl_rev	GCCTGGCTTTGCGCGGATTTC
R4-5´	CCAGAACAGCAGCGACTCCACAAT
R4-5´_n	GAGTCGCAGAGGCTGACGATGAT
R4-3´	TCTGGTACATCATCGTCAGCCTCT
R4-3´_n	GCTGCTGTTCTGGATTGGGTATTTC
R4-fl_fw	GAGTCGCCGCGCTGATAGTCT
R4-fl_rev	TGTCGGTACATGGCACCTGAG

Lind, U., Rosenblad M.A., Hasselberg-Frank, L., Falkbring, S., Brive, L., Laurila, J.M., Pohjanoksa, K., Vuorenpää, A., Kukkonen, J.P., Gunnarsson, L., Scheinin, M., Mårtensson Lindblad, L.G.E., Blomberg, A., Octopamine receptors from the barnacle Balanus improvisus are activated by the α2-adrenoceptor agonist medetomidine, Molecular Pharmacology, Mol#63594

Table S2. PCR programs for cloning of the *B. improvisus* octopamine receptors. The PCR programs used in the RACE reactions and during the cloning of the full-length receptors are displayed. Programs P2, P4 and P11 are touchdown PCRs according to the GeneRacer™ kit manual (Invitrogen), where the annealing step of 65° C is excluded in the first ten 10 cycles. The first 5 cycles are run with a combined elongation/annealing step at 72° C, followed by 5 cycles of 70° C. The next 25 cycles are run with an annealing step at 65° C and an elongation temperature of 72° C.

Program name	Initial denat.	Denat .	Annealing	Elong.	cycles	Final elong.
P1	98°C, 2 min	98° C, 30 s	55° C, 30 s	72° C, 2 min	35	72° C, 4 min,
P2 (touchdown)	98°C, 2 min	98° C, 30 s	65° C, 30 s Excluded in the first 10 cycles	70° C, 72° C 72° C, 2 min	35 (5+5+25)	72° C, 10 min
P3	98°C, 2 min	98° C, 30 s	65° C, 30 s	72° C, 2 min	35	72° C, 10 min
P4 (touchdown)	98°C, 2 min	98° C, 30s	65° C, 30 s Excluded in the first 10 cycles	70° C, 72° C 72° C, 1 min	35 (5+5+25)	72° C, 10 min
P5	96°C, 2 min	96° C, 30 s	55° C, 30 s	72° C, 2 min	35	72°C, 10 min
P6	98°C, 2 min	98° C, 30 s	60° C, 30 s	72° C, 2 min	35	72°C, 10 min
P7	98°C, 2 min	98° C, 30 s	62° C, 30 s	72° C, 2 min	35	72°C ,10 min
P8	98°C, 2 min	98° C, 30 s	64° C, 30 s	72° C, 2 min	35	72°C ,10 min
P9	98°C, 2 min	98° C, 30 s	62° C, 45 s	72° C, 2 min	35	72°C, 10 min
P10	95°C, 2 min	95° C, 30 s	48° C, 45 s	72° C, 1 min	36	72°C, 10 min
P11 (touchdown)	98°C, 2 min	98° C, 30s	65° C, 30s Excluded in the first 10 cycles	70° C, 72° C 72° C, 2.5 min	35 (5+5+25)	72°C, 10 min
P12	98° C, 2 min	98° C, 30 s	62° C, 30 s	72° C, 2.5 min	35	72 °C, 10 min
P13	98° C ,2 min	98° C, 30 s	59° C, 45 s	72° C, 2 min	35	72° C, 10 min

Lind, U., Rosenblad M.A., Hasselberg-Frank, L., Falkbring, S., Brive, L., Laurila, J.M., Pohjanoksa, K., Vuorenpää, A., Kukkonen, J.P., Gunnarsson, L., Scheinin, M., Mårtensson Lindblad, L.G.E., Blomberg, A., Octopamine receptors from the barnacle Balanus improvisus are activated by the α2-adrenoceptor agonist medetomidine, Molecular Pharmacology, Mol#63594

Table S3. Primers for QPCR. Sequence, concentration, localization and annealing temperature of the primers, as well as amplicon length, are shown. TM=transmembrane region, EC=extracellular loop, IC=intracellular loop.

Primer pair	Product length bp	Sequence	Location	Annealing temp. °C	Conc. μM
R0	69	Fw: CGTCACCAACCTGTTCATCG Re: AACGGCAGCACCAGCATC	TM2	59	0.2
R1	86	Fw GGTGGTACACGTACCAGTC Re: GAGCATGTAGGTGCGATTG	EC2	55	0.5
R2	57	Fw: CCGCTGGCAGTGGTAGTG Re: CGAGAGGGCGTTCAGTAGC	C-term.	56.5	0.8
R3	80	Fw: GCCAAGTTCCTCTACAAG Re: TTACGACTCGGCGATATG	IC3	56.5	0.8
R4	90	Fw: GCAGATGCTCGTCACGCAAAAC Re: CGGCGGCTGTGAGAGTGC	IC3	62	0.5
Actin	93	Fw: CATCAAGATCAAGATCATCGC Re: ATCTGCTGGAAGGTGGAC	N/A	59	0.8

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Table S4.
Accession numbers and annotations for the sequences used in the phylogenetic analysis.

ID	Accession nr and description
Tc_OctB3	XP_974238.2| PREDICTED: similar to Octopamine receptor beta-3R (DmOct-beta-3R) [Tribolium castaneum]
Tc_OctB2	XP_974214.1| PREDICTED: similar to beta adrenergic receptor [Tribolium castaneum]
Tc_OctB1	XP_974265.1| PREDICTED: similar to GA19956-PA [Tribolium castaneum]
Tc_Oamb	XP_970007.2| PREDICTED: similar to octopamine receptor [Tribolium castaneum]
Tc_Oct/Tyr	XP_970290.1| PREDICTED: similar to monophenolic amine tyramine [Tribolium castaneum]
Tc_Tyr	XP_001811970.1| PREDICTED: similar to putative GPCR class a orphan receptor 4 (AGAP004034-PA) [Tribolium castaneum]
Tc_Dop2	XP_972779.2| PREDICTED: similar to dopamine receptor 2 [Tribolium castaneum]
Tc_Dop1	XP_971542.2| PREDICTED: similar to dopamine receptor I [Tribolium castaneum]
Tc_Dop	XP_969037.2| PREDICTED: similar to Dopamine 2-like receptor CG33517-PA [Tribolium castaneum]
Tc_Ser7	XP_966577.2| PREDICTED: similar to serotonin receptor 7 [Tribolium castaneum]
Tc_Ser-a	XP_967449.2| PREDICTED: similar to serotonin receptor [Tribolium castaneum]
Tc_Ser-b	XP_972856.2| PREDICTED: similar to putative serotonin receptor [Tribolium castaneum]
Tc_Ser2a	XP_972327.1| PREDICTED: similar to putative serotonin 5HT-2a receptor (AGAP002232-PA) [Tribolium castaneum]
Tc_DopEc	XP_968380.1| PREDICTED: similar to DopEcR CG18314-PA [Tribolium castaneum]
Am_OctB3-b	(ENSAPMP00000022772, AMEL2.0.feb.pep)
Am_OctB3	XP_397077.3| PREDICTED: similar to Octopamine receptor beta-3R (DmOct-beta-3R) [Apis mellifera]
Am_OctB2	XP_396348.3| PREDICTED: similar to Octopamine receptor beta-2 (DmOct-beta-12) [Apis mellifera]
Am_OctB1	XP_397139.2| PREDICTED: similar to octopamine receptor 2 CG6919-PA, isoform A [Apis mellifera]
Am_Oamb	NP_001011565.1| octopamine receptor [Apis mellifera]
Am_Oct/Tyr	NP_001011594.1| G-protein coupled receptor [Apis mellifera]
Am_Tyr	XP_394231.2| PREDICTED: putative tyramine receptor [Apis mellifera]
Am_Dop2	NP_001011567.1| dopamine receptor type D2 [Apis mellifera]
Am_Dop-D2L	NP_001014983.1| D2-like dopamine receptor [Apis mellifera]
Am_Dop1	NP_001011595.1| dopamine receptor, D1 [Apis mellifera]
Am_Ser2	XP_394798.1| PREDICTED: similar to Serotonin receptor 2 CG1056-PB, isoform B [Apis mellifera]
Am_Ser7	NP_001071289.1| serotonin receptor 7 [Apis mellifera]
Am_Ser4	XP_393915.3| PREDICTED: similar to SERotonin/octopamine receptor family member (ser-4) [Apis mellifera]
Am_DopEc	XP_396491.1| PREDICTED: similar to DopEcR CG18314-PA, isoform A isoform 1 [Apis mellifera]
Dm_Oamb-A	NP_732541.1| Octopamine receptor in mushroom bodies CG3856-PA, isoform A [Drosophila melanogaster]
Dm_Oamb-B	NP_524669.2| Octopamine receptor in mushroom bodies CG3856-PB, isoform B [Drosophila melanogaster]
Dm_OctB1	NP_651057.1| octopamine receptor 2 CG6919-PA, isoform A [Drosophila melanogaster]
Dm_OctB3	NP_001034043.1| Octbeta3R CG33959-PD, isoform D [Drosophila melanogaster]
Dm_OctB2	NP_001034049.1| Octbeta2R CG33976-PA [Drosophila melanogaster]
Dm_Oct/Tyr	NP_524419.2| Tyramine receptor CG7485-PA [Drosophila melanogaster]
Dm_Tyr	NP_650652.1| CG7431 CG7431-PA [Drosophila melanogaster]
Dm_DopEc	NP_647897.2| DopEcR CG18314-PA, isoform A [Drosophila melanogaster]
Dm_Dop	NP_477007.1| Dopamine receptor CG9652-PA [Drosophila melanogaster]
Dm_Dop2	NP_733299.1| Dopamine receptor 2 CG18741-PA, isoform A [Drosophila melanogaster]
Dm_Ser7	NP_524599.1| Serotonin receptor 7 CG12073-PA [Drosophila melanogaster]
Dm_Ser2b	NP_523789.3| Serotonin receptor 1B CG15113-PA [Drosophila melanogaster]
Dm_Ser2	NP_524223.2| Serotonin receptor 2 CG1056-PA, isoform A [Drosophila melanogaster]
Dm_Ser1a	NP_476802.1| Serotonin receptor 1A CG16720-PA, isoform A [Drosophila melanogaster]
Bi_R0	GU074418
Bi_R1	GU074419
Bi_R2	GU074420
Bi_R3	GU074421
Bi_R4	GU074422
Ac_OctB	AAF37686.1|AF222978_1 octopamine receptor [Aplysia californica]
Ak_OctB	AAF28802.1|AF117654_1 octopamine receptor [Aplysia kurodai]
Ss_OctB	AAL23575.1| putative G-protein coupled receptor [Spisula solidissima]

continued

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Table S4 continued

ID	Accession nr and description
Dp_OctA	GNO_656044 (DappuDraft_42083)
Dp_OctB	GNO_630044 (DappuDraft_23347)
Dp_Dop2	GNO_324094 (DappuDraft_22045)
Ba_OctA	Q93126|GPR9_BALAM Probable G-protein coupled receptor No9
Ba_Oct/Tyr	Q93127|GPR18_BALAM Probable G-protein coupled receptor No18
Dm_Rh4	NP_476701.1| Rhodopsin 4 CG9668-PA [Drosophila melanogaster]
Dm_Rh6	NP_524368.3| Rhodopsin 6 CG5192-PB [Drosophila melanogaster]
Ls_OctA1	O77408|OAR1_LYMST Octopamine receptor 1 (OA1) [Lymnaea stagnalis]
Ls_OctA2	01670|OAR2_LYMST Octopamine receptor 2 (OA2) [Lymnaea stagnalis]
Pa_OctA	AAP93817.1| octopamine receptor [Periplaneta americana]
Bm_OctA	08JR9|Q08JR9_BOMMO Octopamine receptor [Bombyx mori]
Dm_D2L	Q8IS44|DRD2L_DROME Dopamine D2-like receptor [Drosophila melanogaster]
Hs_HTR2A	P28223|5HT2A_HUMAN 5-hydroxytryptamine receptor 2A
Hs_HTR1A	P08908|5HT1A_HUMAN 5-hydroxytryptamine receptor 1A
Hs_HTR1B	P28222|5HT1B_HUMAN 5-hydroxytryptamine receptor 1B
Hs_ADRA2A	P08913|ADA2A_HUMAN Alpha-2A adrenergic receptor
Hs_DRD2	P14416|DRD2_HUMAN D(2) dopamine receptor
Hs_ADRA1A	P35348|ADA1A_HUMAN Alpha-1A adrenergic receptor
Hs_ADRA1B	P35368|ADA1B_HUMAN Alpha-1B adrenergic receptor
Hs_ADRB3	P13945|ADRB3_HUMAN Beta-3 adrenergic receptor
Hs_ADRB1	P08588|ADRB1_HUMAN Beta-1 adrenergic receptor
Hs_ADRB2	P07550|ADRB2_HUMAN Beta-2 adrenergic receptor
Hs_DRD1	P21728|DRD1_HUMAN D(1A) dopamine receptor
Hs_HRH1	P35367|HRH1_HUMAN Histamine H1 receptor
Bt_Rhod	1u19_a| Crystal Structure Of Bovine Rhodopsin At 2.2 Angstroms Resolution

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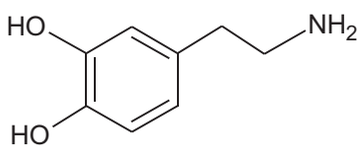
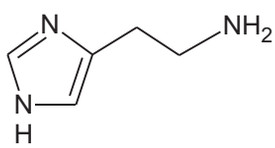
Structures of employed agonist ligands



Dopamine



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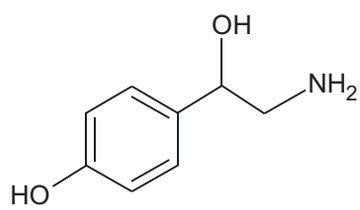
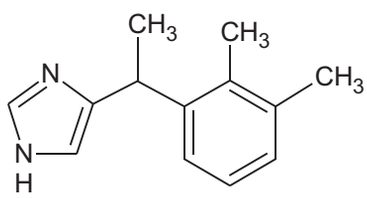
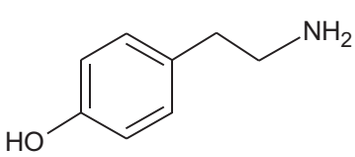


Octopamine



Medetomidine



Tyramine