

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

Ulrika Lind, Magnus Alm Rosenblad, Linda Hasselberg Frank, Susanna Falkbring, Lars Brive, Jonne M. Laurila, Katariina Pohjanoksa, Anne Vuorenpää, Jyrki P. Kukkonen, Lina Gunnarsson, Mika Scheinin, Lena G.E. Mårtensson Lindblad and Anders Blomberg

University of Gothenburg, Gothenburg, Sweden

Department of Cell and Molecular Biology: U.L., M.A.R., S.F., L.B., L.G., A.B.

Department of Zoology: L.H.F., L.G.E.M.L.

Department of Biomedicine: L.B.

University Turku, Turku, Finland

Department of Pharmacology, Drug Development and Therapeutics: J.M.L., K.P., M.S., A.P.

Turku University Hospital (Unit of Clinical Pharmacology), Turku, Finland

M.S.

University of Helsinki, Helsinki, Finland

Department of Basic Veterinary Sciences, Biochemistry: J.P.K.

Minerva Medical Research Institute, Helsinki, Finland

J.P.K.

I-Tech AB, Gothenburg, Sweden

L.G.E.M.L.

Running title: Octopamine receptors from *Balanus improvisus*

Address correspondence to: Anders Blomberg, Department of Cell and Molecular Biology, University of Gothenburg, Box 462, S-405 30 Gothenburg, Sweden, Phone: +46-31-7862589, Fax: +46-31-7862599

Email: Anders.Blomberg@cmb.gu.se

Number of text pages: 33

Number of tables: 2

Number of figures: 9

Number of references: 40

Number of words in abstract: 239

Number of words in introduction: 649

Number of words in discussion: 1520

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

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 (BiOct α) belongs to the α -adrenoceptor-like and the other four (BiOct β -R1, BiOct β -R2, BiOct β -R3 and BiOct β -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.

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

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.

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™ kit (Invitrogen) was used to clone the full-length receptors.

Homology modelling and phylogenetic analysis

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 α -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]GTP γ S.

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).

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

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.

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 savignyi* (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, BiOct β -R1 and BiOct β -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.

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 (DmOct β 1, DmOct β 2 and DmOct β 3) 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 DmOct β 3 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,

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 β 1-, β 2- and β 3-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 carazolol (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

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

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 μ M 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 μ M 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

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 [³⁵S]GTPγS, a non-hydrolyzable GTP analogue often used to investigate activation of G_i-type G proteins (Peltonen et al., 1998). No agonist-stimulated [³⁵S]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 Ca²⁺ levels (Fig. 8). No Ca²⁺ responses were observed in non-transfected cells (results not shown). Mean (± sem) EC₅₀ values of the three tested agonists for the Ca²⁺ 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 Ca²⁺ 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

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).

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 BiOct α . 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 β 1-, β 2- and β 3-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 BiOct β -R1 (R1), BiOct β -R2 (R2), BiOct β -R3 (R3) and BiOct β -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 BiOct α , BiOct β -R1 and BiOct β -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

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

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_{as} -, G_{aq} -, G_{ao} - and G_{ai} -type α -units (Alm Rosenblad *et al.*, unpublished data). G_{as} -containing G-proteins are activated by a large group of GPCRs, and stimulate adenylyl cyclases that convert ATP to cyclic AMP. G_{aq} -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_{as} 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 BiOct α was weak. However, BiOct α was instead shown to stimulate intracellular calcium elevation, indicating signaling via G_{aq} . 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

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 BiOct β -R1, BiOct β -R3 and BiOct β -R4 showed that the EC₅₀ of medetomidine was approximately 30-100 nM. The EC₅₀ 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* EC₅₀ 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 BiOct β -R3 and BiOct β -R4 were found to be more highly expressed in

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.

ACKNOWLEDGEMENTS

Special thanks to Per Jonsson for suggestions and comments on the manuscript, Ronnie Persson for discussions on the 3D model used for ligand-receptor docking, Martin Ogemark for the supply of cyprids and adult individuals, and Homan Alipour for technical help in the initial stages of the project. The Mammalian Protein Expression Core facility at GU is acknowledged for help with initial test transfections. We also thank Gunnar Hansson and Malin Bäckström for access and practical help with the instrumentation for cAMP analysis.

REFERENCES

- Balfanz S, Strunker T, Frings S and Baumann A (2005) A family of octopamine [corrected] receptors that specifically induce cyclic AMP production or Ca²⁺ release in *Drosophila melanogaster*. *J Neurochem* **93**(2):440-451.
- Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein coupled receptors. *Methods Neurosci* **25**:366-428.
- Bischof LJ and Enan EE (2004) Cloning, expression and functional analysis of an octopamine receptor from *Periplaneta americana*. *Insect Biochem Mol Biol* **34**(6):511-521.
- Chang DJ, Li XC, Lee YS, Kim HK, Kim US, Cho NJ, Lo X, Weiss KR, Kandel ER and Kaang BK (2000) Activation of a heterologously expressed octopamine receptor coupled only to adenylyl cyclase produces all the features of presynaptic facilitation in aplysia sensory neurons. *Proc Natl Acad Sci U S A* **97**(4):1829-1834.
- Chatwin HM, Rudling JE, Patel D, Reale V and Evans PD (2003) Site-directed mutagenesis studies on the *Drosophila* octopamine/tyramine receptor. *Insect Biochem Mol Biol* **33**(2):173-184.
- Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK and Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318**(5854):1258-1265.
- Clare A, Thomas R and Rittschof D (1995) Evidence for the involvement of cyclic AMP in the pheromonal modulation of barnacle settlement. *J Exp Biol* **198**(Pt 3):655-664.
- Clare AS (1996) Signal transduction in barnacle settlement: Calcium re-visited. *Biofouling* **10**(1-3):141-159.

- Dahlström M and Elwing H (2006) Adrenoceptor and other pharmacologically active compounds as putative antifoulants. *Prog Mol Subcell Biol* **42**:171-202.
- Dahlström M, Martensson LGE, Jonsson PR, Arnebrant T and Elwing H (2000) Surface active adrenoceptor compounds prevent the settlement of cyprid larvae of *Balanus improvisus*. *Biofouling* **16**(2-4):191-203.
- Evans PD and Maquieira B (2005) Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. *Invert Neurosci* **5**(3-4):111-118.
- Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53**(1):1-24.
- Gerhardt CC, Bakker RA, Piek GJ, Planta RJ, Vreugdenhil E, Leysen JE and Van Heerikhuizen H (1997a) Molecular cloning and pharmacological characterization of a molluscan octopamine receptor. *Mol Pharmacol* **51**(2):293-300.
- Gerhardt CC, Lodder HC, Vincent M, Bakker RA, Planta RJ, Vreugdenhil E, Kits KS and van Heerikhuizen H (1997b) Cloning and expression of a complementary DNA encoding a molluscan octopamine receptor that couples to chloride channels in HEK293 cells. *J Biol Chem* **272**(10):6201-6207.
- Grohmann L, Blenau W, Erber J, Ebert PR, Strunker T and Baumann A (2003) Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. *J Neurochem* **86**(3):725-735.
- Han KA, Millar NS and Davis RL (1998) A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J Neurosci* **18**(10):3650-3658.
- Hauser F, Cazzamali G, Williamson M, Blenau W and Grimmlikhuijzen CJ (2006) A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. *Prog Neurobiol* **80**(1):1-19.

- Hauser F, Cazzamali G, Williamson M, Park Y, Li B, Tanaka Y, Predel R, Neupert S, Schachtner J, Verleyen P and Grimmelikhuijzen CJ (2008) A genome-wide inventory of neurohormone GPCRs in the red flour beetle *Tribolium castaneum*. *Front Neuroendocrinol* **29**(1):142-165.
- Huang J, Hamasaki T, Ozoe F, Ohta H, Enomoto K, Kataoka H, Sawa Y, Hirota A and Ozoe Y (2007) Identification of critical structural determinants responsible for octopamine binding to the alpha-adrenergic-like *Bombyx mori* octopamine receptor. *Biochemistry* **46**(20):5896-5903.
- Huang J, Hamasaki T, Ozoe F and Ozoe Y (2008) Single amino acid of an octopamine receptor as a molecular switch for distinct G protein couplings. *Biochem Biophys Res Commun* **371**(4):610-614.
- Isoai A, Kawahara H, Okazaki Y and Shizuri Y (1996) Molecular cloning of a new member of the putative G protein-coupled receptor gene from barnacle *Balanus amphitrite*. *Gene* **175**(1-2):95-100.
- Kawahara H, Isoai A and Shizuri Y (1997) Molecular cloning of a putative serotonin receptor gene from barnacle, *Balanus amphitrite*. *Gene* **184**(2):245-250.
- Maqueira B, Chatwin H and Evans PD (2005) Identification and characterization of a novel family of *Drosophila* beta-adrenergic-like octopamine G-protein coupled receptors. *J Neurochem* **94**(2):547-560.
- Molaei G, Paluzzi JP, Bendena WG and Lange AB (2005) Isolation, cloning, and tissue expression of a putative octopamine/tyramine receptor from locust visceral muscle tissues. *Arch Insect Biochem Physiol* **59**(3):132-149.
- Nyrönen T, Pihlavisto M, Peltonen JM, Hoffren AM, Varis M, Salminen T, Wurster S, Marjamäki A, Kanerva L, Katainen E, Laaksonen L, Savola JM, Scheinin M and Johnson MS (2001) Molecular mechanism for agonist-promoted alpha(2A)-

- adrenoceptor activation by norepinephrine and epinephrine. *Mol Pharmacol* **59**(5):1343-1354.
- Ohta H, Utsumi T and Ozoe Y (2004) Amino acid residues involved in interaction with tyramine in the Bombyx mori tyramine receptor. *Insect Mol Biol* **13**(5):531-538.
- Ohtani A, Arai Y, Ozoe F, Ohta H, Narusuye K, Huang J, Enomoto K, Kataoka H, Hirota A and Ozoe Y (2006) Molecular cloning and heterologous expression of an alpha-adrenergic-like octopamine receptor from the silkworm Bombyx mori. *Insect Mol Biol* **15**(6):763-772.
- Peltonen JM, Pihlavisto M and Scheinin M (1998) Subtype-specific stimulation of [35S]GTPgammaS binding by recombinant alpha2-adrenoceptors. *Eur J Pharmacol* **355**(2-3):275-279.
- Perez DM (2007) Structure-function of alpha1-adrenergic receptors. *Biochem Pharmacol* **73**(8):1051-1062.
- Rader AJ, Anderson G, Isin B, Khorana HG, Bahar I and Klein-Seetharaman J (2004) Identification of core amino acids stabilizing rhodopsin. *Proc Natl Acad Sci U S A* **101**(19):7246-7251.
- Roeder T (1999) Octopamine in invertebrates. *Prog Neurobiol* **59**(5):533-561.
- Roeder T (2005) Tyramine and octopamine: ruling behavior and metabolism. *Annu Rev Entomol* **50**:447-477.
- Rovati GE, Capra V and Neubig RR (2007) The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* **71**(4):959-964.
- Ruuskanen JO, Peitsaro N, Kaslin JV, Panula P and Scheinin M (2005) Expression and function of alpha-adrenoceptors in zebrafish: drug effects, mRNA and receptor distributions. *J Neurochem* **94**(6):1559-1569.

- Savola JM, Ruskoaho H, Puurunen J, Salonen JS and Karki NT (1986) Evidence for Medetomidine as a Selective and Potent Agonist at Alpha-2-Adrenoceptors. *Journal of Autonomic Pharmacology* **6**(4):275-284.
- Shi L and Javitch JA (2002) The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop. *Annu Rev Pharmacol Toxicol* **42**:437-467.
- Sinclair MD (2003) A review of the physiological effects of alpha2-agonists related to the clinical use of medetomidine in small animal practice. *Can Vet J* **44**(11):885-897.
- Small KS, Brudno M, Hill MM and Sidow A (2007) Extreme genomic variation in a natural population. *Proc Natl Acad Sci U S A* **104**(13):5698-5703.
- Smith MW, Borts TL, Emkey R, Cook CA, Wiggins CJ and Gutierrez JA (2003) Characterization of a novel G-protein coupled receptor from the parasitic nematode *H. contortus* with high affinity for serotonin. *J Neurochem* **86**(1):255-266.
- Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG and Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* **454**(7203):486-491.

FOOTNOTES

This is a subproject within the Marine Paint program financially supported by the Swedish Foundation for Strategic Environmental Research (MISTRA) (2001-012).

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

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 ($\dagger\dagger\dagger P<0.001$) (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.

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 solidissima* (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

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 μ M 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 μ M 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]GTP γ S 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]GTP γ S 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 10 μ M) 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.

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 \pm 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 $83\% \pm 9$ for octopamine and $53\% \pm 8$ 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 ($2^{\text{CT}(\text{actin}) - \text{CT}(\text{receptor})}$) 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, * $p < 0.05$).

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

<i>B. imp.</i> receptor	DmOct β 1	DmOct β 2	DmOct β 3
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)

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. EC₅₀ 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.

	EC₅₀ of receptor-mediated stimulation of cAMP formation (nM, mean \pm sem)		
Receptor	Octopamine	Medetomidine	Tyramine
R1	4.6 \pm 0.2	99 \pm 15	950 \pm 247
R3	1.6 \pm 0.4	41 \pm 23	91 \pm 33
R4	0.99 \pm 0.22	33 \pm 1	260 \pm 63
	EC₅₀ of calcium elevation (nM, mean \pm sem)		
	Octopamine	Medetomidine	Tyramine
R0	1.9 \pm 0.4	0.35 \pm 0.12	160 \pm 30

Figure 1

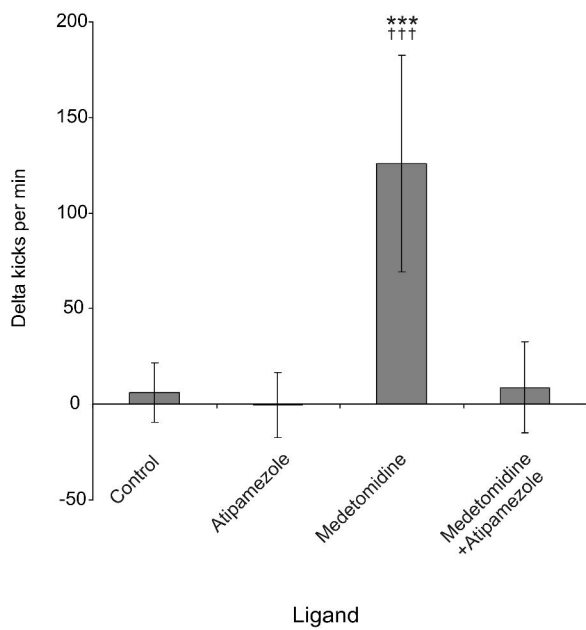
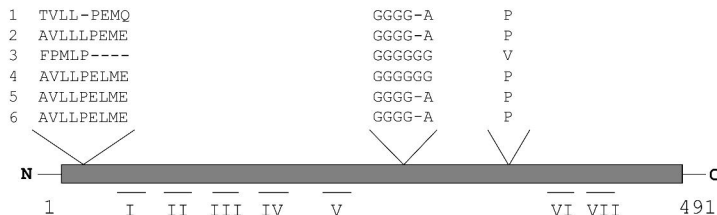


Figure 2

A

Clone



B

Ba_Octa MEGFP LSP-----APADNVTLNVSCGRFATLFDWADHRLISLLALAFNLNMVVA
Bi_R0 MDRELLSPTEAAVLLPELMEEVDNMTVNVS CGQSAF FDWADHRLISLLALAFNLNMVVA

I

Ba_Octa GNLLVVMVAVFVH SKLRTVTNLFIVSLACADLLVGM LVL PFSATLEVLDVWLYGDVWC SVW
Bi_R0 GNLLVVMVAVFVH SKLRTVTNLFIVSLACADLLVGM LVL PFSATLEVLDVWLYGDVWC SVW

II

Ba_Octa LAVDVVMCTSSILNLCAISLD RYLAVSQPISYP SLMSTRRAKQLIAAVVWLSFVICFPFL
Bi_R0 LAVDVVMCTSSILNLCAISLD RYLAVSQPISYP SLMSTRRAKQLIAAVVWLSFVICFPFL

III IV

Ba_Octa VGWNRDPGTLIGSRGSSACRLTCELTNERGYVIYSALGSFFLPSTVMLFFYGRIYRTAVS
Bi_R0 VGWNRDPGTLIGSRGTSACRLTCELTNERGYVIYSALGSFFLPSTVMLFFYGRIYRTAVS

V

Ba_Octa TTRAI AQGFRTTKEDE-EGRLTLRIHRGRSVTORA HQAAAGG--AAHQGVRLTLSEPGA
Bi_R0 TTRAI AQGFRTTKEDEADQRLTLRIHRGRSVTQSAERQAAGGGGARPHQGVRLTLSEPGA

Ba_Octa RRQ NKPSFVVHCREDSRAKNQYEIYTVVEGDSRPGRRVFQ PQRPAKKLSSASQSSEDDSR
Bi_R0 RRQ KKPSPFVVHCREDSRAKNQYEIYTVVEGDSRPGRRAFPQ PQRPAKKLSSASQSSEDETR

Ba_Octa PPRFISRVSRNRNRHQARRFRMETKAAKTVGII VGLFILCWL PFFVVCYLVRGFCADCVPF
Bi_R0 -PPRFISRVSRNRNRHQARRFRMETKAAKTVGII VGLFILCWL PFFVVCYLVRGFCADCVPF

VI

Ba_Octa LLFSVFFWLGVCNSAVNPCVYALCSRDFRFAFSSILCKVCVRRGAMERAFRRTLLVGN-R
Bi_R0 LLFSVFFWLGVCNSAVNPCVYALCSRDFRFAFSSILCKVCVRRGAMERAFRRTLLVQGER

VII

Ba_Octa SQTEEDCEVAD
Bi_R0 SQTEEDA EVAD

Figure 3

R1 MAAPAVTWEPAP--PSGAVTGSVTSEEP--SLLAGVTAAAG-PPPLPLPLPTLLKL
R2 MAEAGVTSDALTRLPLVLLAPLVNASAE--IAAAEISNGTLL-EAELARVPVLAQVLKI
R3 -----MEVNVSASNSS-VLDPAAPPGYMVVLKA
R4 -----MGEFVT--LSVPPTAYNEVISVVQ--DAVPGAGCGAGDMAWHEVTLIVLKA

R1 AAMVAIIVMAVLGNLLVIVSVLLHRRIRVVANCFVVSLAFADMLVALCMTFNASIQTG
R2 TAMTAVILLSVLGNLLVIVSVLLHRRIRVVANYFVMSLAFADMLVALCMTFNASIQTG
R3 ISLSTIIIVMAVLGNLLVVSVYRYRKLRNTNCFVVSLAIADVLVSLIAMPFNASVELSG
R4 LAMVLIIVTSVLGNLLVICSVIRYRRLRVITNYFIVSLALADMLVALGAMTFNASVQLSD

I

II

R1 KWMFGYIMCDMWNSFDVFFSTVSILHLCCISVDRYCAIMRPLEYRSHMNKRTVAAMLIVS
R2 RWMFGYIMCDMWNSFDVFFSTVSILHLCCISVDRYFAILRPLEYRSHMNKRTVAAMLVAS
R3 RWMFGYAMCDVWNSVDVYASTVSIHLCCISIDRYYAIVKPLNYPVITERVVVMMLTFV
R4 RWMFGLVVCDDLWNSLDVYFSTISILHLCCISVDRYVAIVLPLEYPMKMTTRTVLLMILA

III

R1 WTAPVLISFVPIFLGWYTYQSHLDYRARHENTCQFIVNRTYMLVSSTISFWLPGVVMVCM
R2 WTPPAISFVPIFLGWYSTSEHLSYRARHPDCEFIVNEVYMLVSSSVFWIPGVVMVVM
R3 WVFPVSISFVPIFLGWYTTAEHQIFRDENHTCKEFVNNPVAIVSSSISFWIPSTIMCVM
R4 WVAPILISFIPFLGWYATDEYLQSR--HDECTWEVNKWFAAVSSSISFWIPGVIMVCL

IV

V

R1 YSRIYQEARQHRAIRRVPSSARFSS---AHAAVNLADMTDEQLSLALTALNNIREEEFEP
R2 YSRIYREARQHRAISRTPSTINFHSSSTDCGAGGPLDHITTEELTLALSALNNIRDDE--
R3 YFVYQEARQAKFLYKNASASR-----
R4 MLRVFREARDQEQMLHKARGKAQMLV------TQ---

R1 EPAAELVSPLSAGDGNCEGNGNGNGSAEREPRPHREPPLSKKASSVSFGEKLDFS--RK
R2 --SG--G---AGCGGGGGTSPSANGGARGEGAPPPRFLHKKASSVSFSEAATSSDLRK
R3 -----SQIAMNGAISPS-----R
R4 -----NGGSDGGGGTQTT-----H

R1 ASTNSRLGLRSTYSQMLREHKAAKTLGVIMGCFVLCWLPPFSWYATTLCGRACHVPPV
R2 SSNTSRLCRLRFSTSHMLREHKAAKTLGVIMGCFVLCWLPPFTWYAATLCGAACSVPPV
R3 KGPLEGEDRAGETAQKIRHEHKAARTLGIIMGVFILCWLPPFTWYITVSLCREACPCDV
R4 AVDDEHSHSRQ-MHKLRERKAMRTLGLMGALFCWLPPFLWYIIVSLC-DSCHTERI

VI

R1 LVDILFWIGYFNSTLNPMIYAYFNTEFRDAFRQTLSLLCCR-----RRSGSS
R2 LVDILFWIGYLNSTLNPMIYAYFNTEFRDAFRDTLTLVLQC-----RSARSS
R3 VVVVLFWIGYLNCLNPLIYAYFNRDRDAFRQTLQCVFEP-----CCAKAAAE
R4 VESLLFWIGYFNSTLNPIIYAYFNRDFRYAFRSILACVLCHRCCQRDPYHDIYMRHSYP

VII

R1 --LSRPSNLSCPPPMAGDTGNGGAPRPRPCRTPTLQSFPHAARCE-----
R2 GGASYEARNGSQPRADRASGAAGSGSGGAGSSATERPTELAVST-----
R3 -----RSSV-----
R4 TELRRRESVVSQVYQRRSSLSSSELSQVSALVREPAGIHLGSELQPASEFRSPTEQVTLV

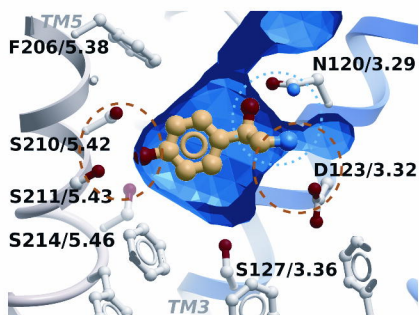
R1 -----
R2 -----
R3 -----
R4 VPPLPAIKPPPQTKQQQQHNQQNQHHPPQQRTPPRSPSRPALRINLLKGQFLNRVPRADK

R1 -----
R2 -----
R3 -----
R4 DKWSGVERMIGLEDLNGRPPQPPAPPPPPPLPLPPHETAAPGS

Phylogenetic tree showing the relationships between various octopamine receptors. The tree is rooted at the top left. Bootstrap values are indicated at the nodes. The tree is divided into two main groups: α-like octopamine receptors (top) and β-like octopamine receptors (bottom). The α-like group includes receptors like 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, 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, and Am_Dop1. The β-like group includes receptors like 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, and Bi_R2.

Figure 5

A



B

α -like receptors		922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040	1041	1042	1043	1044	1045	1046	1047	1048	1049	1050	1051	1052	1053	1054	1055	1056	1057	1058	1059	1060	1061	1062	1063	1064	1065	1066	1067	1068	1069	1070	1071	1072	1073	1074	1075	1076	1077	1078	1079	1080	1081	1082	1083	1084	1085	1086	1087	1088	1089	1090	1091	1092	1093	1094	1095	1096	1097	1098	1099	1100	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130	1131	1132	1133	1134	1135	1136	1137	1138	1139	1140	1141	1142	1143	1144	1145	1146	1147	1148	1149	1150	1151	1152	1153	1154	1155	1156	1157	1158	1159	1160	1161	1162	1163	1164	1165	1166	1167	1168	1169	1170	1171	1172	1173	1174	1175	1176	1177	1178	1179	1180	1181	1182	1183	1184	1185	1186	1187	1188	1189	1190	1191	1192	1193	1194	1195	1196	1197	1198	1199	1200	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210	1211	1212	1213	1214	1215	1216	1217	1218	1219	1220	1221	1222	1223	1224	1225	1226	1227	1228	1229	1230	1231	1232	1233	1234	1235	1236	1237	1238	1239	1240	1241	1242	1243	1244	1245	1246	1247	1248	1249	1250	1251	1252	1253	1254	1255	1256	1257	1258	1259	1260	1261	1262	1263	1264	1265	1266	1267	1268	1269	1270	1271	1272	1273	1274	1275	1276	1277	1278	1279	1280	1281	1282	1283	1284	1285	1286	1287	1288	1289	1290	1291	1292	1293	1294	1295	1296	1297	1298	1299	1300	1301	1302	1303	1304	1305	1306	1307	1308	1309	1310	1311	1312	1313	1314	1315	1316	1317	1318	1319	1320	1321	1322	1323	1324	1325	1326	1327	1328	1329	1330	1331	1332	1333	1334	1335	1336	1337	1338	1339	1340	1341	1342	1343	1344	1345	1346	1347	1348	1349	1350	1351	1352	1353	1354	1355	1356	1357	1358	1359	1360	1361	1362	1363	1364	1365	1366	1367	1368	1369	1370	1371	1372	1373	1374	1375	1376	1377	1378	1379	1380	1381	1382	1383	1384	1385	1386	1387	1388	1389	1390	1391	1392	1393	1394	1395	1396	1397	1398	1399	1400	1401	1402	1403	1404	1405	1406	1407	1408	1409	1410	1411	1412	1413	1414	1415	1416	1417	1418	1419	1420	1421	1422	1423	1424	1425	1426	1427	1428	1429	1430	1431	1432	1433	1434	1435	1436	1437	1438	1439	1440	1441	1442	1443	1444	1445	1446	1447	1448	1449	1450	1451	1452	1453	1454	1455	1456	1457	1458	1459	1460	1461	1462	1463	1464	1465	1466	1467	1468	1469	1470	1471	1472	1473	1474	1475	1476	1477	1478	1479	1480	1481	1482	1483	1484	1485	1486	1487	1488	1489	1490	1491	1492	1493	1494	1495	1496	1497	1498	1499	1500	1501	1502	1503	1504	1505	1506	1507	1508	1509	1510	1511	1512	1513	1514	1515	1516	1517	1518	1519	1520	1521	1522	1523
	Hs_ADRA1A	ADVC	S	LYV	S	S	S	S	F	W	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F																																																							

Figure 6

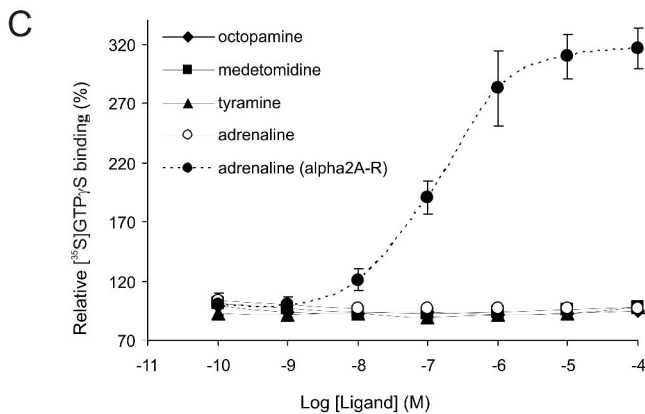
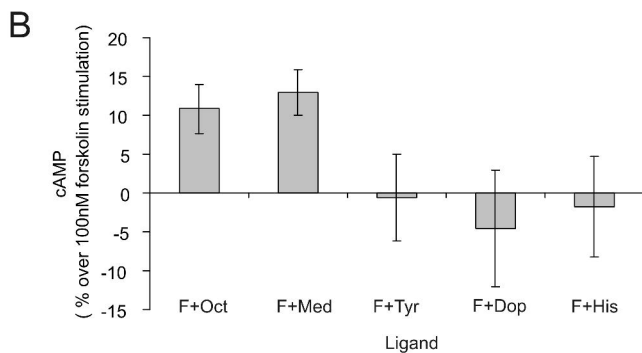
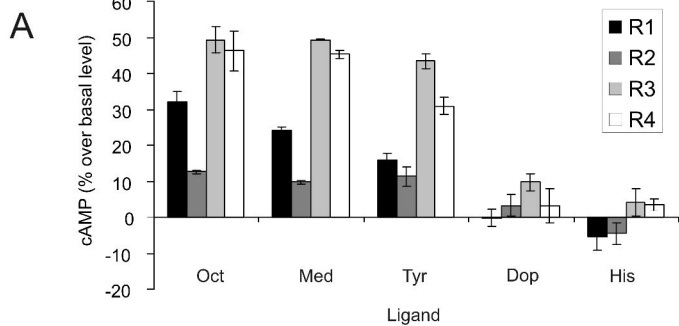


Figure 7

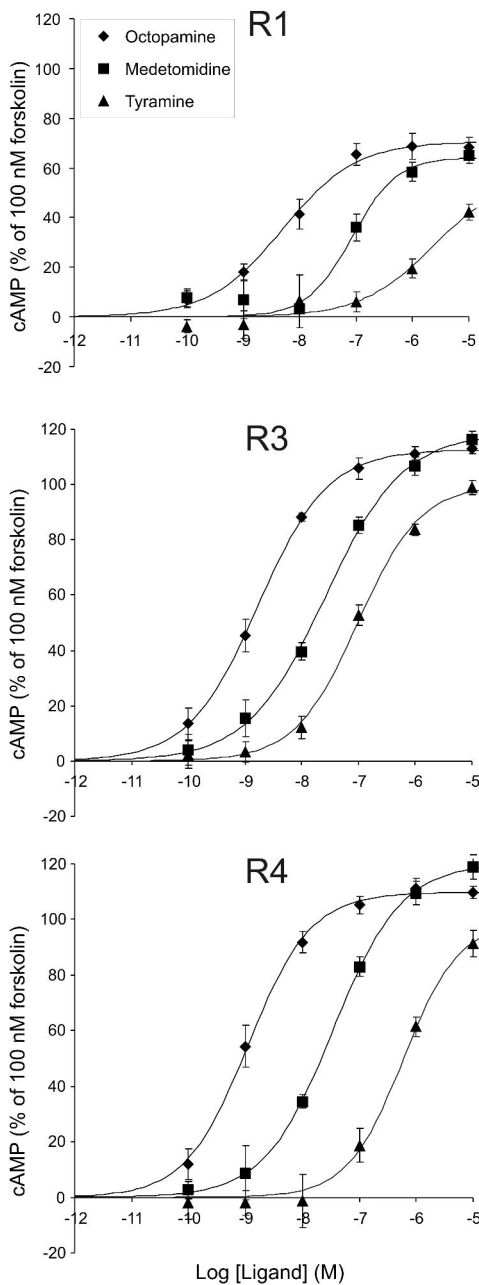
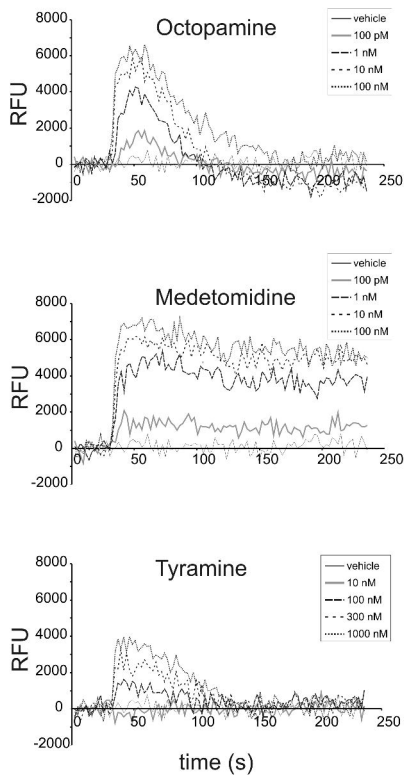


Figure 8

A



B

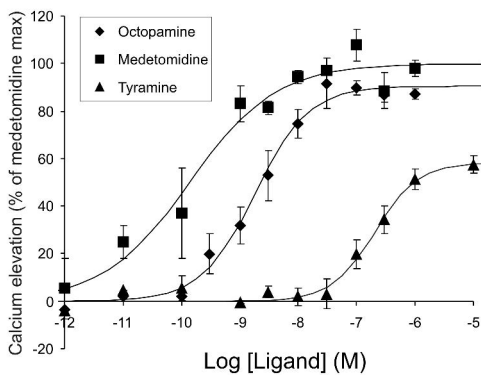


Figure 9

