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Octopamine receptors from the barnacle *Balanus improvisus* are activated by the α_2-adrenoceptor agonist medetomidine

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Running title: Octopamine receptors from *Balanus improvisus*

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Abbreviations used are:

GPCR, G protein-coupled receptor; α-like, α-adrenoceptor like; β-like, β-adrenoceptor like; Oct/Tyr, octopamine/tyramine; TM, transmembrane helix; IC loop, intracellular loop; EC loop, extracellular loop

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ABSTRACT

G protein-coupled octopamine receptors of insects and other invertebrates represent counterparts of adrenoceptors in vertebrate animals. The α_2-adrenoceptor agonist medetomidine, which is in clinical use as a veterinary sedative agent, was discovered to inhibit the settling process of barnacles, an important step in the ontogeny of this crustacean species. Settling of barnacles onto ship hulls leads to biofouling that has many harmful practical consequences, and medetomidine is currently under development as a novel type of antifouling agent. We now report that medetomidine induces hyperactivity in the barnacle larvae to disrupt the settling process. In order to identify the molecular targets of medetomidine, we cloned five octopamine receptors from the barnacle *Balanus improvisus*. We show by phylogenetic analyses that one receptor (BiOctα) belongs to the α-adrenoceptor-like and the other four (BiOctβ-R1, BiOctβ-R2, BiOctβ-R3 and BiOctβ-R4) to the β-adrenoceptor-like octopamine receptor subfamily. Phylogenetic analyses also indicated that *B. improvisus* has a different repertoire of β-adrenoceptor-like octopamine receptors than insects. When expressed in CHO cells, the cloned receptors were activated by both octopamine and medetomidine, resulting in increased intracellular cAMP or calcium levels. Tyramine activated the receptors, but with much lesser potency than octopamine. A hypothesis for receptor discrimination between tyramine and octopamine was generated from a homology 3D model. The characterization of *B. improvisus* octopamine receptors is important for a better functional understanding of these receptors in crustaceans, as well as for practical applications in development of environmentally sustainable antifouling agents.

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INTRODUCTION

Biofouling of ship hulls has many harmful consequences, e.g. increased fuel consumption and carbon dioxide emissions. Barnacles, a group of species of the subphylum Crustacea, are the most problematic biofouling marine organisms. *B. improvisus* is the barnacle species causing the most severe fouling problems in the Baltic Sea, Kattegat and Skagerrak, whereas *B. amphitrite* is more dominant in tropical waters. During the last pelagic stage of the barnacle life cycle (the cyprid), the larvae explore solid surfaces with their antennules in order to find a suitable site for settling and subsequent metamorphosis into a juvenile sessile barnacle. Antifouling marine paints currently on the market contain toxic heavy metal additives to inhibit cyprid larval settlement, which impose negative consequences on the marine environment. Thus, there is currently a great need to develop new environmentally sustainable antifouling substances.

The α_2-adrenoceptor agonist medetomidine was recently discovered to be capable of efficiently inhibiting the settling process of the barnacles *B. improvisus* and *B. amphitrite* already at non-lethal nanomolar concentrations (Dahlström and Elwing, 2006; Dahlström et al., 2000). Panels coated with medetomidine formulated in an acrylate polymer reduced the recruitment of *B. improvisus* to the surface by 96% after 4 weeks of exposure in Swedish waters (Dahlström et al., 2000). Medetomidine is previously known as a pharmacologically active substance in vertebrates, i.e. it is an α_2-adrenoceptor agonist that is used as a sedative agent in veterinary medicine (Savola et al., 1986; Sinclair, 2003). The mechanism for the inhibitory effect of medetomidine on settling of barnacles is not known, but is assumed to be mediated by G protein-coupled octopamine receptors (GPCRs). However, the evidence to support this is so far indirect: i) medetomidine acts on adrenoceptors in vertebrates, and octopamine serves analogous functions in invertebrates to those of adrenaline and noradrenaline in vertebrates (Roeder, 1999), and ii) clonidine, another α_2-adrenoceptor agonist, has been shown to bind to and activate octopamine receptors in other invertebrate species than barnacles (Gerhardt et al., 1997a; Gerhardt et al., 1997b; Maqueira et al., 2005).

Octopamine receptors of insects and other invertebrates are members of the GPCR superfamily and resemble other biogenic amine receptors, which in addition to octopamine and adrenergic receptors also include receptors for serotonin, dopamine, histamine and acetylcholine. The octopamine receptors

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of the fruit fly *Drosophila melanogaster* have been characterized and classified into α-adrenoceptor-like (α-like), β-adrenoceptor-like (β-like) and octopamine/tyramine (Oct/Tyr) receptors, based on their similarity with the vertebrate adrenoceptors. The Oct/Tyr receptors bind both octopamine and tyramine, but with a preference for tyramine. One α-like, three β-like and one Oct/Tyr receptor have been identified in the fruit fly (Evans and Maqueira, 2005). A similar set of octopamine receptor genes has subsequently also been found in other insects whose whole genomes have been sequenced, e.g. *Tribolium castaneum* and *Apis mellifera* (Hauser et al., 2006; Hauser et al., 2008). Relatively little sequence information is available for the octopamine receptor family from other invertebrates than insects and only a few non-insect receptors have been cloned and functionally characterized. Two putative octopamine receptors from the barnacle *B. amphitrite* have been cloned, but not characterized (Isoai et al., 1996; Kawahara et al., 1997); one is an α-like receptor and the other was initially classified as a serotonin receptor, but was later shown to be more similar to Oct/Tyr receptors (Evans and Maqueira, 2005).

In order to learn more about the octopamine receptor family in *B. improvisus* and to better understand the molecular mechanisms of the inhibitory effect of medetomidine on settling, we have cloned and functionally characterized five octopamine receptor family members of this biofouling species. Phylogenetic analysis clearly indicated that four of them belong to the β-like octopamine receptor family, while one is of the α-like type. Interestingly, some of the members were more highly expressed in the cyprid stage as compared to adults. Most importantly from an antifouling perspective, all barnacle octopamine receptors were activated by the antifouling substance medetomidine.

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MATERIALS AND METHODS

For a full description of the materials and methods, see the supplementary information.

Rearing of cyprids and cyprid motility assay

Cypris larvae of *B. improvisus* were produced and reared in a laboratory culture of adult individuals. Cyprid motility was measured by immobilisation of the cyprids in agarose gel and monitoring of the number of leg-kicks under a microscope.

Nucleic acid preparation and cDNA synthesis

RNA or genomic DNA was prepared from the barnacles using the Qiagen RNeasy mini-kit (Qiagen, Valencia, CA, USA) and the E.Z.N.A Blood DNA Kit (Omega Bio-Tek, Norcross, GA, USA), respectively. RNA was used as template for cDNA synthesis using either the iScript kit (BioRad, Hercules, CA, USA) or the SuperscriptIII first strand kit (Invitrogen Life Technologies Inc., Rockville, MD, USA). The genomic DNA and cDNA were then used for cloning of octopamine receptors and for mRNA expression analysis using real-time PCR.

Cloning of the B. improvisus octopamine receptors

To clone the octopamine α-like receptor R0, PCR primers corresponding to regions in transmembrane helices I and VII of the known *B. amphitrite* octopamine receptor were used to amplify a fragment of a putative *B. improvisus* homolog. Fragments of the β-like octopamine receptors R1-4 were cloned using two different degenerate primer pairs annealing to conserved regions in biogenic amine receptors. RACE using the GeneRacer^{TM} kit (Invitrogen) was used to clone the full-length receptors.

Homology modelling and phylogenetic analysis

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A homology model of the *B. improvisus* receptors was built based on the turkey β_1-adrenoceptor structure ((Warne et al., 2008), pdb id: 2vt4). This model was used to identify putative ligand-interacting residues in our barnacle octopamine receptors.

In order to identify potential orthologues of the cloned *B. improvisus* octopamine receptors, phylogenetic trees were built using the maximum likelihood method. Receptor sequences of various lengths, or binding-pocket residues alone, were used.

Expression of cloned receptors in CHO cells and second messenger assays

To functionally characterize the cloned octopamine receptors, receptor cDNAs were stably transfected into CHO cells. Ligand stimulation of cAMP production was monitored using the LANCE kit (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). For the alpha-like receptor R0, ligand stimulation of calcium elevation was measured using the FLIPR Calcium 4 Assay Kit and FlexStation fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). Membrane preparations from transfected CHO cells were used to investigate binding of the α_2-adrenoceptor antagonist [^{3}H]RS79948-197 and in the case of R0, also to monitor possible activation of G_i-type G proteins by measuring agonist-stimulated binding of [^{35}S]GTPγS.

Real-time PCR

Real-time PCR was performed to compare the mRNA expression of the *B. improvisus* receptors in cyprids and adults. PCR reactions containing cDNA, receptor specific primers and iQ SYBR Green supermix (Bio-Rad) were run on an iQ5 iCycler (Bio-Rad).

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RESULTS

Medetomidine activates a kicking response in cyprids

The α_2-adrenoceptor agonist medetomidine is known to result in sedation and locomotor inhibition when given to mammals and fish (Ruuskanen et al., 2005; Sinclair, 2003). Thus, a working hypothesis was that the mode of action of medetomidine as an antifoulant is *via* a sedative action also on barnacles. However, the opposite was found since medetomidine (10 nM) strongly enhanced kicking of the cyprid larvae with more than 100 kicks per minute (Fig. 1). Thus, medetomidine elicits different physiological responses in vertebrates and invertebrates, i.e. sedation/locomotor inhibition in vertebrates and hyperactivity in barnacle cyprids. The medetomidine response in barnacles is most likely elicited via octopamine receptors that are functional counterparts of the vertebrate adrenoceptors. That the effect is receptor-mediated is supported by the finding that the α_2-adrenoceptor antagonist atipamezole (100 nM) suppressed the increased kicking activity elicited by medetomidine to near control levels (Fig. 1). To get a better molecular understanding of the physiological response of cyprids to medetomidine we thus set out to clone and characterize the barnacle octopamine receptor(s).

Cloning of five octopamine receptors from B. improvisus

An α-like octopamine receptor from the barnacle *B. amphitrite* was previously cloned by Isoai *et al*. (Isoai et al., 1996). In order to obtain the homologous receptor from *B. improvisus*, PCR-based cloning was carried out using primers corresponding to sequences in transmembrane helices (TMs) I and VII of the *B. amphitrite* receptor and using as templates either genomic DNA or cDNA from a pool of approximately 1000 cyprids. The cypris larvae were derived from a local Swedish west-coast population of adults maintained in a laboratory aquarium setting. Sequence analysis showed that the fragment obtained represented a TM-I to TM-VII region of a *B. improvisus* homolog of the *B. amphitrite* α-like octopamine receptor.

To obtain the full-length receptor cDNA, rapid amplification of cDNA ends (RACE) was performed to identify the sequences of the 5´ and 3´ ends. An open reading frame comprising 1470

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nucleotides was obtained from both genomic DNA and cDNA, and this receptor gene was termed R0. Comparison of the nucleotide sequences of the coding regions of six different R0 receptor clones revealed relatively high sequence variability. Pair-wise comparisons of the clones gave a nucleotide diversity of 2-3 % within the coding region (data not shown). In contrast, the amino acid sequences encoded by the six clones were much more conserved, with only three variable regions (Fig. 2A). The most N-terminal of these regions, located about ten amino acids downstream of the start methionine and consisting of five to nine amino acids, was the most variable. The other two variable regions are located in the long intracellular (IC) loop 3 between TM-V and TM-VI.

A comparison of the amino acid sequences of the most frequent variant of the *B. improvisus* R0 clones with the published sequence of the *B. amphitrite* α-like octopamine receptor showed that they are 90 % identical at the protein level (Fig. 2B). Interestingly, the earlier mentioned variable region in the N-terminus of the *B. improvisus* receptor has no counterpart in the shorter N-terminus of the *B. amphitrite* receptor. All other 33 amino acid differences between the two receptors are located outside of the TMs, indicating that the receptors might not be different in ligand binding that is believed to be encompassed mainly within the TM portions of the proteins.

To find more members of the *B. improvisus* octopamine receptor family, in addition to the initially cloned α-like receptor, degenerate PCR primers based on conserved sequences of biogenic amine receptors were used. Two different primer pairs were chosen that had previously enabled cloning of an Oct/Tyr receptor from the grasshopper *L. migratoria* and a serotonin receptor from the nematode *H. contortus* (Molaei et al., 2005; Smith et al., 2003). Twenty-nine clones were isolated using the first primer pair. These clones were sequenced and three different receptor sequences were found. One was identical to the already cloned α-like receptor R0 from *B. improvisus.* A BLAST search versus the NCBI non-redundant protein database showed that the other two variants were most similar to β-like octopamine receptors from various insect species. Of the clones obtained using the other primer pair, twelve were sequenced and also shown to contain three different receptor sequences. A BLAST search showed that one was most similar to dopamine receptors whereas the other two were most similar to β-like octopamine receptors.

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Analogously to the isolation of the full-length α-like R0 receptor, RACE was performed on the obtained fragments of β-like octopamine receptors. Two of the receptors, named R1 and R2, were cloned yielding open reading frames of 1500 and 1494 bp, respectively. Identical results were obtained using either cDNA or genomic DNA as template. The final two receptors clones, named R3 and R4, were cloned from cDNA and had open reading frames of 1071 and 1638 bp, respectively. Thus in total four different β-like octopamine receptor genes were identified. Amino acid sequence alignment of the four putative β-like octopamine receptors showed that they were quite similar, especially in their TMs (Fig. 3). The clones R1 and R2 exhibited greatest similarity with each other, with 85 % identical amino acids in their TMs and an overall identity of 63 % for the full-length proteins. Interestingly, R1 and R2 have considerably longer IC3 loops compared to both R3 and R4, and also compared to all other known β-like octopamine receptors. Another interesting structural feature is that R4 has an unusually long C-terminus compared to other cloned octopamine receptors.

Like in the case of the cloned *B. improvisus* α-like receptor R0, we found nucleotide sequence variation in the range of 2-3 % between different clones of each individual receptor gene, probably reflecting population heterogeneity (data not shown). Nucleotide sequence variation in the range 1-5 % is also seen for a number of other genes, e.g. actin and ribosomal components, identified in a cDNA library constructed from the same cyprid population of *B. improvisus* (Alm Rosenblad *et al*., unpublished data). Great genetic variability within populations has also previously been observed in other marine organisms, e.g. the seasquirt *Ciona savigny* (Small et al., 2007). The 2-3 % nucleotide differences between clones of one receptor should be contrasted to the difference between clones representing two different receptors, which is much greater. Of the cloned octopamine receptors, BiOctβ-R1 and BiOctβ-R2 were most similar to each other having a nucleotide diversity of approximately 23 %. We thus regard the variability observed among the individual clones of each of the five octopamine receptors as polymorphism of single genes, although it can not be totally excluded that some of the observed variation could relate to the existence of different but very similar receptor genes.

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Common GPCR sequence features

Analysis of the amino acid sequences of the five cloned *B. improvisus* receptors R0-R4 revealed that they share many common sequence features with other GPCRs. All receptors contained the DRY motif at the end of TM-III, which is well conserved among family A GPCRs and is believed to be involved in receptor activation (Rovati et al., 2007). All receptors contained consensus motifs for PKA and PKC phosphorylation that may be important for receptor desensitization. PKA motifs were found in all receptors in their IC3 loops and/or in their C-termini. Consensus motifs for phosphorylation by PKC were found in the IC2 and IC3 loops of the α-like receptor R0 and in the IC3 loop of R1 and R2. N-linked glycosylation sites were found in the N-termini of R0, R2 and R3 and in the second extracellular (EC) loop of R1. All receptors contained conserved cysteines in their EC loops 1 and 2, which have been suggested to stabilize receptor structure by forming disulfide bonds (Rader et al., 2004). All receptors also contained cysteines in their proximal C-terminal domains, which are possible targets for palmitoylation.

Search for orthologs

In the genome of *D. melanogaster*, a repertoire of three β-like octopamine receptors (DmOctβ1, DmOctβ2 and DmOctβ3) has been found, of which all have been cloned and pharmacologically characterized (Maqueira et al., 2005). An alignment of the full-length *B. improvisus* and *D. melanogaster* β-like octopamine receptors showed that all *B. improvisus* receptors were most similar to DmOctβ3 with an identity of 53-60 % (gaps in the alignments excluded) and a similarity level of 69-74 % (Table 1). In order to identify the *D. melanogaster* orthologs of the cloned *B. improvisus* β-like octopamine receptors, and in that way provide evidence for their evolutionary relationships and possible functional roles, a phylogenetic analysis using invertebrate biogenic amine receptors was performed including either i) N- and C-terminally trimmed (to TM-I and TM-VII, data not shown), ii) only highly conserved (mainly membrane spanning regions, data not shown) or iii) full-length sequences (Fig. 4). The R0 clone clustered with the group of α-like octopamine receptors, as expected,

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whereas R1-R4 clearly clustered with the group of β-like octopamine receptors. However, it was not possible to unambiguously map the *B. improvisus* receptors into the subgroups of the insect β1-, β2- and β3-like receptors, since they were sometimes found within either of the insect subgroups and sometimes found outside, depending on the phylogenetic analysis method and on the sequence regions of the receptors that were used. In addition to the *B. improvisus* receptors identified in this study and the two previously cloned receptors from *B. amphitrite*, the only crustacean for which octopamine receptor sequences are currently available is *Daphnia pulex*. In our phylogenetic studies, one of the putative *D. pulex* octopamine receptors clustered with the β-like receptor group and was usually found on the same branch as the *B. improvisus* R3 receptor.

Functional predictions based on 3D modelling of the ligand-binding pocket

The classification of receptors is most commonly based on the ligands that activate them or that activate a homolog. The conservation of receptor sequences between different species, however, is dependent on many functional and structural determinants, such as ligand binding, interactions with G-proteins and other proteins, modification sites, localization signals etc. Thus, sequence regions related to other functions might perturb a phylogenetic analysis targeting ligand specificity. Therefore, we also classified the receptors based on the putative binding pocket residues alone.

No octopamine receptor protein structure has been experimentally determined. However, related structures of the human β_2-adrenoceptor in complex with the inverse agonist ligand carozolol (Cherezov et al., 2007) and the turkey β_1-adrenoceptor in complex with the antagonist cyanopindolol (Warne et al., 2008) are available. In order to identify putative ligand-binding pocket residues in our *B. improvisus* octopamine receptors, the turkey β_1-adrenoceptor structure was used as a template to build 3D homology models of the *B. improvisus* β-like octopamine receptors. Several receptor-ligand complex models were then generated by the *in silico* docking of octopamine (Fig. 5A). Many of the receptor-ligand interactions that have been shown to be important for ligand binding in functional studies of biogenic amine receptors are present in the models. These include an aspartate in TM-III (D3.32), which is believed to interact with the protonated amine group of the ligand, one or more

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serines in TM-V that make hydrogen bonds with the hydroxyl groups of the catecholamine ring (S5.42, S5.43 and S5.46) and a cluster of aromatic amino acids in TM-VI that most likely interact with the aromatic ring of the ligand (W6.48, F6.51 and F6.52) (Shi and Javitch, 2002). In one of the models, where N3.29 made a hydrogen bond with the octopamine β-hydroxyl group, 18 residues in the TMs are within 5 Å of the docked ligand and were chosen to represent the binding pocket.

The corresponding 18 positions were extracted from a multiple sequence alignment including our barnacle receptors, the two structurally determined β-adrenoceptors mentioned above, and sequences of selected functionally characterized biogenic amine receptors from humans and various invertebrates. Within the α-like receptor group, all eighteen putative ligand-interacting amino acids were completely conserved between the insect octopamine receptors and the *B. improvisus* R0 receptor (Fig. 5B, upper part). The human α-adrenoceptors and the two octopamine receptors from the mollusc *L. stagnalis* differed at 1-5 positions as compared to the insect and the *B. improvisus* α-like octopamine receptors. The ligand-binding sites of the four *B. improvisus* β-like octopamine receptors R1-4 were highly similar to the characterized *D. melanogaster* receptors (Fig. 5B, lower part). R3 was identical to the *D. melanogaster* β-like receptors, whereas the ligand binding sites of R1, R2 and R4 differed from those only at one position. A phylogenetic analysis based on the eighteen putative ligand-interacting amino acids clearly separated the different classes of biogenic amine receptors into different clades (Fig. S1). As in the phylogenetic analyses of the full-length receptors, R1-R4 clustered in this binding pocket analysis with the β-like octopamine receptors, and R0 clustered with the α-like octopamine receptors, supporting their receptor family identity and functional classification.

Functional studies of the octopamine receptors

GPCRs can activate different cellular signaling pathways leading to modulation of e.g. cAMP and calcium levels. The previously cloned β-like octopamine receptors from *D. melanogaster* and *A. californica* have been shown to mediate stimulation of cAMP formation, whereas most of the cloned α-like octopamine receptors have been reported to signal both via increased cAMP production and increased intracellular calcium concentrations (Balfanz et al., 2005; Bischof and Enan, 2004; Chang et

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al., 2000; Gerhardt et al., 1997a; Grohmann et al., 2003; Han et al., 1998; Maqueira et al., 2005; Ohtani et al., 2006).

In order to confirm the phylogenetic analyses indicating that the cloned *B. improvisus* receptors belong to the octopamine receptor family and to investigate whether they are targets for the antifouling substance medetomidine, the five receptors were expressed in stably transfected CHO cells for functional characterization. Ligand-induced changes in cAMP concentrations were analyzed by treating the cells with 1 μM of either medetomidine, octopamine, tyramine, dopamine or histamine. The β-like receptors R1, R3 and R4 showed relatively strong cAMP responses to octopamine, medetomidine and tyramine, whereas the responses of cells expressing R2 were weaker, but still clearly detectable (Fig. 6A). For the α-like receptor R0, small but significant cAMP increases with medetomidine and octopamine were obtained, but only after co-administration of 100 nM forskolin that activates adenylyl cyclase to a submaximal level (Fig. 6B). No increases in cAMP levels were observed when octopamine, medetomidine and tyramine were added to non-transfected CHO cells, showing that the obtained responses were mediated by the expressed octopamine receptors (data not shown). Dopamine and histamine activated the receptors only to a small extent or not at all (Fig. 6A and 6B). We conclude that the β-like receptors were clearly activated by medetomidine, resulting in increased cellular cAMP production.

In order to more thoroughly investigate the pharmacology of the β-like octopamine receptors, dose-response curves were generated with the three receptor types that showed the greatest responses to 1 μM concentrations of the investigated ligands (R1, R3 and R4). All three receptors were most sensitive to octopamine with an EC_{50} of about 1 nM, followed by medetomidine with an EC_{50} approximately 25 times greater and tyramine with an EC_{50} that is 50-200 times greater than that of octopamine (Fig. 7; Table 2). The only obvious difference between the three receptors was that R3 seemed to be somewhat more sensitive to tyramine than R1 and R4.

The weak medetomidine-evoked cAMP signal mediated by the α-like receptor R0 was examined further. We first confirmed proper expression of the R0 receptor in the CHO cells with a receptor binding assay using the α_2-adrenoceptor antagonist radioligand [^{3}H]RS79948-197. Membranes

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prepared from the transfected CHO cells that were used in the functional assay were expressing R0 receptors at a density of approximately 2.4 pmol/mg membrane protein, which is in the range of other recombinant GPCRs expressed in CHO cells. Thus, the weak medetomidine-induced stimulation of cAMP production was apparently not caused by low levels of receptor expression. Next, we monitored agonist-stimulated binding of [^{35}S]GTPγS, a non-hydrolyzable GTP analogue often used to investigate activation of G_i-type G proteins (Peltonen et al., 1998). No agonist-stimulated [^{35}S]GTPγS binding was detectable for tyramine, adrenaline, medetomidine or octopamine in membranes from the R0-transfected CHO cells (Fig. 6C). We concluded that signaling via R0 is not mediated through activation of G_i-type G proteins.

Since α-like octopamine receptors of other invertebrates have been shown to signal by stimulating intracellular calcium release, in addition to cAMP formation, this possibility was also investigated for R0. Addition of octopamine, medetomidine and tyramine to R0-expressing CHO cells, resulted in concentration-dependent increases in intracellular Ca^{2+} levels (Fig. 8). No Ca^{2+} responses were observed in non-transfected cells (results not shown). Mean (± sem) EC_{50} values of the three tested agonists for the Ca^{2+} responses were 0.35 ± 0.12 nM for medetomidine, 1.9 ± 0.4 nM for octopamine and 160 ± 30 nM for tyramine (Table 2). On visual inspection of the Ca^{2+} response data (Fig. 8A), it is evident that the responses to medetomidine were more sustained than the responses to octopamine and tyramine. This difference in response kinetics is potentially highly interesting, but its molecular mechanisms were not investigated in this study.

In conclusion, all five cloned *B. improvisus* receptors clearly belong to the octopamine receptor family. All of them are activated by the antifouling substance medetomidine. Our analysis suggests that for the β-like receptors R1-R4, receptor signaling is mediated via stimulation of cAMP formation. For the α-like receptor R0, a small cAMP signal was obtained after activation with octopamine and medetomidine, but only with concomitant forskolin treatment. However, a clear ligand-dependent elevation of calcium levels was detected, indicating that R0 appears to mainly mediate cellular signaling via increasing intracellular calcium concentrations.

Differential expression of the B. improvisus octopamine receptors

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The free-swimming cyprid larvae and the permanently attached adults of *B. improvisus* are physiologically very different organisms. Thus, their octopamine receptors might have quite different functional roles. In particular, gene expression in the cyprid stage is of biotechnological interest since this stage responds to medetomidine exposure by not settling. To compare the mRNA expression levels of the cloned *B. improvisus* receptors in cyprids and adults, quantitative real-time PCR was performed on both life stages. It was found that all five octopamine receptor mRNA species were expressed in both cyprids and adults. However, while R0, R1 and R2 were about equally expressed in both life stages, R3 and R4 were differentially expressed, being significantly more abundant in the cyprid stage (Fig. 9).

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DISCUSSION

Cloning and classification of barnacle α- and β-like octopamine receptors

Octopamine is the invertebrate counterpart of the vertebrate adrenergic transmitters, and it modulates a great variety of invertebrate behaviours. In an attempt to identify the cellular targets in barnacles of the antifouling substance medetomidine, one α-like and four β-like octopamine receptors from *B. improvisus* were cloned and functionally characterized. These are the first functionally characterized crustacean octopamine receptors. The R0 clone is of the α-like type, and we propose this *B. improvisus* receptor to be named BiOctα. Although the four octopamine receptors R1-R4 clearly belong to the β-like octopamine receptor family, they could not unambiguously be mapped into the subgroups of the insect β1-, β2- and β3-like receptors. *B. improvisus* might thus have a different repertoire of β-like octopamine receptors than insects. We therefore refrain from an orthology classification and propose to name these cloned receptors BiOctβ-R1 (R1), BiOctβ-R2 (R2), BiOctβ-R3 (R3) and BiOctβ-R4 (R4). The identification of four β-like octopamine receptors is interesting, considering that *D. melanogaster* has only three variants. In this context it should be noted that *D. melanogaster* achieves greater transcript/protein variability via alternative splicing of at least two of its β-like octopamine receptor genes (Maqueira et al., 2005). The option of alternative splicing to increase the receptor repertoire seems less obvious in *B. improvisus*, since at least BiOctα, BiOctβ-R1 and BiOctβ-R2 are encoded by intronless genes, as was shown by cloning of identical receptor sequences from both genomic DNA and cDNA. It will be interesting to further investigate the octopamine receptor repertoire in *B. improvisus* and to compare it to that of other crustaceans as soon as more genome sequences become available.

Binding selectivity for octopamine receptors

The sequence-based octopamine receptor classification was supported by our functional studies since all receptors were activated by octopamine with high efficacy and potency. However, they were also activated by tyramine, but with much lower potency. This is in line with both α- and β-like

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octopamine receptors from *D. melanogaster* (Han et al., 1998; Maqueira et al., 2005). A hypothesis for receptor discrimination between tyramine and octopamine was generated from the homology 3D model of the ligand-binding pocket. Octopamine has a hydroxyl group in the β-position, which corresponds to a hydrogen in tyramine. A potential hydrogen-bonding partner for the β-hydroxyl group of octopamine is asparagine N3.29, which is conserved in the β-like octopamine receptor family (Fig. 5B). The corresponding residue in the tyramine binding receptors is an aliphatic hydrophobic residue (Ile, Leu, Val), which would agree well with the absence of a β-hydroxyl group in tyramine. The lack of hydrogen bonding between position 3.29 and tyramine might thus explain the lower potency of tyramine as compared to octopamine in β-like octopamine receptors.

However, α-like octopamine receptors contain hydrophobic residues at position 3.29, so recognition of the β-hydroxyl group may be different in different receptor types. In support of this, mutational studies of adrenoceptors or octopamine receptors to identify specific ligand interactions have not been conclusive (Chatwin et al., 2003; Huang et al., 2007; Ohta et al., 2004; Perez, 2007). Docking simulations of human α_{2A}-adrenoceptors have indicated that the β-hydroxyl groups in the R-enantiomers of noradrenaline-like compounds could form hydrogen bonds with Asp113 (D3.32), whereas the S-enantiomers of noradrenaline and its analogs cannot form that interaction. Consistent with this, the affinities of the S-enantiomers are much weaker than those of the R-enantiomers. In this model the β-hydroxyl group would form a hydrogen bond with one of the two side-chain oxygens of Asp113 (D3.32) of α_{2A}-adrenoceptors, while the other side-chain oxygen would bind the charged nitrogen of the ligand (Nyrönen et al., 2001). In addition, Huang *et al.* have suggested Y412/6.55 for recognition of the octopamine β-hydroxyl in the α-like octopamine receptor of *Bombyx mori* (Huang et al., 2008). However, this Y6.55 residue is at the periphery of the binding pocket in both our and their model, and its involvement in ligand binding would require a conformational change in the receptor or a water-mediated interaction. Thus, D3.32, N3.29 and Y6.55 could potentially serve similar functions in different octopamine receptors for binding of the β-hydroxyl of octopamine.

Down-stream signaling of the B. improvisus octopamine receptors

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In contrast to the well-investigated mammalian G-proteins, knowledge about the G-protein families of invertebrates is still rather fragmentary. Four barnacle G_{α} subunits are expressed in the cyprids, and they resemble vertebrate $G_{\alpha s}$-, $G_{\alpha q}$-, $G_{\alpha o}$- and $G_{\alpha i}$–type α-units (Alm Rosenblad *et al*., unpublished data). $G_{\alpha s}$–containing G-proteins are activated by a large group of GPCRs, and stimulate adenylyl cyclases that convert ATP to cyclic AMP. $G_{\alpha q}$–containing G-proteins interact with phospholipase C leading to a signaling cascade that ends with an increase in cytosolic calcium levels.

Given the clear increase in cAMP production upon ligand activation, $G_{\alpha s}$ is most likely the G-protein isoform that transmits the signal from the *B. improvisus* β-like octopamine receptors. The cAMP response of the α-like receptor BiOctα was weak. However, BiOctα was instead shown to stimulate intracellular calcium elevation, indicating signaling via $G_{\alpha q.}$ Previously characterized α-like octopamine receptors have been reported to signal both via increased cAMP production and calcium release (Balfanz et al., 2005; Bischof and Enan, 2004; Gerhardt et al., 1997a; Han et al., 1998; Ohtani et al., 2006). It has, however, been suggested that the main signaling pathway of α-like octopamine receptors is via G_q-mediated increases in cytosolic calcium concentrations (Balfanz et al., 2005), which would be in accordance with our results.

Earlier attempts to investigate the effect in *vivo* of the second messengers cAMP and calcium in cyprid settling have demonstrated the difficulty of conducting such whole-animal experiments, and the results have been inconclusive. Exposure to added second messengers and the use of potent pharmacological inhibitors or activators have in many cases produced opposing results (Clare et al., 1995; Clare, 1996). In addition, such experiments were conducted on whole animals, while ideally, relevant functional organs of the larvae should be investigated, which is technically difficult in these small cyprid larvae.

The antifouling substance medetomidine activates the B. improvisus octopamine receptors

We here show that the antifouling substance medetomidine induces a locomotor activation response in barnacle cyprids, which most likely is the cause of settling inhibition. Earlier studies have indicated that one of the most important aspects of octopamine signaling in insects is a fight-or-flight

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response induced by a number of stressful stimuli, e.g. encounters with predators leading to increased muscle performance, increased sensory perception and increased energy supply (Roeder, 2005). In addition, octopaminergic insect repellants enhance the motor activity of insects, making them leave the plant (Roeder, 2005).

Our most important finding in the context of biofouling is that octopamine receptors from *B. improvisus* were activated by the antifouling substance medetomidine. In addition, we found that all five octopamine receptor subtypes were expressed in the cyprids and can thus be regarded as potential candidates to mediate the inhibitory effects of medetomidine on the settling process. Dose-response experiments in the cAMP assay with the three β-like octopamine receptors BiOctβ-R1, BiOctβ-R3 and BiOctβ-R4 showed that the EC_{50} of medetomidine was approximately 30-100 nM. The EC_{50} of medetomidine for R0 in the calcium assay was about 0.3 nM. Thus, the *in vitro* assay results were in rather good agreement with the *in vivo* EC_{50} estimate previously obtained for cyprid settling inhibition of *B. improvisus*, which is 1 nM (Dahlström and Elwing, 2006). In this perspective it is worth noting that medetomidine can be docked in a similar orientation as octopamine in our 3D homology model of the octopamine receptors (Fig. 5C). One protonated imidazole-ring nitrogen forms a hydrogen bond-stabilized charge interaction with D3.32, and the other nitrogen is in a position to form a hydrogen bond with N3.29. The aromatic six-membered ring, however, is tilted by 70 degrees in our model with respect to the octopamine ring to avoid steric clashes between the ring methyl groups of the ligand and residues of the receptor.

It is also of practical interest that all of the cloned octopamine receptors appear sensitive to medetomidine. This should make the development of resistance, caused by mutations in the binding sites in each of the octopamine receptors, a less likely event. The somewhat higher sensitivity of R0 to medetomidine compared to the β-like receptors could suggest that R0 might be the main anti-fouling target. Still, in addition to ligand potency, also other factors determine the functional importance of the different receptor types as antifouling targets. Spatial localization in the cyprids could play a role, and expression in the antennules, which are used by the cyprids to explore surfaces, might be important. The relative expression levels of different receptors might also influence their importance as antifouling targets. Both BiOctβ-R3 and BiOctβ-R4 were found to be more highly expressed in

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cyprids than in adults and one could speculate that these two are more important than the other receptors in some cyprid-specific processes such as settling and thus more likely to be functionally important targets for medetomidine. Our results open up the possibility for population-based analysis of medetomidine sensitivity, for a better understanding of the development of resistance, as well as for developing novel efficacious and environmentally sustainable antifouling substances.

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FOOTNOTES

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The nucleotide sequences for the *B. improvisus* octopamine receptors have been deposited in the GenBank database under GenBankAccession Numbers: BiOctA=GU074418, BiOctB-R1= GU074419, BiOctB-R2=GU074420**,** BiOctB-R3=GU074421**,** BiOctB-R4=GU074422

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FIGURE LEGENDS

Figure 1. Medetomidine induces increased leg kicking in cyprids.

Cyprids were immobilized in agarose gel and numbers of leg-kicks were recorded before and after the addition of 10 nM medetomidine, 100 nM of the α_2-adrenoceptor antagonist atipamezole or a combination of these ligands. The difference in the number of kicks before and after ligand addition is shown. The numbers of kicks were significantly greater in the medetomidine-treated cyprids as compared to the controls (***P<0.001) and to the combination of medetomidine and atipamezole (†††P<0.001) (Anova with Newman-Keuls *post hoc* test).

Figure 2. Variable regions of *B. improvisus* R0 α-like octopamine receptor clones and sequence comparison with the *B. amphitrite* α-like octopamine receptor. A. The amino acid sequences of four genomic DNA and two cDNA R0 clones were aligned. The three regions where the amino acid sequences differ between the clones are indicated. **B**. The amino acid sequence of the *B. improvisus* R0 receptor and its *B. amphitrite* ortholog were aligned and showed to be 90 % identical at the protein level. The *B. amphitrite* receptor has a shorter N-terminus than R0 of *B. improvisus* and there are another 33 amino acid differences, all located outside of the TMs. Identical residues are shaded in dark grey and functionally similar residues are shaded in grey. TMs in the structure of the crystallized β_1-adrenoceptor were used to predict the TMs of R0 by sequence alignment. Putative TMs are underlined.

Figure 3. Amino acid sequence alignment of the *B. improvisus* β-like receptors. The amino acid sequences of the *B. improvisus* β-like octopamine receptors were aligned. Identical residues are shaded in dark grey and functionally similar residues are shaded in grey. TMs in the structure of the crystallized β_1-adrenoceptor were used to predict the TMs of R1-4 by sequence alignment. Putative TMs are underlined.

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Figure 4. **Phylogenetic tree including *B. improvisus* octopamine receptors and other selected invertebrate full-length biogenic amine receptors.** A phylogenetic tree was constructed with PhyML (WAG option) using sequences of the cloned *B. improvisus* (Bi) octopamine receptors, full-length biogenic amine receptors from the three fully sequenced insect species *D. melanogaster* (Dm), the red flour beetle *T. castaneum* (Tc) and the honey bee *A. mellifera* (Am), two octopamine receptors from *B. amphitrite* (Ba), β-like octopamine receptors from the seahares *A. californica* (Ac) and *A. kurodai* (Ak), the Atlantic surf clam *Spisula sollidisisma* (Ss) as well as three putative octopamine receptors from the waterflea *D. pulex* (Dp). One of the *D. pulex* receptors was in our analysis clearly classified as a dopamine receptor (Dp_Dop2). *D. pulex* labels are chosen according to results from the phylogenetic analyses. *D. melanogaster* Rhodopsin 4 and 6 were used as an outgroup. The clusters of α-like and β-like receptors are indicated with frames. The positions of the *B. improvisus* receptors are indicated with arrows. Bootstrap values are shown at the branches. For accession numbers to the used sequences see Table S4.

Figure 5. β-like octopamine receptor homology model. An octopamine receptor structure model was built using the turkey β_1-adrenoceptor 3D structure as template and putative ligand-interacting residues were identified. **A.** The model is shown as ribbons and thin ball-and-stick representations. The binding pocket shape is shown as a blue surface, and selected residues and helices are labeled. Residues and pocket surfaces close to the viewer are hidden for clarity, and the extracellular side of the membrane is up. Residues in the TMs are referred to by residue number and the nomenclature of Ballesteros and Weinstein (Ballesteros and Weinstein, 1995). (R)-Octopamine is suggested to bind primarily in two orientations and both include established hydrogen bonding interactions and an ion interaction between the ligand amine and D123/3.32 (orange circles). The model shown also includes a hydrogen bond interaction to N120/3.29 (blue circle), which may explain the β-like octopamine receptors' binding preference of octopamine over tyramine. The other plausible hydrogen bond interacting residue is also shown (S127/3.36). **B.** By identifying all protein side-chain atoms within 5 Å from octopamine in the R4 receptor homology model, 18 binding site residues were selected. The

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corresponding amino acids in selected biogenic amine receptors were extracted from a multiple sequence alignment. Putative ligand-interacting residues of the α-like receptors are shown in the upper part and of the β-like receptors in the lower part. **C.** Medetomidine docks in a conformation similar to that of octopamine (panel A), with electrostatic and hydrogen bonding interactions between the imidazole ring and D123/3.32.

Figure 6. Ligand-evoked cAMP responses of the cloned octopamine receptors. A. 1 μM of either octopamine, medetomidine, tyramine, dopamine or histamine were added to CHO cells expressing the β-like octopamine receptors R1, R2, R3 and R4. The percent increase in cAMP compared to basal levels is displayed. One experiment with technical duplicates is shown. **B.** 1 μM of either octopamine, medetomidine, tyramine, dopamine or histamine were added together with 100 nM forskolin to CHO cells expressing the α-like receptor R0. The percent increase over cAMP levels stimulated by 100 nM forskolin alone is displayed. The average of two experiments with six replicates each is shown. In each experiment the stimulation by octopamine and medetomidine was statistically significant as compared to the response induced by forskolin alone (Students two-tailed t-test with Bonferroni correction, **P<0.01). **C**. Agonist-stimulated [^{35}S]GTPγS binding to CHO cell membranes expressing the α-like octopamine receptor R0 was measured. Recombinant human α_{2A}-adrenoceptors that signal via G_i-type G proteins in CHO cells (Peltonen *et al*., 1998) were used as control to detect adrenaline-stimulated [^{35}S]GTPγS binding and the expected results were obtained (EC_{50}=140 nM, E_{max}=210 % over basal).

Figure 7. Dose-response curves for R1, R3 and R4 with octopamine, medetomidine and tyramine. A wide concentration range (0.1 nM to 10μM) of the ligands octopamine, medetomidine and tyramine were added to CHO cells expressing the receptors R1, R3 and R4 (no forskolin was added in these experiments). The experiment was run three times, two times in duplicate and one time in triplicate, and the average in % of the response obtained with 100 nM forskolin alone is shown. The error bars show the standard error mean.

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Figure 8. Ligand-evoked calcium responses of the cloned α-like receptor R0

Different concentrations of octopamine, medetomidine and tyramine were added to CHO cells expressing the R0 receptor. Increases of intracellular calcium levels were monitored using the FLIPR Calcium 4 Assay Kit (Molecular Devices) and the FlexStation automated fluorescence plate reader. **A.** Typical Ca^{2+} response results from individual wells in a single experiment are displayed. Results are shown as relative fluorescence units (RFU) from individual wells exposed to different concentrations of agonists (baseline subtracted).

B. Concentration-response curves using quadruplicate samples were created. Means ± sem from 3-5 experiments are shown after normalization of the Ca^{2+} responses in relation to the maximum response obtained with 100 nM medetomidine. The maximum responses compared to medetomidine were 83% ± 9 for octopamine and 53% ± 8 for tyramine.

Figure 9. mRNA expression of the cloned *B. improvisus* receptors. A. RNA was prepared from six batches of cyprids and from 6 adult animals, followed by cDNA synthesis. Q-PCR was performed with primers for the cloned *B. improvisus* receptors. The average of the relative gene expression ($2^{CT\ (actin)\ -\ CT\ (receptor)}$) from the six samples of cyprids or adults are shown. Error bars display the standard error. Significant differences between adults and cyprids are indicated (t-test,*$p<0.05$).

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Tables

Table 1. Identity and similarity of the cloned β-like octopamine receptors from *B. improvisus* with the β-like octopamine receptors from *D. melanogaster*. The table shows the percentage of identical residues among all aligned positions (alignment using program ClustalW). The percentage of functionally similar residues is shown in parenthesis. *B. imp.*, *Balanus improvisus; DmOct*β, *Drosophila melanogaster* β-adrenoceptor like receptor

***B. imp.* receptor**	**DmOctβ1**	**DmOctβ2**	**DmOctβ3**
R1	45 (61)	43 (61)	52 (70)
R2	44 (62)	41 (60)	52 (69)
R3	53 (71)	54 (69)	62 (75)
R4	46 (65)	49 (68)	55 (72)

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Table 2

Ligand-dependent second messenger responses of the cloned *B. improvisus* octopamine receptors. Receptors were expressed in CHO cells and different concentrations of octopamine, medetomidine and tyramine were added. EC_{50} values of stimulated cAMP formation for the β-like receptors R1, R3 and R4 (n=3) and of intracellular calcium elevation for the α-like receptor R0 (n=3-5) are shown.

	EC_{50} of receptor-mediated stimulation of cAMP formation (nM, mean ± sem)		
Receptor	**Octopamine**	**Medetomidine**	**Tyramine**
R1	4.6 ± 0.2	99 ± 15	950 ± 247
R3	1.6 ± 0.4	41 ± 23	91 ± 33
R4	0.99 ± 0.22	33 ± 1	260 ± 63
	EC_{50} of calcium elevation (nM, mean ± sem)		
	Octopamine	**Medetomidine**	**Tyramine**
R0	1.9 ± 0.4	0.35 ± 0.12	160 ± 30

Figure 1

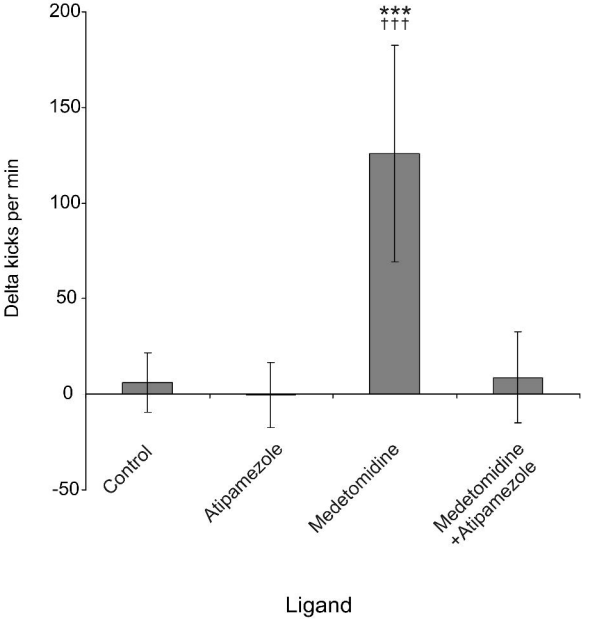
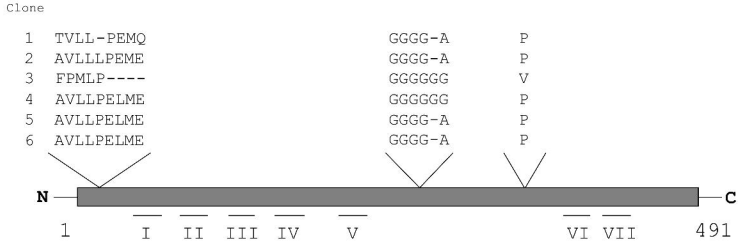

200
150
100
50
0
-50
Delta kicks per min
***
†††
Control
Atipamezole
Medetomidine
Medetomidine +Atipamezole
Ligand

Figure 2

A


Clone
1 TVLL-PEMQ GGGG-A P
2 AVLLLPEME GGGG-A P
3 FPMLP---- GGGGGG V
4 AVLLPELME GGGGGG P
5 AVLLPELME GGGG-A P
6 AVLLPELME GGGG-A P
N
C
1
I
II
III
IV
V
VI
VII
491

B

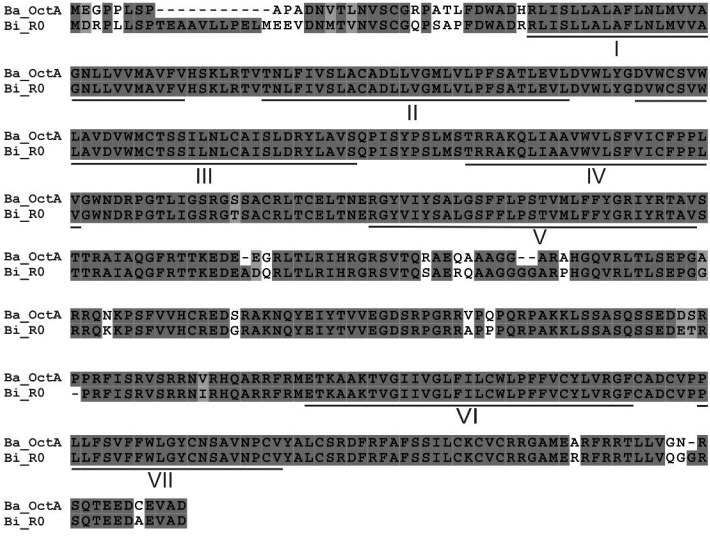

Ba_OctA MEGPPLSP----------APADNVTLNVSCGRPATLFDWADHRLISLLALAFLNLMVVA
Bi_R0 MDRPLLSPTEAAVLLPELMEEVDNMTVNVSCGQPSAPFDWADRRLISLLALAFLNLMVVA
I
Ba_OctA GNLLVVMAVFVHSKLRTVTNLFIVSLACADLLVGMLVLPFSATLEVLDVWLYGDVWCSVW
Bi_R0 GNLLVVMAVFVHSKLRTVTNLFIVSLACADLLVGMLVLPFSATLEVLDVWLYGDVWCSVW
II
Ba_OctA LAVDVWMCTSSILNLCAISLDRYLAVSQPISYPSLMSTRRAKQLIAAVWVLSFVICFPPL
Bi_R0 LAVDVWMCTSSILNLCAISLDRYLAVSQPISYPSLMSTRRAKQLIAAVWVLSFVICFPPL
III
IV
Ba_OctA VGWNDRPGTLIGSRGSSACRLTCELTNERGYVIYSALGSFFLPSTVMLFFYGRIYRTAVS
Bi_R0 VGWNDRPGTLIGSRGTSACRLTCELTNERGYVIYSALGSFFLPSTVMLFFYGRIYRTAVS
V
Ba_OctA TTRAIAQGFRTTKEDE-EGRLTLRIHRGRSVTQRAEQAAAGG--ARAHGQVRLTLSEPGA
Bi_R0 TTRAIAQGFRTTKEDEADQRLTLRIHRGRSVTQSAERQAAGGGGARPHGQVRLTLSEPGG
Ba_OctA RRQNKPSFVVHCREDSRAKNQYEIYTVVEGDSRPGRRVPQPQRPAKKLSSASQSSEDDSR
Bi_R0 RRQKKPSFVVHCREDGRAKNQYEIYTVVEGDSRPGRRAPPPQRPAKKLSSASQSSEDETR
Ba_OctA PPRFISRVSRRNVRHQARRFRMETKAAKTVGIIVGLFILCWLPFFVCYLVRGFCADCVPP
Bi_R0 -PRFISRVSRRNIRHQARRFRMETKAAKTVGIIVGLFILCWLPFFVCYLVRGFCADCVPP
VI
Ba_OctA LLFSVFFWLGYCNSAVNPCVYALCSRDFRFAFSSILCKCVCRRGAMEARFRRTLLVGN-R
Bi_R0 LLFSVFFWLGYCNSAVNPCVYALCSRDFRFAFSSILCKCVCRRGAMERRFRRTLLVQGGR
VII
Ba_OctA SQTEEDCEVAD
Bi_R0 SQTEEDAEVAD

Figure 3

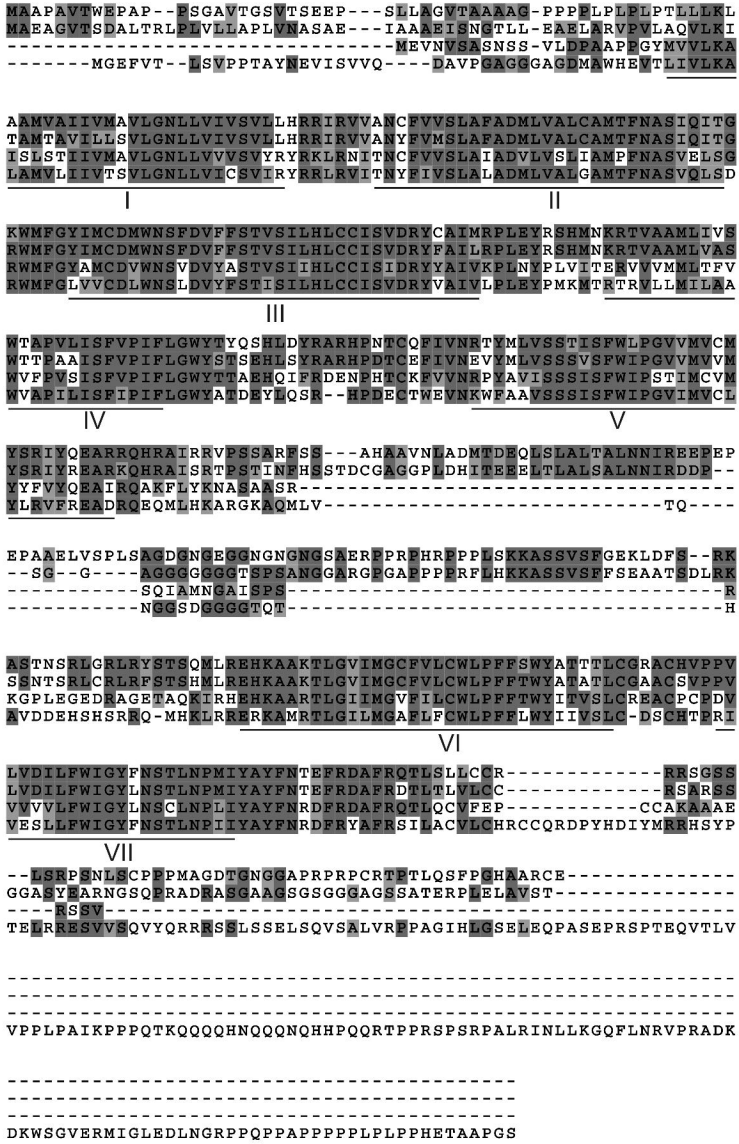

R1 MAAPAVTWEPAP--PSGAVTGSVTSEEP---SLLAGVTAAAAG-PPPPLPLPLPTLLLKL
R2 MAEAGVTSDALTRLPLVLLAPLVNASAE---IAAAEISNGTLL-EAELARVPVLAQVLKI
R3 ----------MEVNVSASNSS-VLDPAAPPGYMVVLKA
R4 -------MGEFVT--LSVPPTAYNEVISVVQ----DAVPGAGGGAGDMAWHEVTLIVLKA
R1 AAMVAIIVMAVLGNLLVIVSVLLHRRIRVVANCFVVSLAFADMLVALCAMTFNASIQITG
R2 TAMTAVILLSVLGNLLVIVSVLLHRRIRVVANYFVMSLAFADMLVALCAMTFNASIQITG
R3 ISLSTIIVMAVLGNLLVVVSVYRYRKLRNITNCFVVSLAIADVLVSLIAMPFNASVELSG
R4 LAMVLIIVTSVLGNLLVICSVIRYRRLRVITNYFIVSLALADMLVALGAMTFNASVQLSD
I
II
R1 KWMFGYIMCDMWNSFDVFFSTVSILHLCCISVDRYCAIMRPLEYRSHMNKRTVAAMLIVS
R2 RWMFGYIMCDMWNSFDVFFSTVSILHLCCISVDRYFAILRPLEYRSHMNKRTVAAMLVAS
R3 RWMFGYAMCDVWNSVDVYASTVSIIHLCCISIDRYYAIVKPLNYPLVITERVVVMMLTFV
R4 RWMFGLVVCDLWNSLDVYFSTISILHLCCISVDRYVAIVLPLEYPMKMTRTRVLLMILAA
III
R1 WTAPVLISFVPIFLGWYTYQSHLDYRARHPNTCQFIVNRTYMLVSSTISFWLPGVVMVCM
R2 WTTPAAISFVPIFLGWYSTSEHLSYRARHPDTCEFIVNEVYMLVSSSVSFWIPGVVMVVM
R3 WVFPVSISFVPIFLGWYTTAEHQIFRDENPHTCKFVVNRPYAVISSSISFWIPSTIMCVM
R4 WVAPILISFIPIFLGWYATDEYLQSR--HPIECTWEVNKWFAAVSSSISFWIPGVIMVCL
IV
V
R1 YSRIYQEARRQHRAIRRVPSSARFSS---AHAAVNLADMTDEQLSLALTALNNIREEPEP
R2 YSRIYREARKQHRAISRTPSTINFHSSTDCGAGGPLDHITEEELTLALSALNNIRDDP--
R3 YYFVYQEAIRQAKFLYKNASANSR----------
R4 YLRVFREADRQEQMLHKARGKAQMLV----------TQ----
R1 EPAAELVSPLSAGDGNGEGGNGNGGSAERPRPHRPPLSKKASSVSFGEKLDFS--RK
R2 --SG--G----AGGGGGGGTSPSANGGARGRGAPPPRFLHKKASSVSFFSEAATSDLRK
R3 ----------SQIAMNGAISPS----------R
R4 ----------NGGSDGGGGTQT----------H
R1 ASTNSRLGRLRYSTSQMLREHKAAKTLGVIMGCFVLCWLPFFSWYATTTLCGRACHVPPV
R2 SSNTSRLCRLRFSTSHMLREHKAAKTLGVIMGCFVLCWLPFFTWYATATLCGAACSVPPV
R3 KGPLEGEDRAGETAQKIRHEHKAARTLGIIMGVFILCWLPFFTWYITVSLCREACPCPDV
R4 AVDDEHSHSRRQ-MHKLRRERKAMRTLGILMGAFLFCWLPFFLWYIIVSLC-DSCHTPRI
VI
R1 LVDILFWIGYFNSTLNPMIYAYFNTEFRDAFRQTLSLLCCR----------RRSGSS
R2 LVDILFWIGYLNSTLNPMIYAYFNTEFRDAFRDTLTLVLCC----------RSARSS
R3 VVVVLFWIGYLNSCLNPLIYAYFNRDFRDAFRQTLQCVFEP----------CCAKAAAE
R4 VESLLFWIGYFNSTLNPIIYAYFNRDFRYAFRSILACVLCHRCCQRDPYHDIYMRRHSYP
VII
R1 --LSRPSNLSCPPPMAGDTGNGGAPRPRPCRTPTLQSFPGHAARCE----------
R2 GGASYEARNGSQPRADRASGAAGSGSGGGAGSSATERPLELAVST----------
R3 ----RSSV----------
R4 TELRRESVVSQVYQRRRSSLSSELSQVSALVRPPAGIHLGSELEQPASEPRSPTEQVTLV
R1 ----------
R2 ----------
R3 ----------
R4 VPPLPAIKPPPQTKQQQQHNQQQNQHHPQQRTPPRSPSRPALRINLLKGQFLNRVPRADK
R1 ----------
R2 ----------
R3 ----------
R4 DKWSGVERMIGLEDLNGRPPQPPAPPPPPLPLPPHETAAPGS

Figure 4

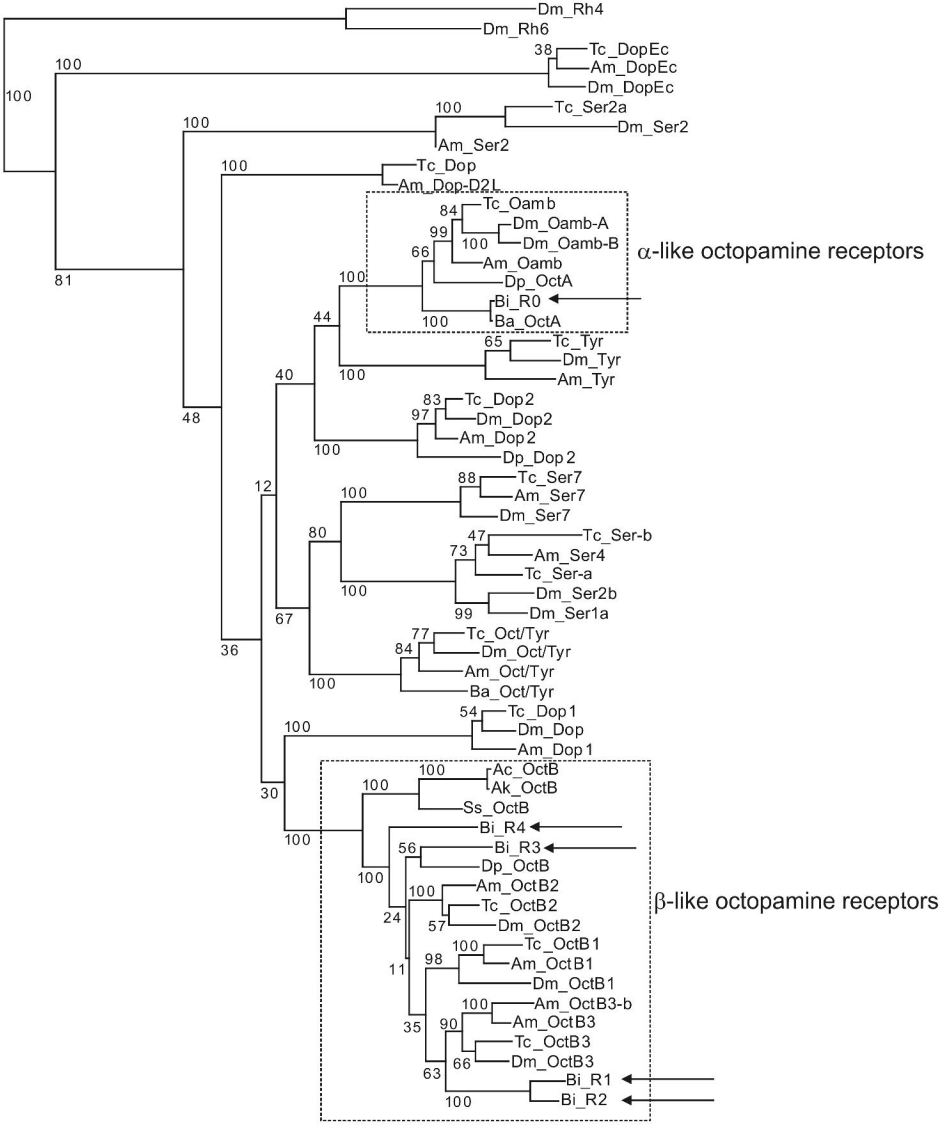
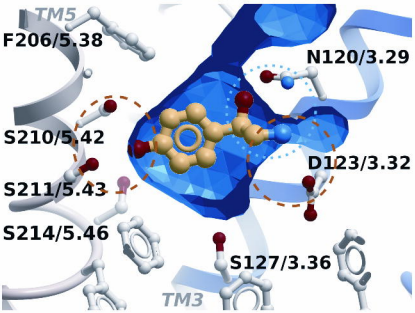

Dm_Rh4
Dm_Rh6
Tc_DopEc
Am_DopEc
Dm_DopEc
Tc_Ser2a
Dm_Ser2
Am_Ser2
Tc_Dop
Am_Dop-D2L
Tc_Oamb
Dm_Oamb-A
Dm_Oamb-B
Am_Oamb
Dp_OctA
Bi_R0
Ba_OctA
α-like octopamine receptors
Tc_Tyr
Dm_Tyr
Am_Tyr
Tc_Dop2
Dm_Dop2
Am_Dop2
Dp_Dop2
Tc_Ser7
Am_Ser7
Dm_Ser7
Tc_Ser-b
Am_Ser4
Tc_Ser-a
Dm_Ser2b
Dm_Ser1a
Tc_Oct/Tyr
Dm_Oct/Tyr
Am_Oct/Tyr
Ba_Oct/Tyr
Tc_Dop1
Dm_Dop
Am_Dop1
Ac_OctB
Ak_OctB
Ss_OctB
Bi_R4
Bi_R3
Dp_OctB
Am_OctB2
Tc_OctB2
Dm_OctB2
Tc_OctB1
Am_OctB1
Dm_OctB1
Am_OctB3-b
Am_OctB3
Tc_OctB3
Dm_OctB3
Bi_R1
Bi_R2
β-like octopamine receptors

Figure 5

A



B

α-like receptors

	3.29	3.32	3.33	3.36	4.57	4.61	5.38	5.39	5.42	5.43	5.46	5.47	6.48	6.51	6.52	6.55	7.39	7.43
Hs_ADRA1A	A	D	V	C	S	L	Y	V	S	A	S	F	W	F	F	M	F	Y
Hs_ADRA1B	A	D	V	C	S	L	Y	A	S	S	F	W	F	F	L	F	Y	
Hs_ADRA2A	L	D	V	C	S	L	Y	V	S	C	S	F	W	F	F	Y	F	Y
Ls_OctA2	L	D	V	C	S	F	Y	V	S	S	F	W	F	L	Y	I	Y	
Ls_OctA1	L	D	V	C	C	L	Y	R	A	A	S	F	W	F	F	Y	F	Y
Bi_R0	L	D	V	C	C	L	Y	V	S	A	S	F	W	F	F	Y	F	Y
Am_Oamb	L	D	V	C	C	L	Y	V	S	A	S	F	W	F	F	Y	F	Y
Bm_OctA	L	D	V	C	C	L	Y	V	S	A	S	F	W	F	F	Y	F	Y
Dm_Oamb	L	D	V	C	C	L	Y	V	S	A	S	F	W	F	F	Y	F	Y
Pa_OctA	L	D	V	C	C	L	Y	V	S	A	S	F	W	F	F	Y	F	Y

β-like receptors

Hs_ADRB1	T	D	V	V	S	I	Y	A	S	S	S	F	W	F	F	N	N	Y
Hs_ADRB2	T	D	V	V	S	I	Y	A	S	S	S	F	W	F	F	N	N	Y
Hs_ADRB3	T	D	V	V	S	I	Y	V	S	S	S	F	W	F	F	N	N	Y
Ac_OctB	N	D	V	S	S	V	Y	A	S	S	S	F	F	F	Y	F	Y	
Bi_R1	N	D	V	S	S	I	Y	M	S	S	S	F	W	F	F	Y	F	Y
Bi_R2	N	D	V	S	S	I	Y	M	S	S	S	F	W	F	F	Y	F	Y
Bi_R3	N	D	V	S	S	I	Y	A	S	S	S	F	W	F	F	Y	F	Y
Bi_R4	N	D	V	S	S	I	F	A	S	S	S	F	W	F	F	Y	F	Y
Dm_OctB1	N	D	V	S	S	I	Y	A	S	S	S	F	W	F	F	Y	F	Y
Dm_OctB2	N	D	V	S	S	I	Y	A	S	S	S	F	W	F	F	Y	F	Y
Dm_OctB3	N	D	V	S	S	I	Y	A	S	S	S	F	W	F	F	Y	F	Y

C

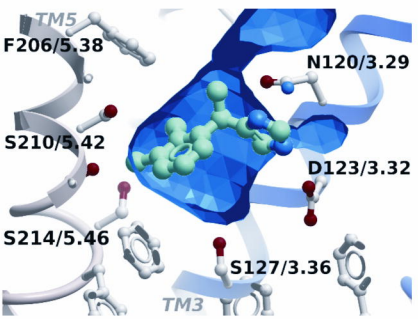
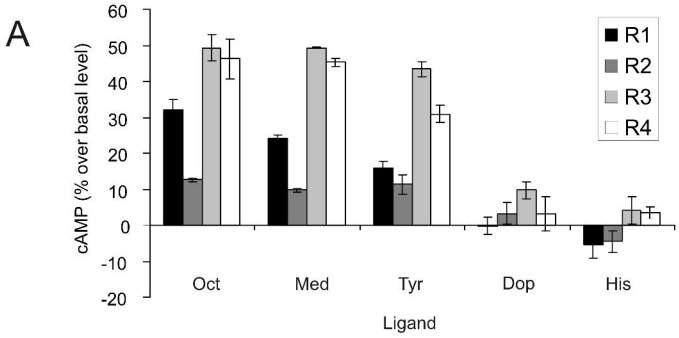
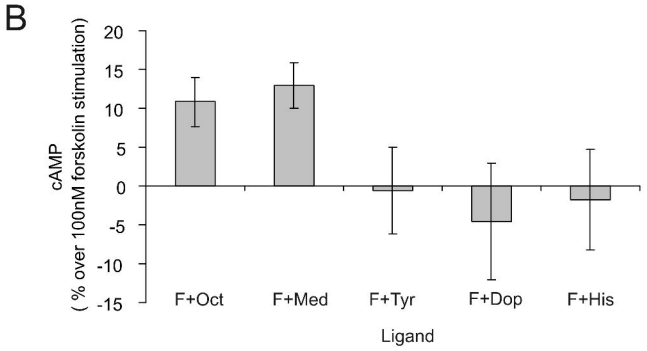


Figure 6


A
cAMP (% over basal level)
60
50
40
30
20
10
0
-10
-20
R1
R2
R3
R4
Oct
Med
Tyr
Dop
His
Ligand


B
cAMP
(% over 100nM forskolin stimulation)
20
15
10
5
0
-5
-10
-15
F+Oct
F+Med
F+Tyr
F+Dop
F+His
Ligand

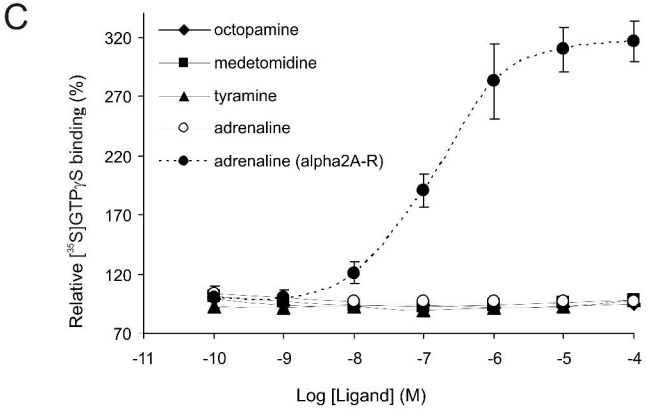

C
Relative [35S]GTPγS binding (%)
320
270
220
170
120
70
octopamine
medetomidine
tyramine
adrenaline
adrenaline (alpha2A-R)
-11
-10
-9
-8
-7
-6
-5
-4
Log [Ligand] (M)

Figure 7

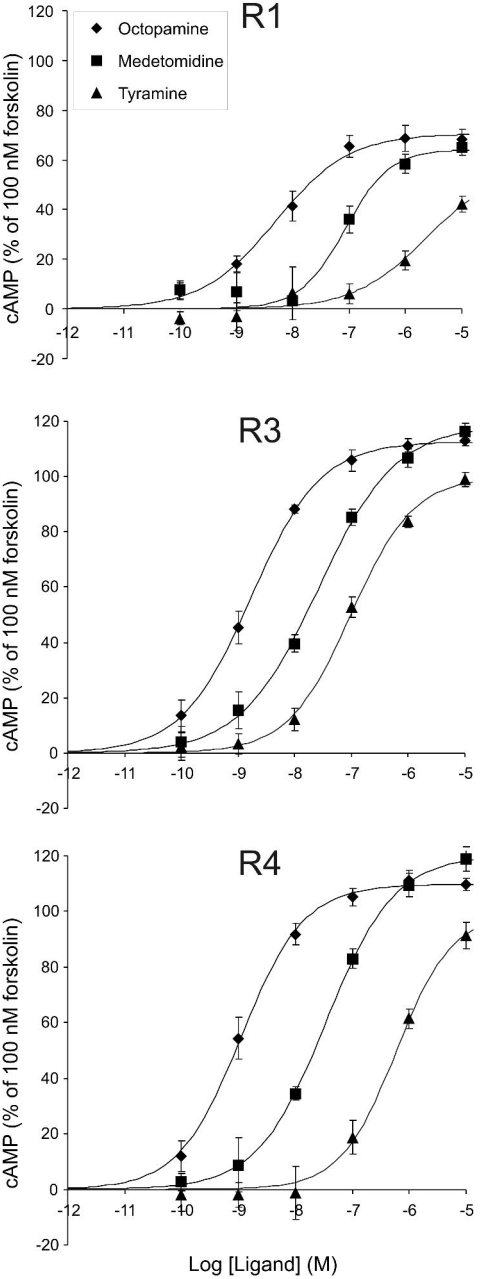
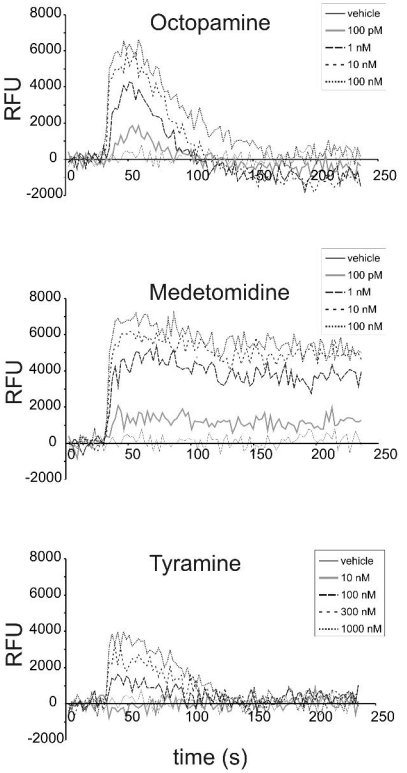

R1
Octopamine
Medetomidine
Tyramine
cAMP (% of 100 nM forskolin)
120
100
80
60
40
20
0
-20
-12
-11
-10
-9
-8
-7
-6
-5
R3
cAMP (% of 100 nM forskolin)
R4
cAMP (% of 100 nM forskolin)
Log [Ligand] (M)

Figure 8

A


Octopamine
vehicle
100 pM
1 nM
10 nM
100 nM
RFU
8000
6000
4000
2000
0
-2000
0
50
100
150
200
250
Medetomidine
vehicle
100 pM
1 nM
10 nM
100 nM
RFU
Tyramine
vehicle
10 nM
100 nM
300 nM
1000 nM
RFU
time (s)

B

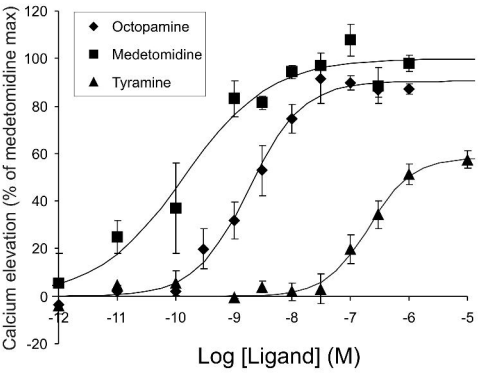
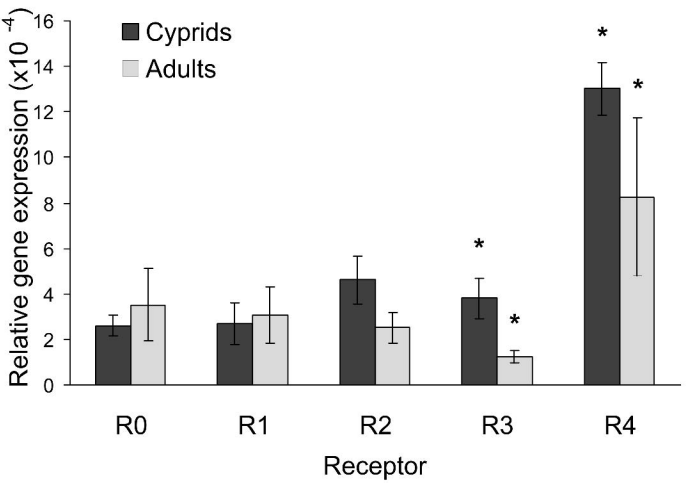

Octopamine
Medetomidine
Tyramine
Calcium elevation (% of medetomidine max)
120
100
80
60
40
20
0
-20
-12
-11
-10
-9
-8
-7
-6
-5
Log [Ligand] (M)

Figure 9


Cyprids
Adults
Relative gene expression (x10 -4)
16
14
12
10
8
6
4
2
0
*
*
*
*
R0
R1
R2
R3
R4
Receptor

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

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

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

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

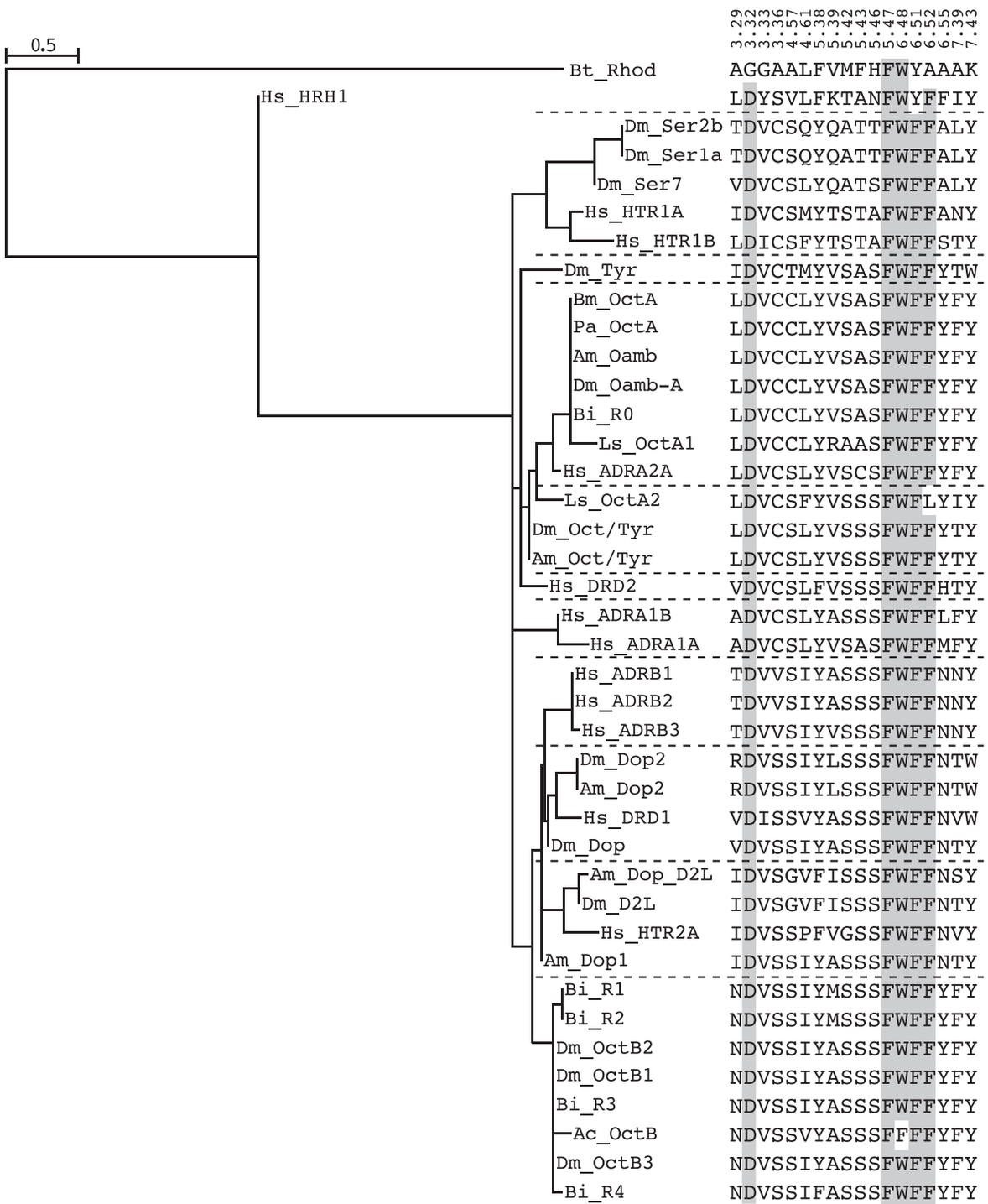

Figure S1. Phylogenetic tree based on sequence alignments of putative ligand-binding residues in functionally characterized biogenic amine receptors. A phylogenetic maximum likelihood tree, using PhyML, was built based on 18 putative ligand-binding residues, identified from the 3D model, in selected 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.

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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

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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

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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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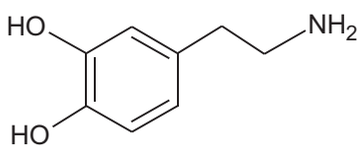
Structures of employed agonist ligands



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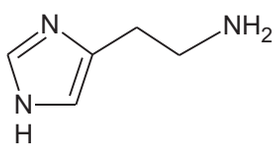

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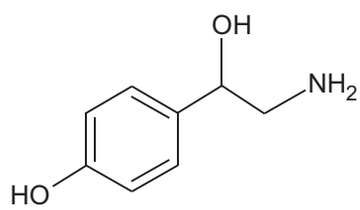

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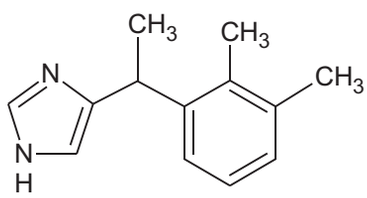
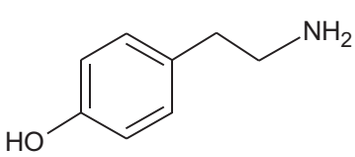


Medetomidine



Tyramine