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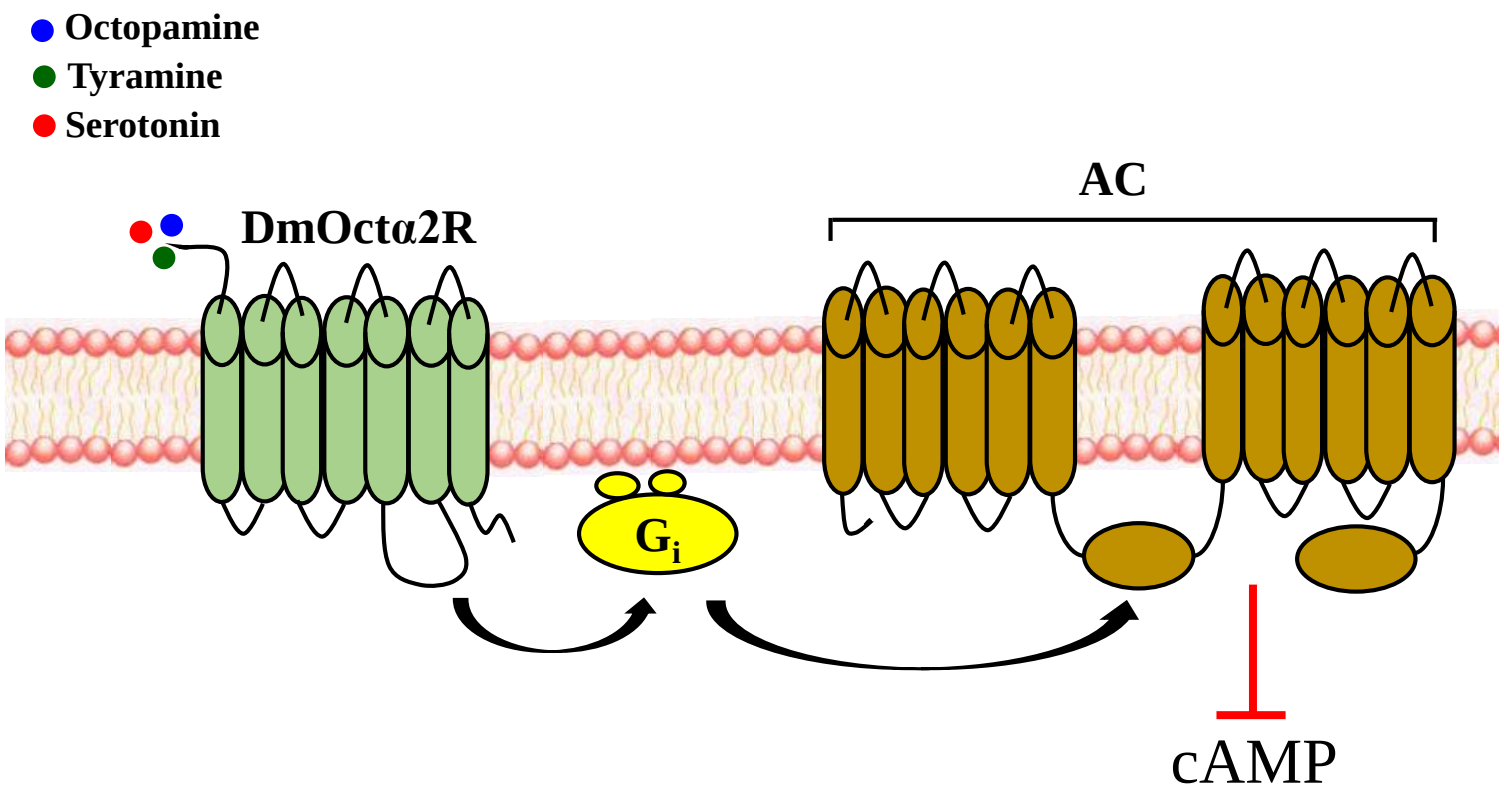
Octopamine
Tyramine
Serotonin
DmOctα2R
AC
G_i
cAMP

A new *Drosophila* octopamine receptor responds to serotonin

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Abstract

As the counterparts of the vertebrate adrenergic transmitters, octopamine and tyramine are important physiological regulators in invertebrates. They control and modulate many physiological and behavioral functions in insects. In this study, we reported the pharmacological properties of a new α2-adrenergic-like octopamine receptor (CG18208) from *Drosophila melanogaster*, named DmOctα2R. This new receptor gene encodes two transcripts by alternative splicing. The long isoform DmOctα2R-L differs from the short isoform DmOctα2R-S by the presence of an additional 29 amino acids within the third intracellular loop. When heterologously expressed in mammalian cell lines, both receptors were activated by octopamine, tyramine, epinephrine and norepinephrine, resulting in the inhibition of cAMP production in a dose-dependent manner. The long form is more sensitive to the above ligands than the short form. The adrenergic agonists naphazoline, tolazoline and clonidine can stimulate DmOctα2R as full agonists. Surprisingly, serotonin and serotoninergic agonists can also activate DmOctα2R. Several tested adrenergic antagonists and serotonin antagonists blocked the action of octopamine or serotonin on DmOctα2R. The data presented here reported an adrenergic-like G protein-coupled receptor activated by serotonin, suggesting that the neurotransmission and neuromodulation in the nervous system could be more complex than previously thought.

Keywords

octopamine, tyramine, serotonin, cAMP, pharmacology, adrenergic receptor, GPCR

Abbreviations

OA, Octopamine; TA, Tyramine; 5-HT, serotonin; DA, Dopamine; GPCR, G protein-coupled receptor; $[cAMP]_i$: intracellular cyclic adenosine monophosphate level; $[Ca^{2+}]_i$: intracellular calcium level; HEK 293 : human embryonic kidney 293; CHO-K1: Chinese hamster ovary K1.

Introduction

Neurotransmitters and neuromodulators like epinephrine (also called adrenaline), norepinephrine (also called noradrenaline), dopamine (DA) and serotonin (5-HT) are important neuroactive molecules to control and regulate many aspects of behaviors and physiology in vertebrates. DA and 5-HT are also important neuroactive substances in invertebrates. However, epinephrine and norepinephrine have no known physiological relevance in protostomes and their roles are considered to be fulfilled by their invertebrate counterparts, octopamine (OA) and tyramine (TA) (Roeder, 2005). Norepinephrine, epinephrine, OA, TA and DA are all derived from tyrosine and their chemical structures are similar (Blenau and Baumann, 2001). 5-HT is derived from tryptophan and structurally quite different from other monoamine neurotransmitters.

Biogenic amines mainly exert their effects through G-protein-coupled receptors (GPCRs). Based on the structural and signaling similarities to vertebrate adrenergic receptors, insect octopamine receptors are grouped into three classes: α-adrenergic-like receptors (OctαR, also known as OAMB or OA1), β-adrenergic-like receptors (OctßR, also known as OA2) and octopamine/tyramine or tyramine receptors (Oct-TyrR or TAR1) (Evans and Maqueira, 2005). Activation of OctαR primarily leads to the elevation of $[Ca^{2+}]_i$ when expressed in cell lines (Balfanz et al., 2005). A total of three receptor genes have been characterized from *Drosophila* encoding ß-type adrenergic-like receptors. Activation of this group of receptors, DmOctß1R, DmOctß2R and DmOctß3R all leads to increased intracellular cAMP levels (Balfanz et al., 2005; Maqueira et al., 2005). Both OctαR and OctßR show high specificity to OA over other biogenic amines such as TA and DA. The TAR1 can be stimulated by both TA and OA to reduce forskolin-stimulated cAMP levels. Based on the pharmacological properties of CG7431 from *Drosophila melanogaster* (Cazzamali et al., 2005) and an orthologous receptor from *Bombyx mori* (Huang et al., 2009), a new class of tyramine receptors (TAR2) was added to the classification (Farooqui, 2012). The group of TAR2 is specifically activated by TA to increase $[Ca^{2+}]_i$ (Huang et al., 2009) or exclusively causes an increase in $[cAMP]_i$ (Reim et al., 2017). Bayliss *et al.* revealed that the receptor encoded by gene *CG16766* is activated by a number of biogenic amines, including TA, OA and DA. It is not only coupled to intracellular Ca^{2+} mobilization, but also reduces forskolin-stimulated cAMP level upon activation (Bayliss et al., 2013). Hence, CG16766 represents a new group of tyramine receptors which is designated the tyramine 3 receptors (TAR3).

Recently, a novel octopamine receptor (*CsOA3*) was cloned from the rice stem borer, *Chilo suppressalis* (Wu et al., 2014). In this study, we isolated the orthologous gene of *CsOA3* from *D. melanogaster* (*CG18208*), named *DmOctα2R*. The *DmOctα2R* gene gives rise to two transcripts by alternative splicing (*DmOctα2R-L* and *DmOctα2R-S*). In stably transfected human embryonic kidney 293 (HEK 293) cells or Chinese hamster ovary K1 (CHO-K1) cells, activation of either DmOctα2R-L or DmOctα2R-S resulted in inhibition of forskolin-stimulated cAMP synthesis. In addition to OA and TA, 5-HT also activates DmOctα2R in a dose dependent manner.

Methods

Chemicals

(±)-Octopamine hydrochloride (OA), tyramine hydrochloride (TA), dopamine hydrochloride (DA), epinephrine hydrochloride (E), norepinephrine hydrochloride (NE), serotonin hydrochloride (5-HT), 5-carboxamidotryptamine maleate salt (5-CT), 5-methoxytryptamine (5-MT), α-methylserotonin maleate salt (αm-5-HT), 8-Hydroxy-DPAT hydrobromide (8-OH-DPAT), lisuride, yohimbine (YH), epinastine (EP); chlorpromazine (CH); phentolamine (PA); Methiothepin mesylate salt (MT), Ketanserin (+)-tartrate salt (KS), G418 disulphate salt, forskolin, 3-isobutyl-1-methylxanthine (IBMX) were all obtained from Sigma-Aldrich (St Louis, MO, USA). Tolazoline hydrochloride, clonidine hydrochloride, naphazoline hydrochloride were purchased from Selleck Chemicals (Houston, TX, USA).

Insects

Flies were reared at 25 ^{0}C and ambient humidity under a 12:12 h light: dark photoperiod using standard cornmeal medium.

Cloning of *CG18208*

Total RNA was isolated from mixed whole bodies of male and female flies using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Single-strand cDNA synthesized from RNA using a ReverTra Ace-α-kit (Toyobo, Osaka, Japan) was used as a template for PCRs. Primers were designed based on

the sequence data of *CG18208* published in FlyBase (http://www.flybase.org). The forward primer CG18208-compF, located upstream of the putative start codon and the reverse primer CG18208-compR, located downstream of the putative stop codon, were used to amplify the full-length coding sequence of the CG18208 cDNA. The PCR program started with a denaturation step for 30 sec at 98 ^{0}C, followed by 38 repeats of the following cycle: one step for 10 sec at 98 ^{0}C, one step for 30 sec at 60 ^{0}C, one step for 1 min at 72 ^{0}C, and a final extension of 10 min at 72 ^{0}C using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA). PCR products were separated to check the size by electrophoresis on a 1.0% agarose gel. The purified PCR product was cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and then sequenced using a 3730 XL DNA analyzer (Applied Biosystems, Carlsbad, CA, USA).

Multiple sequence alignment and phylogenetic analysis

The primary structure of the protein was deduced from the cDNA sequence and used for phylogenetic analysis. Sequences used for the alignments were identified by BLAST programs from the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments of the complete amino acid sequences were performed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The results were displayed by BioEdit. The transmembrane segments were predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The phylogenetic tree and molecular evolutionary analyses were performed using MEGA 5.05 software with the neighbor joining method, using the divergent *Drosophila* FMRFamide receptor (DmFR) as out-group.

Construction of expression plasmids

An expression plasmid containing the Kozak consensus sequence (Kozak, 1987) was constructed by PCR with specific primers CG18208-*Hind III* and CG18208-*XhoI*. The PCR product was digested with *Hind III* and *XhoI*. The digested DNA fragments were purified using PCR Clean-Up Kit (Axygen, Union City, CA, USA) and then subcloned into hemagglutinin epitope-Tagged (HA-Tagged) pcDNA 3.0 vector (Invitrogen) yielding pcCG18208. The correct insertion was confirmed by DNA sequencing.

Cell culture, transfection, and creation of stable cell lines

Human Embryonic Kidney 293 (HEK 293) cells and Chinese hamster ovary K1 (CHO-K1) cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HEK 293 cells and CHO-K1 cells were grown in Dulbecco's modified Eagle's medium (D-MEM) (Gibco BRL, Gaithersburg, MD, USA) and Dulbecco's modified Eagle medium Nutrient Mixture F-12 (1:1) (DMEM/F-12) (Gibco BRL, Gaithersburg, MD, USA) respectively. Both cell lines were supplemented with 10% fetal bovine serum (Gibco BRL) at 37°C and 5% CO_2. After transfection of the pcCG18208 plasmid into the cells using Lipofectamine 2000 (Invitrogen), the antibiotic G418 (0.8 mg/mL) was added to the medium to select for cells that constitutively expressed the receptor. After 2 weeks of G418 selection, G418-resistant colonies were trypsinized in cloning cylinders and transferred to 12-well plastic plates for expansion. Four to six individual cell lines were analyzed for determination of the receptor mRNA expression by RT-PCR and localization of the protein by immunofluorescence ((Fig. S1). Cells were grown on Lab-Tek™ II Chambered Coverglass (Nunc) and fixed for 20 min in 4% paraformaldehyde. Fixed cells were incubated in phosphate buffer containing 5% normal goat serum for 2 hours at 25 ℃, followed by incubation for overnight with anti-HA tag mouse monoclonal antibodies (dilution 1:500, Abcam) at 4 ℃. Samples were rinsed with PBS and incubated with goat anti-mouse secondary antibodies labeled with FITC (dilution 1:500, Abcam) for 2 hours at 25 ℃. After several rinses with PBS, the nucleus was stained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Sigma). Images were recorded using a Zeiss LSM 800 confocal microscope (Carl Zeiss SAS, Germany).The clonal cell line that most efficiently expressed pcCG18208 was chosen for this study.

cAMP determination

cAMP levels were measured as previously described (Qi et al., 2017). Cells were plated into 12-well tissue culture plates (Nunc, Roskilde, Denmark) at a density of 1×10^6 cells per well and incubated at 37°C with 5% CO_2 in a humidified incubator. The cells were preincubated in Dulbecco's phosphate-buffered saline (DPBS; Gibco-Invitrogen) containing 100 uM phosphodiesterase inhibitor IBMX for 20 min at room temperature. After the preincubation, a 50 ul aliquot of D-PBS containing various concentrations of reagents was added. The culture was then incubated for 20 min at room

temperature. The reaction was stopped by aspirating the solution and then adding 250 ul ice-cold cell lysis buffer immediately to lysis the cells. The cell lysate was scraped into 1.5 ml Eppendorf tubes for collections and stored at −70°C until use. The solution was centrifugated and $[cAMP]_i$ in the supernatant was determined using a cAMP ELISA kit (R&D Systems, Minne-apolis, MN) according to the manufacturer's instructions.

Ca^{2+} mobilization

Ca^{2+} levels were measured as previously described (Huang et al., 2012). Briefly, DmOctα2R-S- or DmOctα2R-L- expressing HEK 293 cells were seeded on the coverslip with Dulbecco's modified Eagle's medium and incubated for overnight at 37°C in 5% CO_2. After incubation, the cells were subsequently washed twice with a bathing solution (152 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 10 mM HEPES, pH 7.4). Then, the cells were loaded with Fura2-AM (Dojindo Laboratories, Kumamoto, Japan) using 0.2% Cremophor EL (Sigma–Aldrich) for 30 min. The coverslip was transferred to a microscopic chamber that was constantly perfused with a bathing solution at approximately 2 mL/min. The fluorescence at 510 nm by excitation at 340 or 380 nm with a xenon lamp was measured with individual cells using an Easy Ratio Pro calcium imaging system (PTI, Birmingham, NJ, USA). Recordings were taken from at least 10 cells per field of view. Each experiment was repeated three times or more.

Tissue distribution of CG18208 expression by qRT-PCR

Male and female adults of mixed age were dissected into different parts including heads, thoraxes, abdomens (without wings and legs), wings and legs. Total RNA of the dissected tissues were isolated with Trizol reagent (Invitrogen) according to manufacturer's instructions. Residual genomic DNA was removed by RQ1 RNase-Free DNase (Promega). Quantity of RNA was measured by using a Nanodrop 2000 spectrophotomete (Thermo Scientific Inc., Bremen, Germany). RNA (1 μg) was reverse transcribed to cDNA with the Rever Tra Ace qPCR RT kit (Toyobo, Osaka, Japan). Real-time quantitative PCR (qPCR) with gene-specific primers (Supplementary Table S1) were performed to investigate relative expression in selected tissues. The *RPL 11* gene was used as a reference gene (Collin et al., 2013). Relative expression of CG 18208 was normalized to the reference gene using the

176 $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

177 ## Results

178 ### Cloning and sequence analysis of DmOctα2R

179 The amplification of the full length cDNA of *DmOctα2R* (*CG18208*) resulted in two fragments,
180 *DmOctα2R-S* and *DmOctα2R-L* that originate from alternative splicing of the gene. *DmOctα2R-S*
181 contains an open reading frame (ORF) of 2, 013 bp coding for a protein of 670 amino acid residues,
182 with a calculated molecular weight of 72.1 kDa. The ORF of *DmOctα2R-L* consists of 2, 100 bp and
183 codes for a protein of 699 amino-acid residues, with a calculated molecular weight of 75.3 kDa (Fig. 1).
184 DmOctα2R-L differs from DmOctα2R-S by the presence of an additional 29 amino acids within the
185 third intracellular loop (Fig. 1). The amino acid sequence of DmOctα2R consists of seven hydrophobic
186 transmembrane domains (TM1–TM7) (Fig. 1B) connected by intra- and extracellular loops. Some
187 conserved motifs that are essential for ligand binding and signal transduction are found in both the long
188 and short isoforms of DmOctα2R. The eight consensus motifs for potential N-glycosylation (N-x-[S/T])
189 located in the N-terminus (Fig. 1B) (Swarz and Aebi, 2011). Four consensus sites for phosphorylation
190 by protein kinase C (PKC) ([S/T]-x-[R/K]) are found within the first and the third intracellular loop
191 (T_{142}, S_{145}, S_{427}, T_{439}) (Woodgett et al., 1986). The aspartic acid residue in TM3 ($D^{3.32}$) which might
192 bind to the protonated amino group of the ligand is well conserved in DmOctα2R. At the C-terminus of
193 TM3, the D_{212}-R-Y ($D^{3.49}R^{3.50}Y^{3.51}$) motif is required for G protein coupling (Moro et al., 1993). Serine
194 residues $S_{278/282}$ in TM5 ($S^{5.42/5.46}$) can improve ligand-binding by forming hydrogen bonds. The
195 conserved sequence of F_{636}-X-X-X-W_{640}-X-P_{642} found in the TM6 of DmOctα2R is a common feature
196 among many non-peptide receptors. The second phenylalanine residue ($F^{6.52}$) after the motif is a unique
197 feature of aminergic receptors.

198 Amino acid sequence comparisons between DmOctα2R and other OA receptors in *D. melanogaster*
199 showed 66% similarity between DmOctα2R and DmOA1, 56% between DmOctα2R and DmOctß1R.
200 Unlike DmOA1, DmOctα2R and DmOctß1R have a long N-terminal and a short second extracellular
201 loop between TM4 and TM5 (Fig. 1B). Compared with DmOA1 and DmOctß1R, DmOctα2R have a
202 shorter C-terminal. A phylogenetic analysis of invertebrate biogenic-amine receptors with human
203 catecholamine receptors revealed that DmOctα2R clustered with its orthologues AmOctα2R, CsOA3

and TcOctα2R assembled in a distinct clade, which is close to the clade of human α2-adrenergic receptors and insect TAR1 (Fig. 2).

Functional characterization of DmOctα2R

Biogenic amines specificity

To investigate the intracellular signalling pathways activated by the cloned DmOctα2R-L/S receptors, we generated stable HEK 293 cell lines expressing either DmOctα2R-L or DmOctα2R-S. The ligand specificity of the DmOctα2R receptor was tested by the application of various biogenic amines including octopamine (OA), tyramine (TA), dopamine (DA) and serotonin (5-HT) at a final concentration of 10 μM. The results show that the four biogenic amines had no significant effects on cAMP production in both receptor-expressing cells (Fig. 3A and B). However, except DA, all other biogenic amines reduced forskolin-stimulated cAMP levels in DmOctα2R-S- and DmOctα2R-L-expressing cells (Fig. 3A and B). Such effect was not found in non-transfected control cells (Supplementary Fig. S2). The effects of OA, TA and 5-HT were concentration-dependent and saturable, resulting in a sigmoidal dose–response curve (Fig. 3C and D). Half-maximal reduction of cAMP production (EC_{50}) in DmOctα2R-L-expressing cells was achieved at OA concentration of 1.18×10^{-10} M (log EC_{50} = -9.93±0.061, mean ± SEM), TA concentration of 3.48×10^{-9} M (log EC_{50} = -8.46±0.079), 5-HT concentration of 1.04×10^{-6} M (log EC_{50} =-5.98±0.017). OA and TA was ≈8800- and 300-fold more potent than 5-HT in inhibition of forskolin-stimulated cAMP level. The EC_{50} value of DmOctα2R-S-expressing cells was achieved at OA concentration of 1.38×10^{-9} M (log EC_{50} =-8.86±0.11), TA concentration of 1.08×10^{-7} M (log EC_{50} =-6.97±0.033), 5-HT concentration of 4.93×10^{-6} M (log EC_{50} =-5.31±0.19). Thus, OA is much more potent and efficacious than TA and 5-HT to activate DmOctα2R.

DA can significantly increase forskolin-induced cAMP levels in both non-transfected and DmOctα2R-expressing cells (Supplementary Fig. S2), probably due to the activation of endogenous dopamine receptors in HEK 293 cells. Meanwhile, HEK 293 cells express endogenous β-adrenergic receptors (Gerhardt et al., 1997). To test the effect of dopamine, norepinephrine and epinephrine on DmOctα2R, we generated stable CHO-K1 cell lines expressing either the long form or the short form of DmOctα2R. Neither form of DmOctα2R can be activated by DA (Fig. 4A). The effects of

232 octopamine, epinephrine and norepinephrine on DmOctα2R-L-expressing CHO-K1 cells were
233 concentration-dependent and saturable, with EC_{50} values of 4.89×10^{-10} M (log EC_{50} = -8.31±0.175),
234 1.70×10^{-7} (log EC_{50} =-6.77±0.290) and 2.06×10^{-7} (log EC_{50} =-6.69±0.290) respectively (Fig. 4B) (Table
235 1). Such effect was not found in non-transfected control cells (Supplementary Fig. S3). The EC_{50} value
236 of DmOctα2R-S-expressing CHO-K1 cells was achieved at OA concentration of 1.45×10^{-8} M (log EC_{50}
237 =-7.84±0.134). However, both norepinephrine and epinephrine displayed poor agonistic activity at
238 DmOctα2R-S receptor (Fig. 4C). The results indicate that OA is much more potent and efficacious than
239 epinephrine and norepinephrine to activate DmOctα2R. When heterologously expressed in HEK 293
240 cells or CHO-K1 cells, the long form is more sensitive to the ligands than the short form.
241 We also examined the effects of OA, TA, DA and 5-HT on intracellular Ca^{2+} level in DmOctα2R-L-
242 and DmOctα2R-S- expressing cells. The results show that none of the tested amines were able to
243 generate intracellular Ca^{2+} response (supplemental Fig. S4). Application of adenosine triphosphate
244 (ATP) served as a positive control to demonstrate that the Ca^{2+} detection system worked properly.

245 **Action of agonists and antagonists on modulation of intracellular cAMP levels**

246 To characterize the pharmacological profile of DmOctα2R, we examined the effects of various
247 potential agonists on DmOctα2R-L-expressing cells. As show in Fig. 5A, the adrenergic agonists
248 naphazoline, tolazoline and clonidine decreased forskolin-stimulated cAMP levels in
249 DmOctα2R-L-expressing cells. The EC_{50} values for naphazoline, tolazoline and clonidine were
250 4.49×10^{-12} M (log EC_{50} =-11.35±0.057), 1.3×10^{-10} M (log EC_{50} =-9.89±0.027) and 7.28×10^{-11} M (log
251 EC_{50} =-10.14±0.067) respectively. Lisuride, a dopamine and a partial agonist for several serotonin
252 receptors, exhibited high potency on DmOctα2R-L, with an EC_{50} of 1.05×10^{-10} M (log EC_{50}
253 =-9.98±0.079). We also tested the serotonin receptor agonists including 5-methoxytryptamine (5-MT),
254 α-methylserotonin (αm5-HT), 8-Hydroxy-DPAT (8-OH-DPAT), 5-carboxamidotryptamine (5-CT) and
255 AS 19 on DmOctα2R-L-expressing cells. The EC_{50} values for 5-MT, αm5-HT and 8-OH-DPAT were
256 1.29×10^{-6} M (log EC_{50} =-5.89±0.038), 9.20×10^{-7} M (log EC_{50} =-6.04±0.032) and 1.01×10^{-7} M (log
257 EC_{50} =-6.70±0.034) respectively. However, both 5-CT and AS 19 displayed poor agonistic activity.
258 Control experiments indicated that none of the tested agonists were able to decrease
259 forskolin-stimulated cAMP levels in non-transfected wild-type HEK 293 cells (data not shown). Our

data indicated that both adrenergic agonists and serotonergic agonists can stimulate DmOctα2R.

Furthermore, the effects of putative antagonists on OA or 5-HT induced attenuation of cAMP synthesis were assayed. Potential antagonists were tested by simultaneously applying OA (300 pM) or 5-HT (1 μM) and a high dose of antagonist (1 μM or 10 μM) on DmOctα2R-L- expressing cells. The effects of OA could be strongly blocked by the octopamine receptor blocker epinastine (EP). The typical adrenergic antagonist phentolamine (PA) also inhibited OA-induced effects, significantly (Fig. 6A). The α2-adrenergic antagonist yohimbine (YH), which also shows affinity for serotonin receptors, displayed strong inhibition of OA-induced responses. The broad-spectrum antagonist chlorpromazine (CH) inhibited OA effects significantly (Fig. 6A). However, SCH 23390, a dopaminergic antagonists, displayed no detectable blocking effect on the receptor (data not shown). Methiothepin (MT), a non-selective antagonists of mammalian 5-HT receptors and ketanserin (KS), a selective antagonist of mammalian 5-HT_2 were able to block the 5-HT effect (Fig. 6B). Interestingly, epinastine, phentolamine, yohimbine and chlorpromazine were all able to antagonize 5-HT induced attenuation of cAMP synthesis (Fig. 6C). Furthermore, the serotonin receptor antagonist methiothepin and ketanserin inhibit the OA-induced effects, significantly (Fig. 6D). All tested antagonists had no significant effect on $[cAMP]_i$ in non-transfected cells (data not shown).

Tissue-specific expression of DmOctα2R

To compare the mRNA expression levels of the two isoforms, *DmOctα2R-L* and *DmOctα2R-S*, quantitative real-time PCR was performed with cDNA obtained from different body parts of adult males and females. It should be noted *DmOctα2R* amplified with the primers contained two isoforms, which coexpressed in a ratio strongly favoring the long isoform, *DmOctα2R-L* (Fig. 7). Male flies express much higher mRNA levels of *DmOctα2R* and *DmOctα2R-L* than females. *DmOctα2R* and *DmOctα2R-L* are mainly expressed in the heads, thoraxes and legs of adult flies (Fig. 7).

Discussion

In this study, we cloned and functionally characterized an orphan gene *CG18208* from the fruit fly, *Drosophila melanogaster*. Structural and pharmacological studies as well as phylogenetic analysis demonstrated that this gene encodes a α2-adrenergic-like octopamine receptor, which we named it

DmOctα2R. Alternative splicing of *CG18208* generated two protein isoforms which share the typical seven transmembrane architecture of GPCRs. Activation of DmOctα2R by OA, TA, epinephrine or norepinephrine led to a substantial decrease of forskolin-induced cAMP synthesis. Surprisingly, 5-HT also attenuated the forskolin-induced production of cAMP in DmOctα2R-expressing cells. DA had no significant effect on DmOctα2R. To date, octopamine receptors have been cloned and characterized from several invertebrate species, all of them are activated by both OA and TA and some of them are also stimulated by DA (Li et al., 2016; Wu et al., 2014). OA, TA and DA are monoamines synthesized from the same precursor, the amino acid tyrosine (Roeder, 2005) and their structures are similar. However, the chemical structure of 5-HT is quite different from other biogenic amines. To our knowledge, there is no known adrenergic GPCR which can be activated by 5-HT at the range of physiological concentrations.

DmOctα2R exhibits unique pharmacological properties which are distinct from the other two classes of octopamine receptors. For tested synthetic agonists, naphazoline is more potent than clonidine and tolazoline is the least potent. Serotonin receptor agonists also activate DmOctα2R with less potency except lisuride, which showed comparable activity with adrenergic agonists. Epinastine is found to be a highly selective antagonist for insect octopamine receptors in nervous tissue homogenates (Roeder et al., 1998), however, it is not effective on other cloned octopamine receptors (Huang et al., 2010; Wu et al., 2012). In our antagonist assays, epinastine blocked the actions of OA or 5-HT on DmOctα2R-expressing cells, suggesting that it may be specific for Octα2R family. Chlorpromazine seems to be a general octopamine receptor antagonist since it is also effective on two silkworm octopamine receptors, BmOAR1 and BmOAR2 (Chen et al., 2010; Huang et al., 2010; Ohtani et al., 2006). Two α2-adrenergic blockers phentolamine and yohimbine antagonize DmOctα2R activity, indicating the potential pharmacological similarity between α2-adrenergic receptors and DmOctα2R. The inhibition effect of serotonergic antagonists methiothepin and ketaserin on OA/5-HT-induced cAMP decreases in DmOctα2R-expressing cells further prove that DmOctα2R shares pharmacological properties with serotonin receptors.

Bauknecht and Jekely recently revealed that the orthologous genes of Octα2R from the marine annelid *Platynereis dumerilii* and a hemichordate deuterostome *Saccoglossus kowalevskii* are more sensitive to norepinephrine than octopamine, hence, Octα2R was considered as an adrenergic α2 receptor and is absent in most insects (Bauknecht and Jekely, 2016). However, our results indicate that

Octα2R of *Drosophlia* is more sensitive to octopamine than norepinephrine. We also found that all the arthropods with a sequenced genome that we investigated contain Octα2R-type receptors (Table 2). In other protostomes, such as the Priapulida, Nematoda, Annelida and Mollusca also contain Octα2R-type receptor, suggesting that the presence of Octα2R receptor is a general phenomenon in protostomia phyla. Actually, these so-called adrenergic α1 and α2 receptors (Bauknecht and Jekely, 2016) may function as octopamine receptors in vivo, because protostomes contain substantial amounts of octopamine, but only traces of norepinephrine and epinephrine (Pflüger and Stevenson, 2005). We also found the homologous genes of Octα2R in cnidarians by BLAST analyses, but they do not cluster with orthologues in protostomes (Supplementary Fig. S4). Without the pharmacological data, whether these receptors are adrenergic-like remains uncertain. The GPCR family is believed to have evolved about 1.2 billion years ago (Peroutka and Howell, 1994). Serotonin receptors appear to be among the oldest receptors within the rhodopsin-like family (Römpler et al., 2007). It has been speculated that the primordial serotonin receptor of the rhodopsin-GPCR family may have first appeared more than 700-750 million years ago, a time that likely predates the evolution of muscarinic, dopaminergic, and adrenergic receptor systems (Peroutka and Howell, 1994). Since DmOctα2R is responsive to serotonin, we suppose that Octα2R could be one of the oldest adrenergic-like receptors evolved from serotonin receptors.

Different biogenic amines may have opposite or similar modulatory effects on animal behaviors. TA and OA have opposite effects on the locomotion of *Drosophila* larvae (Saraswati et al., 2004). OA and 5-HT have opposite effects on antipredator behavior in the orb-weaving spider, *Larinioides cornutus* (Jones et al., 2011). In *Drosophila*, dispensable, redundant, complementary, and cooperative roles of OA, DA and 5-HT were found in modulating distinct behaviors (Chen et al., 2013). Since DmOctα2R is stimulated by three kinds of biogenic amines, the neurotransmission and neuromodulation in the nervous system may be more complex than previously thought.

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479 359, 81-86.

480

481

Figures and legends

Fig. 1. Sequence analysis of the two isoforms of DmOctα2R. (**A**) Cartoon illustration of the two transcripts from the *DmOctα2R*. The longer transcript contains three exons and the shorter transcript have a truncated exon 3, originating from the use of an alternative splice donor site. Alternatively spliced location was indicated by black box and the nucleotide sequences surrounding the spliced sites were shown**.** (**B**) Comparison of deduced amino acid sequences of DmOA1A, DmOctß1R, DmOctα2R-L and DmOctα2R-S receptors. The last residue in each line is indicated at the right. Identical residues in all sequences are shown as white letters against black, whereas conservatively substituted residues are shaded. Dashes indicate gaps that were introduced to maximize homologies. The predicted seven transmembrane regions are indicated by TM1 – 7. Potential N-glycosylation sites (●) and potential phosphorylation sites (★) for protein kinase C are labeled. The aspartic acid residue ($D^{3.32}$) and the serine residues ($S^{5.42}$, $S^{5.46}$) that are predicted to be involved in agonist binding are labeled with filled triangles and quadrilateral, respectively. The second phenylalanine (◊) after the FxxxWxP motif in TM6 is a unique feature of aminergic receptors ($F^{6.52}$). The red box indicates the amino acid residues originating from alternative splicing of the *DmOctα2R* gene.

Fig. 2. Phylogenetic analysis of DmOctα2R-S/L and other selected invertebrate biogenic amine receptors with human catecholamine receptors. Neighbor joining trees were constructed using MEGA 5.05 software with 1000-fold bootstrap re-sampling. The numbers at the nodes of the branches represent the level of bootstrap support for each branch. *Drosophila melanogaster* FMRF (DmFR) amide receptor was used as outgroup. For accession numbers to the used sequences, see Supplementary Table S2.

Fig. 3. Effects of biogenic amines on intracellular cAMP levels in DmOctα2R-L-expressing (A, C) and DmOctα2R-S-expressing (B, D) HEK293 cells. For **A** and **B**, concentration of the reagents used were 10 μM. The amount of cAMP is given as the percentage of the value obtained with 10 μM forskolin (= 100%). Data are expressed as the mean ± SEM of at least three independent experiments, each performed in triplicate. Asterisks indicate values significantly different from the forskolin treatment value using one-way ANOVA followed by Dunnett's multiple comparison test (*$p < 0.05$,

511 **p < 0.01, ***p < 0.001). Abbreviations: FK: forskolin; OA, octopamine; TA, Tyramine; DA,
512 Dopamine.
513
514 **Fig. 4. Effects of dopamine, octopamine (OA), norepinephrine (NE) and epinephrine (E) on**
515 **intracellular cAMP levels in DmOctα2R-expressing CHO-K1 cells.** (**A**) Effects of dopamine on the
516 long isoform (DmOctα2R-L) and short isoform (DmOctα2R-S) of DmOctα2R. (**B**)
517 Concentration-response curves of OA, NE and E on intracellular cAMP levels in
518 DmOctα2R-L-expressing CHO-K1 cells. (**C**) Concentration-response curves of OA, NE and E on
519 intracellular cAMP levels in DmOctα2R-S-expressing CHO-K1 cells. The amount of cAMP is given as
520 the percentage of the value obtained with 10 μM forskolin (= 100%). Data are expressed as the mean ±
521 SEM of two independent experiments, each performed in triplicate.
522
523 **Fig. 5. Effects of various agonists on forskolin-stimulated intracellular cAMP levels in**
524 **DmOctα2R-L-expressing cells.** (**A**) Concentration-response curves of several adrenergic-receptor
525 agonists on $[cAMP]_i$. (**B**) Concentration-response curves of several serotonin receptor agonists on
526 $[cAMP]_i$. The amount of cAMP is given as the percentage of the value obtained with 10 μM forskolin
527 (= 100%). Data are expressed as the mean ± SEM of at least three independent experiments, each
528 performed in triplicate. Abbreviations: 5-MT, 5-methoxytryptamine; αm-5-HT, α-methylserotonin;
529 8-OH-DPAT, 8-Hydroxy-DPAT hydrobromide.
530
531 **Fig. 6. Effects of several antagonists on the long isoform of DmOctα2R.** (**A**) Effects of
532 adrenergic-receptor antagonists on OA attenuation of forskolin-stimulated intracellular cAMP levels in
533 DmOctα2R-L-expressing HEK 293 cells. (**B**) Effects of serotonin receptor antagonists on 5-HT
534 attenuation of forskolin-stimulated intracellular cAMP levels in DmOctα2R-L -expressing HEK 293
535 cells. (**C**) Effects of adrenergic-receptor antagonists on 5-HT attenuation of forskolin-stimulated
536 intracellular cAMP levels in DmOctα2R-L-expressing HEK 293 cells. (**D**) Effects of serotonin receptor
537 antagonists on OA-mediated attenuation of forskolin-stimulated intracellular cAMP levels. The amount
538 of cAMP is given as the percentage of the value obtained with 10 μM forskolin (= 100%). Data
539 represent means ± SEM of at least three independent experiments performed in triplicate. Asterisks
540 indicate values significantly different from the control value using one-way ANOVA followed by

Dunnett's multiple comparison test (*p < 0.05, **p < 0.01, ***p < 0.001). Abbreviations: FK: forskolin; OA, octopamine; EP: epinastine; CH: chlorpromazine; PA, phentolamine; YH: yohimbine; 5-HT: serotonin; MT: methiothepin; KS: ketanserin.

Fig. 7. mRNA expression levels of the *DmOctα2R* gene in different body parts of adult flies. The tissues were pooled for each mRNA isolation. Data represent means ± SEM of two independent experiments performed in triplicate.

Table 2 Representatives of adrenergic-like receptors identified in protostomia phyla.

Group/Species name	Octα1R/OA1 (gene/protein ID)	OctβR/OA2 (gene/protein ID)	α1-adrenergic	Octα2R /α2-adrenergic/OA3 (gene/protein ID)	TAR1 (gene/protein ID)	TAR2 (gene/protein ID)	TAR3 (gene/ protein ID)
Arthropoda							
Drosophila melanogaster	CG3856	Octβ1R: CG6919 Octβ2R: CG6989 Octβ3R: CG31351		**CG18208**	CG7485	CG7431	CG16766
Tribolium castaneum	XP_008198470	Octβ1R: XP_015838945 Octβ2R: NP_001280501 Octβ3R: NP_001280505		**XP_015839170**	NP_001164311	XP_015838738	
Apis mellifera	NP_001011565	Octβ1R: CCO13922 Octβ2R: CCO13923 Octβ3R: CCO13924 Octβ4R: CCO13925		**XP_001122075**	NP_524419	NP_650652	
Acyrthosiphon pisum	XP_016658387	Octβ1R: XP_001947781 Octβ2R: XP_001944827 Octβ3R: XP_001948521		**XP_003247818**	XP_001944003	XP_008188216	
Bombyx mori	NP_001091748	Octβ1R: XP_004922133 Octβ2R: NP_001171666		**XP_012551639**	NP_001037504	NP_001164649	
Pediculus humanus	XP_002429940	Octβ1R: XP_002422997 Octβ2R: XP_002430734 Octβ3R: XP_002431813		**XP_002430048**	XP_002426131	XP_002429476	
Daphnia pulex	EFX88331	EFX87996	EFX64650	**EFX76926**			
Ixodes scapularis	XP_002408812	XP_002411135	XP_002399655	**XP_002400445**	XP_002415939		
Priapulida							

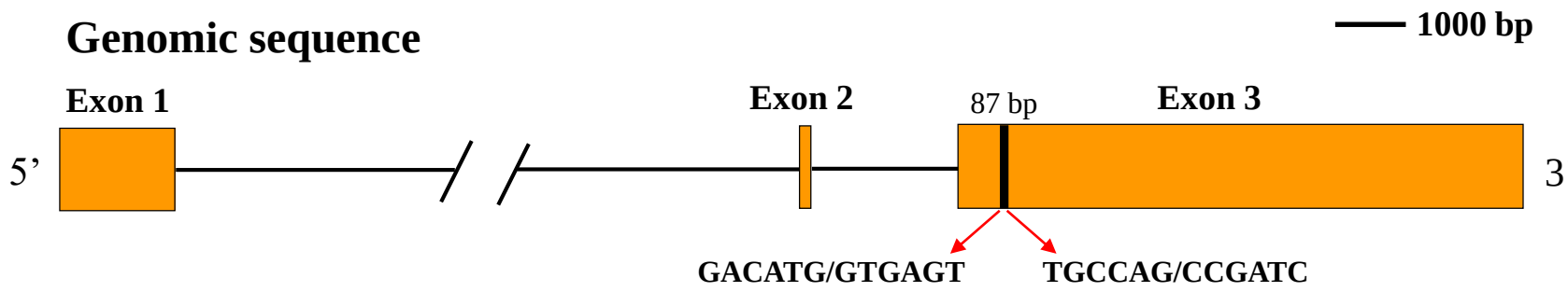
Priapulus caudatus	XP_014675454	XP_014671143	XP_014662992	**XP_014681069**	XP_014672793	XP_014678717
Nematoda						
Caenorhabditis elegans	ser-3 （NP_491954）			**OCTR-1 （NP_001024568）**	ser-2 (NP_001024339)	Tyra-2 (NP_001033537)
Annelida						
Platynereis dumerilii	APC23183	APC23841	APC23842	**APC23843**	AKQ63052	APC23184
Mollusca						
Lottia gigantea	XP_009058244	XP_009052442	XP_009050255	**XP_009067029**		
Cnidaria						
Exaiptasia pallida				KXJ16327 (？)		
Acropora digitifera				XP_015757865 (？)		

Table 1 cAMP formation in cells expressing DmOctα2R-L or DmOctα2R-S induced by various agonists in a concentration-dependent manner.

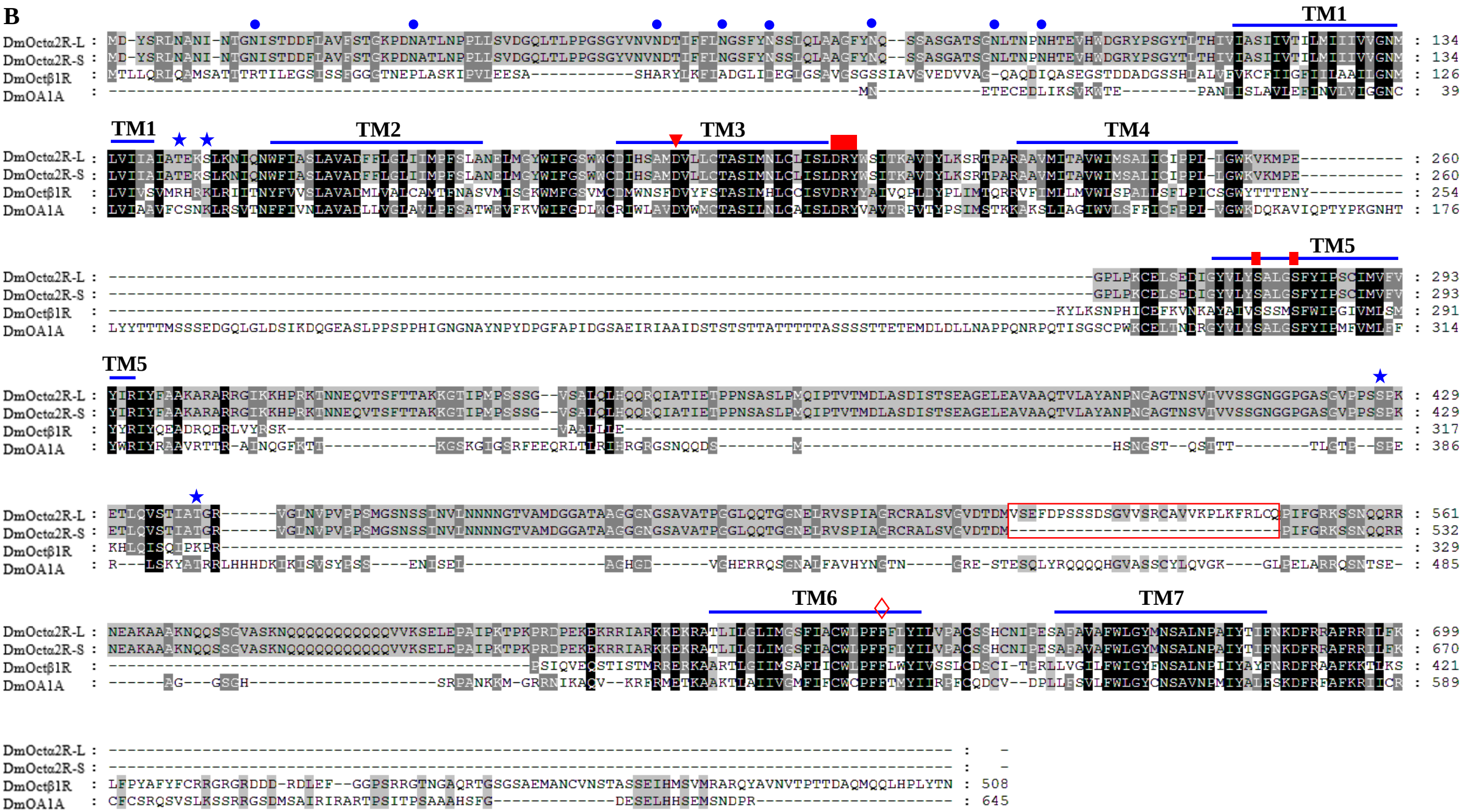
Agonist	DmOctα2R-L		DmOctα2R-S	
	EC_{50} (M)	Log EC_{50} (mean±SEM)	EC_{50} (M)	Log EC_{50} (mean±SEM)
HEK 293 cells				
OA	1.18×10^{-10}	-9.93±0.061	1.38×10^{-9}	-8.86±0.11
TA	3.48×10^{-9}	-8.46±0.079	1.08×10^{-7}	-6.97±0.033
5-HT	1.04×10^{-6}	-5.98±0.017	4.93×10^{-6}	-5.31±0.19
tolazoline	1.3×10^{-10}	-9.89±0.027	ND	
clonidine	7.28×10^{-11}	-10.14±0.067	ND	
naphazoline	4.49×10^{-12}	-11.35±0.057	ND	
lisuride	1.05×10^{-10}	-9.98±0.079	ND	
5-MT	1.29×10^{-6}	-5.89±0.038	ND	
αm-5-HT	9.20×10^{-7}	-6.04±0.032	ND	
8-OH-DPAT	1.01×10^{-7}	-6.70±0.034	ND	
5-CT	–	–	ND	
AS 19	–	–	ND	
CHO-K1 cells				
OA	4.89×10^{-10}	-8.31±0.175	1.45×10^{-8}	-7.84±0.134
Norepinephrine	2.06×10^{-7}	-6.69±0.290	–	–
Epinephrine	1.70×10^{-7}	-6.77±0.290	–	–

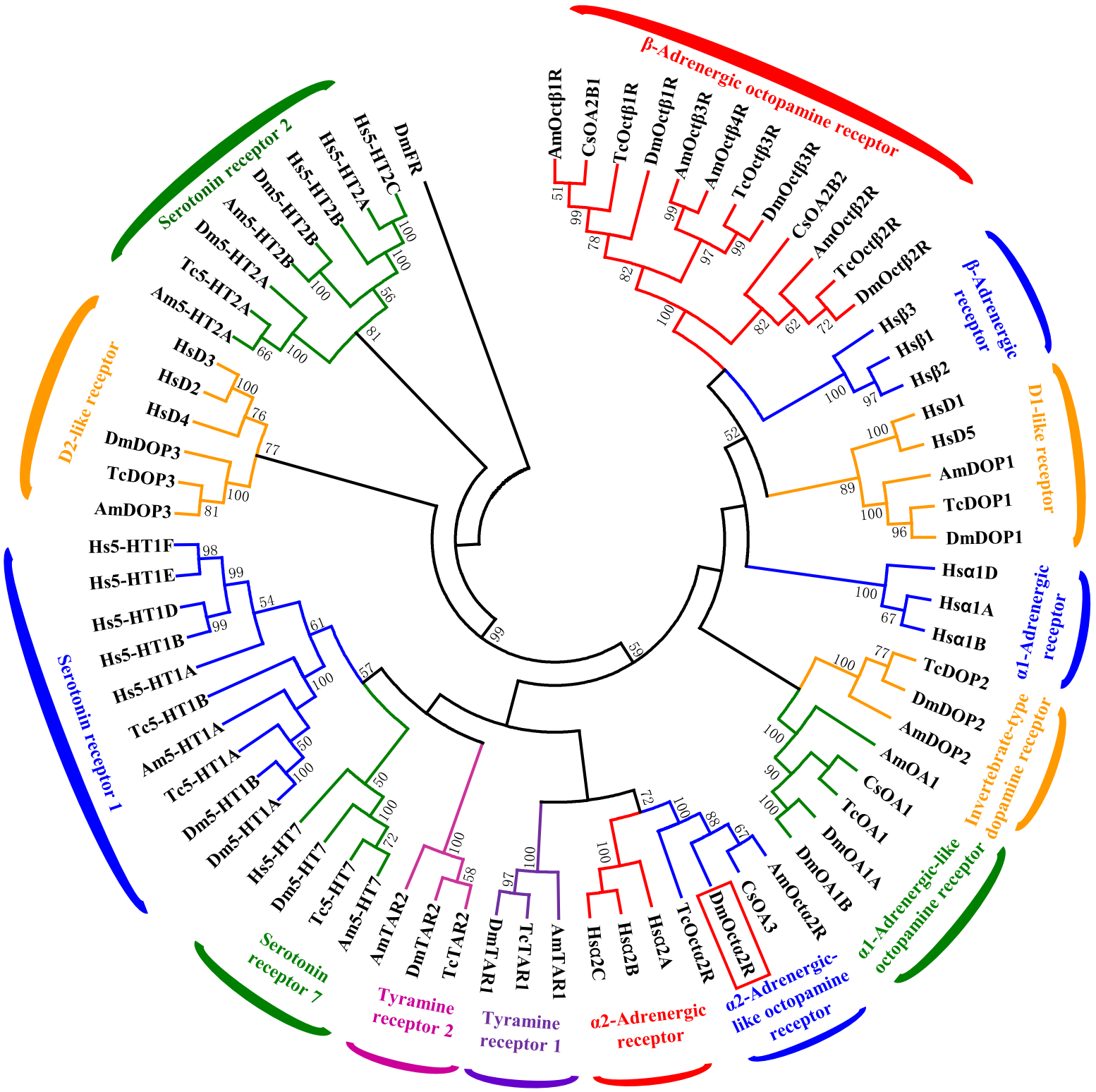
A

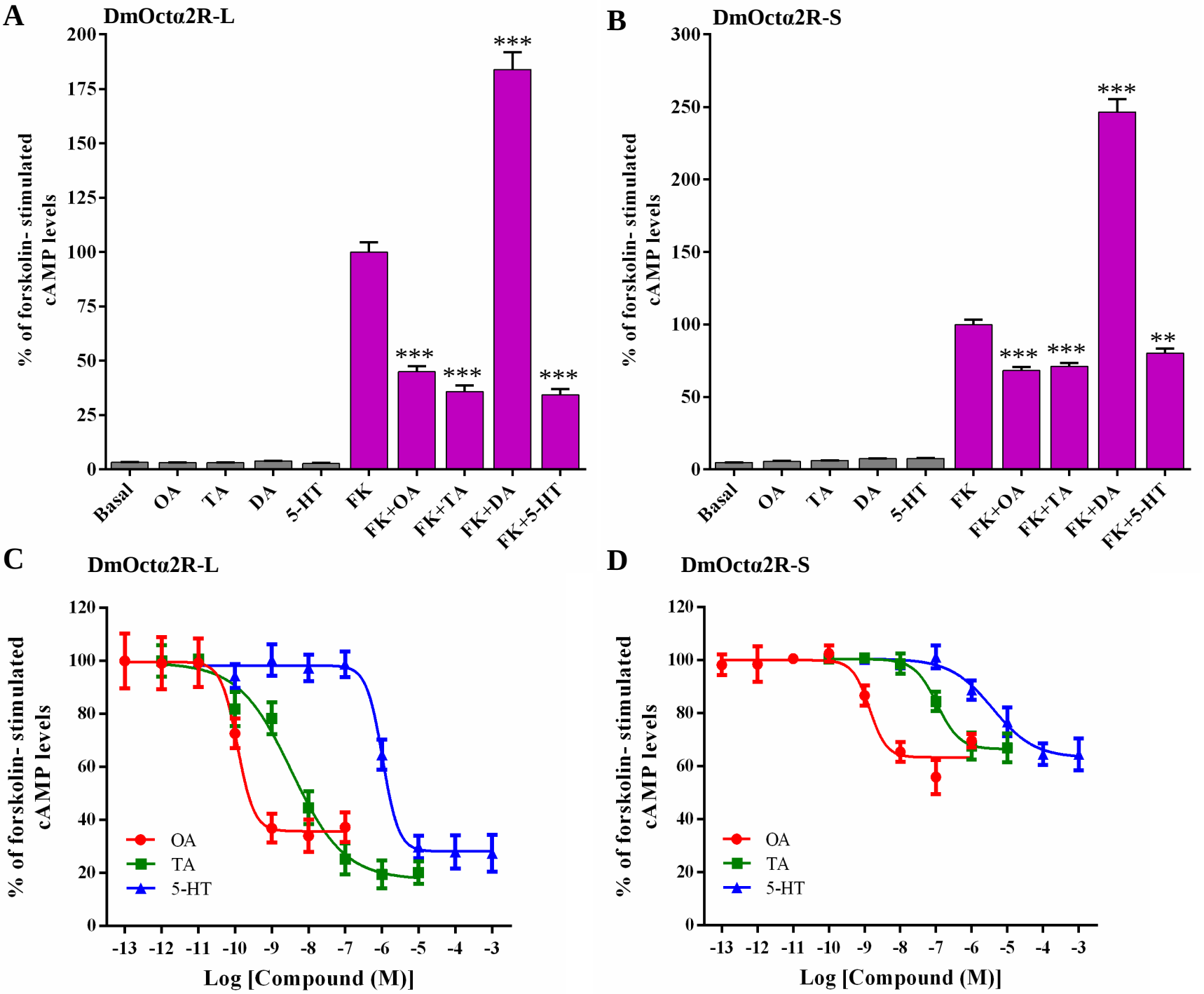
DmOctα2R (CG18208)

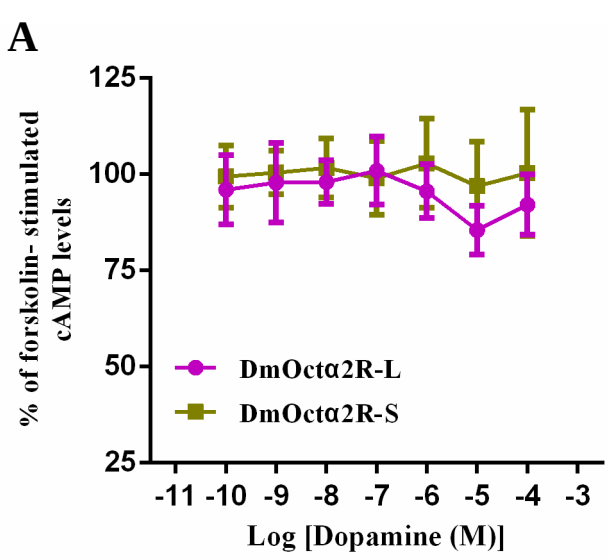


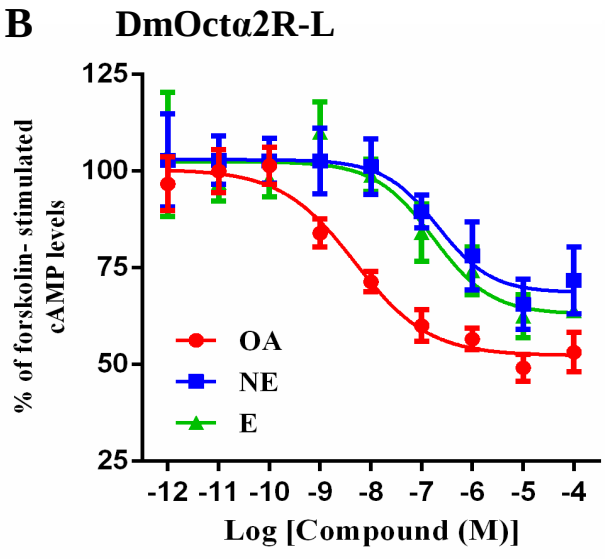
B

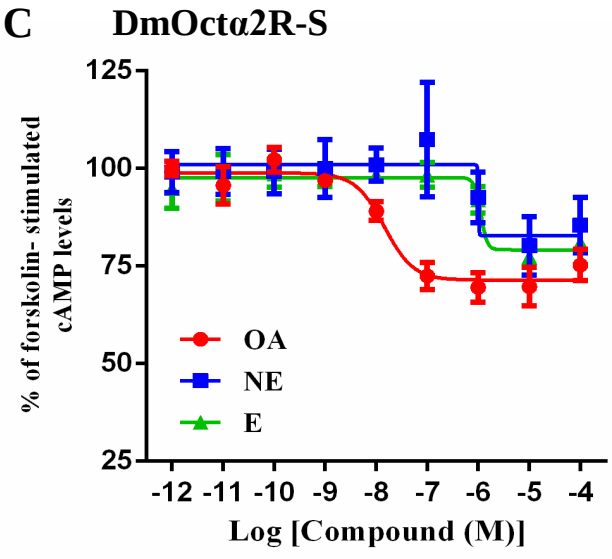



β-Adrenergic octopamine receptor
AmOctβ1R
CsOA2B1
TcOctβ1R
DmOctβ1R
AmOctβ3R
AmOctβ4R
TcOctβ3R
DmOctβ3R
CsOA2B2
AmOctβ2R
TcOctβ2R
DmOctβ2R
β-Adrenergic receptor
Hsβ3
Hsβ1
Hsβ2
D1-like receptor
HsD1
HsD5
AmDOP1
TcDOP1
DmDOP1
α1-Adrenergic receptor
Hsα1D
Hsα1A
Hsα1B
Invertebrate-type dopamine receptor
TcDOP2
DmDOP2
AmDOP2
α1-Adrenergic-like octopamine receptor
AmOA1
CsOA1
TcOA1
DmOA1A
DmOA1B
α2-Adrenergic-like octopamine receptor
AmOctα2R
CsOA3
DmOctα2R
TcOctα2R
α2-Adrenergic receptor
Hsα2A
Hsα2B
Hsα2C
Tyramine receptor 1
AmTAR1
TcTAR1
DmTAR1
Tyramine receptor 2
AmTAR2
DmTAR2
TcTAR2
Serotonin receptor 7
Am5-HT7
Tc5-HT7
Dm5-HT7
Hs5-HT7
Serotonin receptor 1
Hs5-HT1F
Hs5-HT1E
Hs5-HT1D
Hs5-HT1B
Hs5-HT1A
Tc5-HT1B
Am5-HT1A
Tc5-HT1A
Dm5-HT1B
Dm5-HT1A
D2-like receptor
AmDOP3
TcDOP3
DmDOP3
HsD4
HsD2
HsD3
Serotonin receptor 2
Am5-HT2A
Tc5-HT2A
Dm5-HT2A
Am5-HT2B
Dm5-HT2B
Hs5-HT2B
Hs5-HT2A
Hs5-HT2C
DmFR

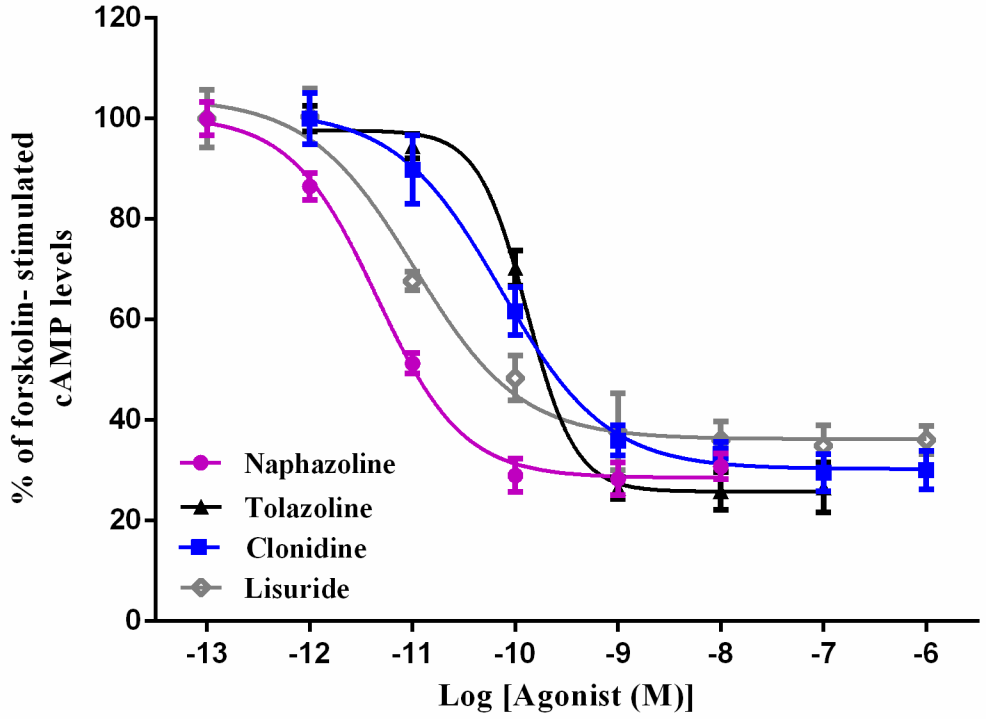

A
DmOctα2R-L
% of forskolin- stimulated cAMP levels
200
175
150
125
100
75
50
25
0
***
***
***
***
Basal
OA
TA
DA
5-HT
FK
FK+OA
FK+TA
FK+DA
FK+5-HT
B
DmOctα2R-S
% of forskolin- stimulated cAMP levels
300
250
200
150
100
50
0
***
***
***
**
Basal
OA
TA
DA
5-HT
FK
FK+OA
FK+TA
FK+DA
FK+5-HT
C
DmOctα2R-L
% of forskolin- stimulated cAMP levels
120
100
80
60
40
20
0
OA
TA
5-HT
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log [Compound (M)]
D
DmOctα2R-S
% of forskolin- stimulated cAMP levels
120
100
80
60
40
20
0
OA
TA
5-HT
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log [Compound (M)]


A
% of forskolin- stimulated cAMP levels
125
100
75
50
25
-11 -10 -9 -8 -7 -6 -5 -4 -3
Log [Dopamine (M)]
DmOctα2R-L
DmOctα2R-S

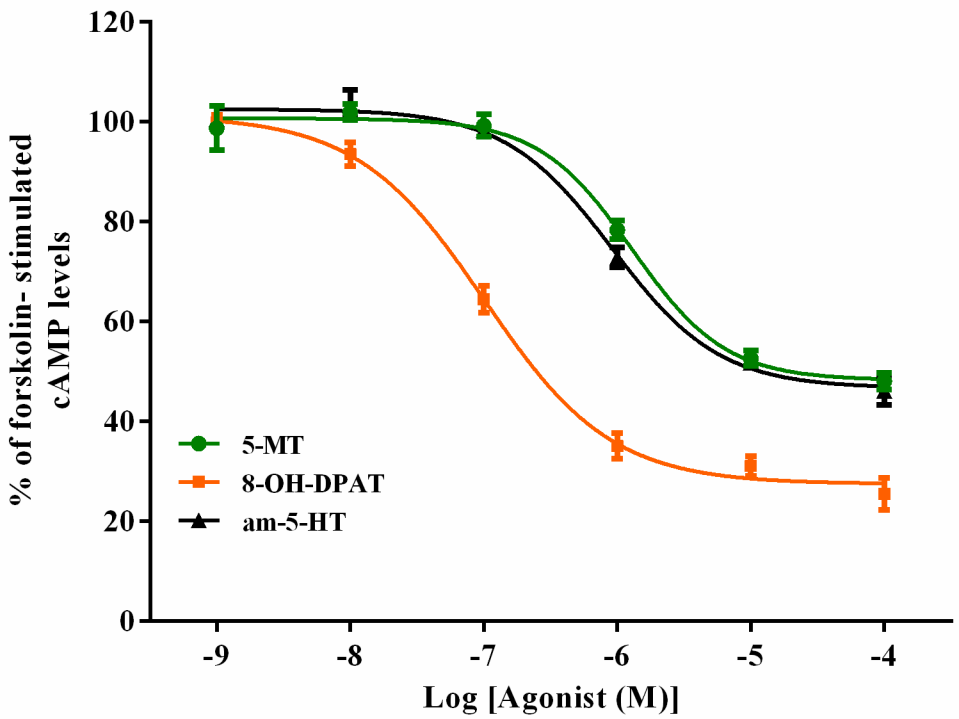

B
DmOctα2R-L
% of forskolin- stimulated cAMP levels
125
100
75
50
25
-12 -11 -10 -9 -8 -7 -6 -5 -4
Log [Compound (M)]
OA
NE
E

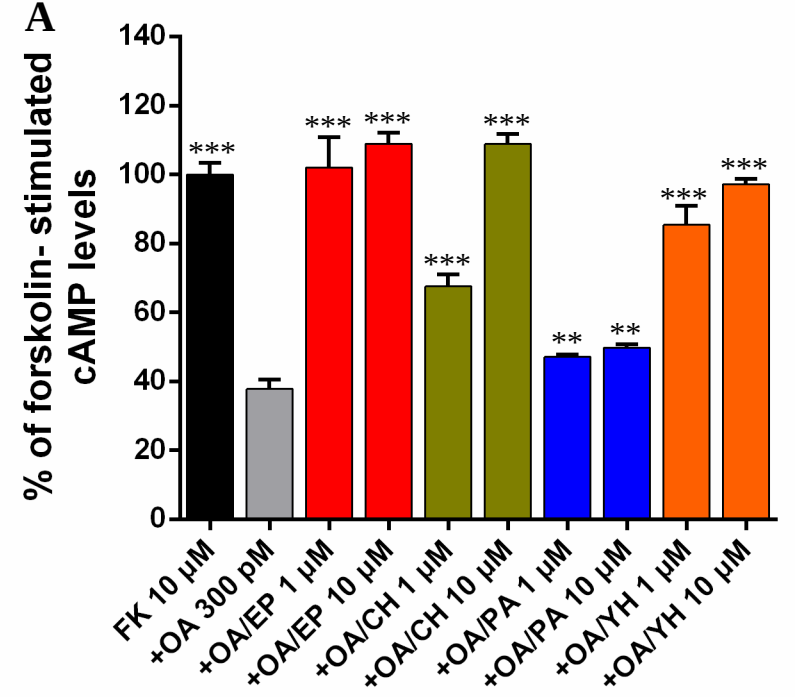

C
DmOctα2R-S
% of forskolin- stimulated cAMP levels
125
100
75
50
25
-12 -11 -10 -9 -8 -7 -6 -5 -4
Log [Compound (M)]
OA
NE
E

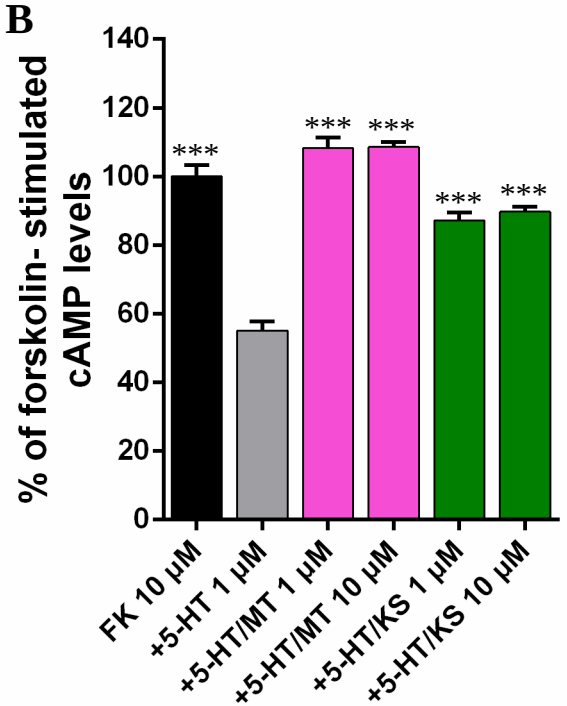
A

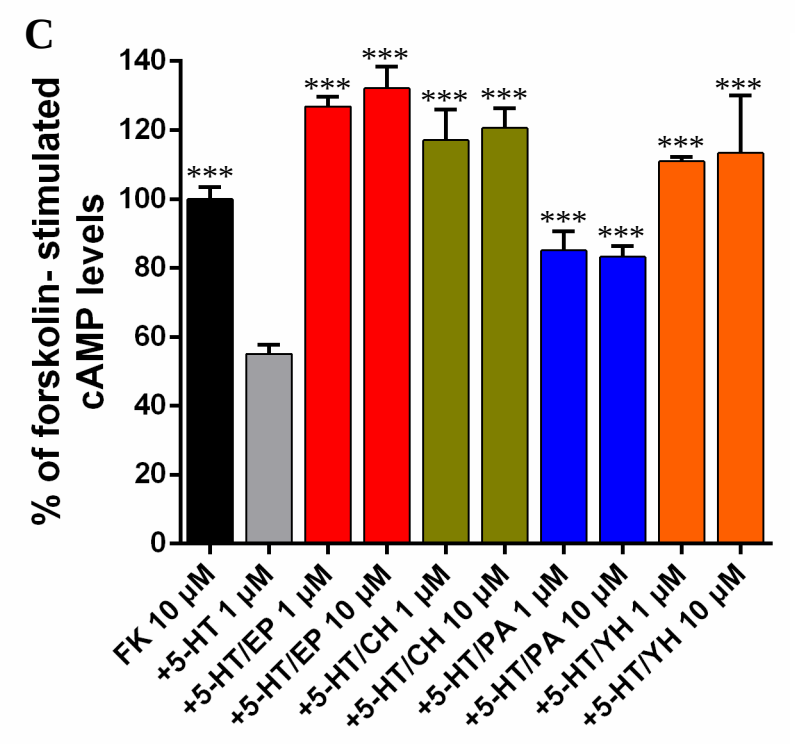

% of forskolin- stimulated cAMP levels
120
100
80
60
40
20
0
-13
-12
-11
-10
-9
-8
-7
-6
Log [Agonist (M)]
Naphazoline
Tolazoline
Clonidine
Lisuride

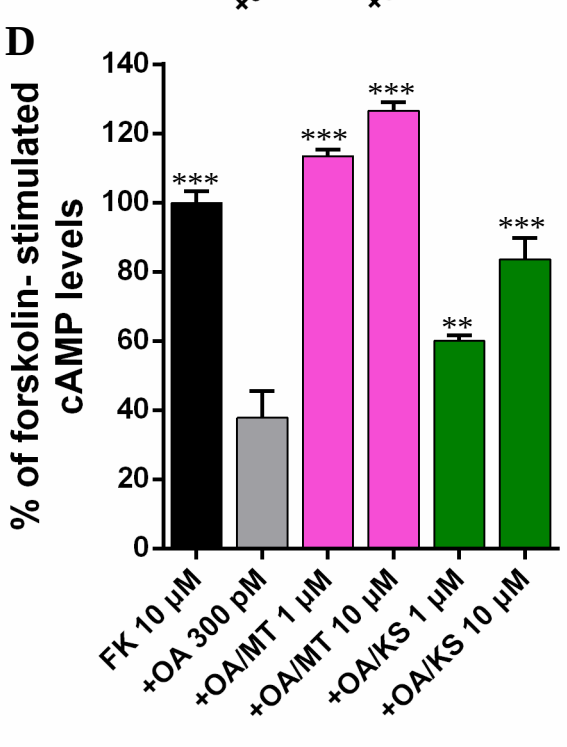
B

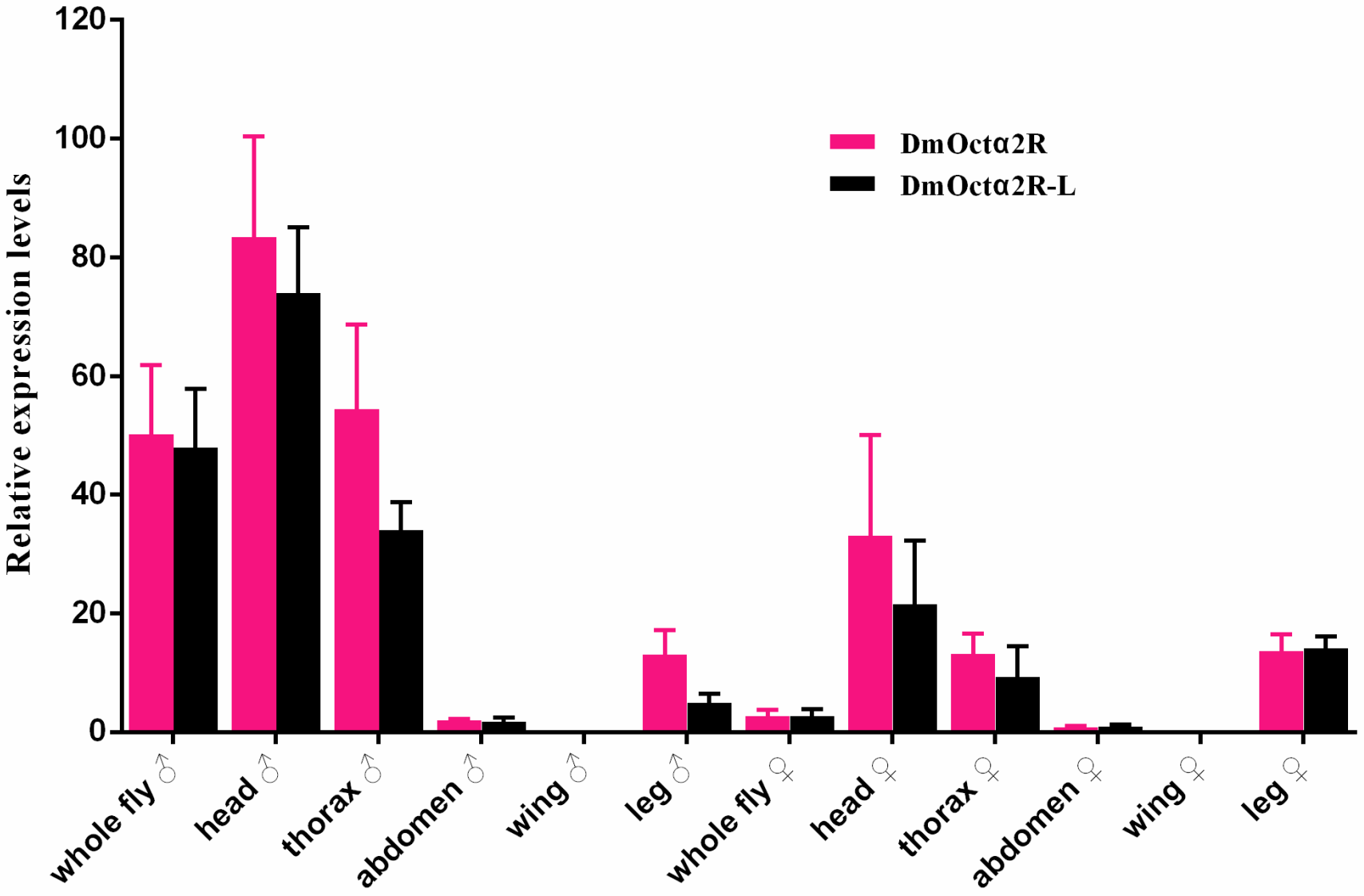

% of forskolin- stimulated cAMP levels
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log [Agonist (M)]
5-MT
8-OH-DPAT
am-5-HT


A
% of forskolin- stimulated cAMP levels
140
120
100
80
60
40
20
0
***
***
***
***
***
**
**
***
***
FK 10 µM
+OA 300 pM
+OA/EP 1 µM
+OA/EP 10 µM
+OA/CH 1 µM
+OA/CH 10 µM
+OA/PA 1 µM
+OA/PA 10 µM
+OA/YH 1 µM
+OA/YH 10 µM


B
% of forskolin- stimulated cAMP levels
140
120
100
80
60
40
20
0
***
***
***
***
***
FK 10 µM
+5-HT 1 µM
+5-HT/MT 1 µM
+5-HT/MT 10 µM
+5-HT/KS 1 µM
+5-HT/KS 10 µM


C
% of forskolin- stimulated cAMP levels
140
120
100
80
60
40
20
0
***
***
***
***
***
***
***
***
***
FK 10 µM
+5-HT 1 µM
+5-HT/EP 1 µM
+5-HT/EP 10 µM
+5-HT/CH 1 µM
+5-HT/CH 10 µM
+5-HT/PA 1 µM
+5-HT/PA 10 µM
+5-HT/YH 1 µM
+5-HT/YH 10 µM


D
% of forskolin- stimulated cAMP levels
140
120
100
80
60
40
20
0
***
***
***
**
***
FK 10 µM
+OA 300 pM
+OA/MT 1 µM
+OA/MT 10 µM
+OA/KS 1 µM
+OA/KS 10 µM


Relative expression levels
120
100
80
60
40
20
0
DmOctα2R
DmOctα2R-L
whole fly ♂
head ♂
thorax ♂
abdomen ♂
wing ♂
leg ♂
whole fly ♀
head ♀
thorax ♀
abdomen ♀
wing ♀
leg ♀

Highlights

1. An orphan gene *CG18208* from the fruit fly, *Drosophila melanogaster* was characterized.
2. *CG18208* encodes two transcripts by alternative splicing to generate *DmOctα2R-L* and *DmOctα2R-S*.
3. Octopamine, tyramine and serotonin can activate DmOctα2R to inhibit cAMP production.
4. Adrenergic and serotoninergic agonists and antagonists are effective on DmOctα2R.