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Identification and characterisation of the dopamine receptor II from the cat flea *Ctenocephalides felis* (CfDopRII)

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Abstract

G protein-coupled receptors (GPCRs) represent a protein family with a wide range of functions. Approximately 30% of human drug targets are GPCRs, illustrating their pharmaceutical relevance. In contrast, the knowledge about invertebrate GPCRs is limited and is mainly restricted to model organisms like *Drosophila melanogaster* and *Caenorhabditis elegans*. Especially in ectoparasites like ticks and fleas, only few GPCRs are characterised. From the cat flea *Ctenocephalides felis*, a relevant parasite of cats and dogs, no GPCRs are known so far. Thus, we performed a bioinformatic analysis of available insect GPCR sequences from the honeybee *Apis mellifera*, the mosquito *Anopheles gambiae*, the fruit fly *Drosophila melanogaste*r and genomic sequences from insect species. Aim of this analysis was the identification of highly conserved GPCRs in order to clone orthologs of these candidates from *Ctenocephalides felis*. It was found that the dopamine receptor family revealed highest conservation levels and thus was chosen for further characterisation. In this work, the identification, full-length cloning and functional expression of the first GPCR from *Ctenocephalides felis*, the dopamine receptor II (CfDopRII), are described.

Keywords: *Ctenocephalides felis*; G protein-coupled receptors; Dopamine receptors; *Drosophila melanogaster*; RNAinterference; Functional characterisation

1. Introduction

The cat flea *Ctenocephalides felis* is an ectoparasite with a life cycle comparable to that of *Drosophila melanogaster* which affects cats and dogs. Fleas are not restricted to one host, e.g. they can change their habitat from companion animals to humans and vice versa. Larvae feed on danders and dried blood excreted by the adult fleas. Flea bites can transmit infections or induce severe allergies (Dryden and Rust, 1994). The genome of the cat flea is not sequenced yet and only a small number of proteins (75 entries in Genbank, NCBI http://www.ncbi.nlm.nih.gov/Taxonomy) is already known. So far, no G protein-coupled receptors (GPCRs) from *Ctenocephalides felis* have been described.

GPCRs represent a protein family with up to 300 members in insects. They all share a common topology of seven transmembrane helices. GPCRs transduce extracellular signals into the intracellular cytoplasm (Brody and Cravchik, 2000). Heterotrimeric G proteins couple to GPCRs and affect different cell signaling pathways (Bissantz, 2003; Broeck, 2001). For instance, $G\alpha_s$ activates the adenylyl cyclase, whereas $G\alpha_i$ inhibits this enzyme (Neves et al., 2002). To facilitate identification of the first GPCR from *Ctenocephalides felis*, we decided to select a gene family of high conservation. Therefore, we examined the degree of similarity between insect GPCRs employing a bioinformatic analysis. This investigation revealed that dopamine receptors belong to the GPCR sub-family

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showing the highest degree of conservation. The three known dopamine receptors from *Drosophila melanogaster* (Dm) and *Apis mellifera* (Am) are divided into two classes: The DopRI and DopRII are functionally D1-like and activate the adenylyl cyclase (Genbank entries: AmDopRI/*Am*DOP1, CAA73841, Mustard et al., 2003; AmDopRII/*Am*DOP2, AAM19330, Mustard et al., 2003; DmDopRI/dDA1, AAA85716, Gotzes et al., 1994 and Sugamori et al., 1995; DmDopRII/DAMB/DopR99B, Q24563, Han et al., 1996 and Feng et al., 1996). In contrast, dopamine receptors III are related to D2-like receptors inhibiting the adenylyl cyclase (Genbank entries: AmDopRIII/*Am*DOP3,AY921573, Beggs et al., 2005; DmDopRIII/DD2R, AAN15955, Hearn et al., 2002). The pharmacology of invertebrate dopamine receptors was investigated in different expression systems: Dopamine receptors from *Drosophila melanogaster* were characterised in COS7- and Sf9-cells (DmDopRI, Sugamori et al., 1995), in *Xenopus laevis* oocytes (DmDopRII, Feng et al., 1996) and in HEK293 cells (DmDopRII, Han et al., 1996 and DmDopRIII, Hearn et al., 2002). AmDopRI was functionally expressed in HEK293 cells, Sf21- and Sf9-cells (Blenau et al., 1998; Mustard et al., 2003), AmDopRII in Sf21- and Sf9-cells (Humphries et al., 2003; Mustard et al., 2003) and AmDopRIII in HEK293 cells (Beggs et al., 2005). Moreover, GPCRs from *Anopheles gambiae* were predicted with bioinformatic tools: All three dopamine receptors, orthologs of the receptors from *Drosophila melanogaster* and *Apis mellifera,* have been identified in the genomic sequences from *Anopheles gambiae,* but these genes have not been cloned and characterised yet (Hill et al., 2002).

Here, we describe a bioinformatic analysis of conservation levels between insect GPCR sequences and the cloning of the first GPCR from the cat flea *Ctenocephalides felis*. The new dopamine receptor (CfDopRII) was functionally characterised by the expression in HEK293 cells and in *Xenopus laevis* oocytes. Moreover, the in vivo function of the receptor was investigated in the model organism *Drosophila melanogaster* by RNAi gene knock-down.

2. Materials and methods

2.1. Bioinformatic analysis

The genome comparison tool Genlight (Beckstette et al., 2004) was used for the analysis of different sequence sets. GPCR sequences from *Drosophila melanogaster* were obtained from FlyBase (batch download from http://flybase.bio.indiana.edu/; Brody and Cravchik, 2000). Additionally, GPCR sequences from *Anopheles gambiae* (Hill et al., 2002) and the genomic sequence data from *Aedes aegypti* (www.ensemble.org; Aedes_aegypti. AEDES1.august.pep_tigr.fa and Aedes_aegypti.AEDES1.august.pep_vector base.fa), *Anopheles gambiae* (www.ensemble.org; Anopheles_gambiae.A gamP3-feb.pep.fa), *Apis mellifera* (www.ensemble.org; Apis_mellifera.AMEL 2.0.feb.pep.fa), *Bombyx mori* (http://silkworm.genomics.org.cn; SW_ge2k_BGF.pep) and from *Drosophila melanogaster* (www.ensemble.org; Drosophila_melanogaster.BDGP4.feb.pep.fa) were used.

Predictions of transmembrane helices were performed with TMHMM server version 2.0 (www.cbs.dtu.dk/services/TMHMM/; Krogh et al., 2001). Alignments were created with ClustalW version 1.82 (www.ebi.ac.uk/clustalw/; Thompson et al., 1994) and edited in GeneDoc version 2.6.001 (www.psc.edu/biomed/genedoc/; Nicholas et al., 1997). BLAST searches with single sequences were accomplished at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul et al.,1990). The phylogenetic tree was created with the software Metalife Trinity 2006 version 2.2, Metalife AG, Winden, Germany (www.metalife.de/index.html). Identities between different sequences were determined with Vector NTI Advance version 9.0, Invitrogen, Carlsbad, USA; scoring matrix BLOSUM62.

2.2. Design of degenerate primers and cloning of CfDopRII

Degenerate primers were designed for highly conserved regions with a low level of degeneracy (Kwok et al., 1994). The degenerate primers (first PCR forward primer: CAYACIGCNACIAAYTAYTT and first PCR reverse primer: CCARCAIGCRTADATIACIGGRTTCAT; second PCR forward primer: ATGCCITTYWSIGCIYTNTA and second PCR reverse primer: TTDATCCAICCIARCCANGT) were used for nested PCR to amplify gene fragments from cDNA which was derived from reverse transcription of RNA from *Ctenocephalides felis*.

The sequence of the fragments was determined and the degree of similarity with other insect dopamine receptors was investigated using BLAST searches. After identification of a first fragment, the 5′- and 3′-ends of the gene were cloned by nested RACE-PCR (BD SMART RACE cDNA Amplification Kit, BD Biosciences, Franklin Lakes, NJ, USA; first PCR 3′-end: TCCTATCTGGGTTGTGCGTGAATTGTATCC; second PCR 3′-end: AACACGAAGAAATAGTATCGGCGGTAGTCA; first PCR 5′-end: AACAAGACATCCAGGGAGCGCCAAATGTCA; second PCR 5′-end: CACCAGTCGGAGCCAAAGAACCAAGTGTTG). The open reading frame for CfDopRII was predicted with Vector NTI Advance version 9.0 (Invitrogen, Carlsbad, USA). Afterwards the full length open reading frame was amplified from cDNA of *Ctenocephalides felis* and optimized according to the Kozak sequence upstream of the start codon (Kozak, 1987). The primers for the amplification contained an *Eco*RI and *Bgl*II overhang for suitable cloning (forward primer: GAATTCAGATCTGCCATGAATATCAGTTTCAAC; reverse primer: GAATTCAGATCTATGCTAAATGTAGGATTGTCCATAG). The CfDopRII was cloned into the vector pCRIITOPO (Invitrogen, Carlsbad, USA). The sequences of four independent clones were determined in order to obtain a sequence free of PCR errors. For expression in *Xenopus laevis* oocytes CfDopRII was inserted in the *Bgl*II site of pSP64 T (Krieg and

Melton, 1984). The gene was transferred from the pCRIITOPO (Invitrogen, Carlsbad, USA) into the pcDNA3.1(+) (Invitrogen, Carlsbad, USA) using the *Eco*RI restriction sites for expression in HEK293 cells.

2.3. Cloning of DopRII from Drosophila melanogaster

The dopamine receptor II from *Drosophila melanogaster* (DmDopRII, Genbank accession number: NM_079824; Feng et al., 1996) was PCR-amplified from cDNA of adult flies (forward primer: GAATTCTGATCAACAGCACTGAGTTGCCATGGTGGACG; reverse primer: GAATTCTGATCACTAAACTATATGTAGGTCTG CTCGC) and inserted in the *Bgl*II site of pSP64 T. Afterwards, it was cloned into pcDNA3.1 using PCR amplification with primers possessing *Eco*RI and *Not*I overhangs, respectively (forward primer: GGATCCGAATTCTGCAATGG TGGACGACAATGGCTC; reverse primer: GGATCCGCGGCCGCCTATATG TAGGTCTGCTCGC).

2.4. Expression in Xenopus oocytes

After linearization of the *Ctenocephalides felis* DopRII-pSP64 T construct with *Xba*I, capped sense cRNA (with a cap analogon) was obtained by using the mMESSAGE mMachine Kit (Ambion, Austin, TX, USA). Stage V and VI oocytes from virgin female adult *Xenopus laevis* were manually separated and placed in sterile ND96 [in mM: NaCl 96,KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES buffer (pH 7.6) 5, containing 2.4 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.2 mg/ml gentamycin]. The oocytes were defolliculated enzymatically by incubation in ND96 [without $CaCl_2$] containing collagenase (2 mg/ml; Sigma-Aldrich, München, Germany) for 1 h. Afterwards oocytes were injected with 50 ng of CfDopRII sense cRNA and incubated for 5 d at 19 °C. Non-injected and water-injected oocytes were used as controls. The protocol was performed according to Feng et al., 1996.

2.4.1. Detection of DopRII activation

cRNA-injected, water-injected and non-injected oocytes were preincubated in ND96 with 100 µM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, München, Germany) for 30 min. For stimulation, oocytes were incubated for additional 30 min in 100 µM IBMX-ND96 solution containing dopamine in concentrations ranging from 1 mM to 1 nM. Meanwhile control oocytes were incubated in parallel in the same medium without dopamine. As additional controls non-injected and water-injected oocytes were incubated with dopamine. After incubation each single oocyte was homogenized in 200 µl lysis buffer (cAMP Biotrak Enzyme Immunoassay System, Amersham Biosciences, Buckinghamshire, UK) and shaken for 10 min at room temperature. In the assay 100 µl of the cell suspension were used to measure cAMP levels.

2.5. Expression in HEK 293 cells

Plasmid preparation of pcDNA3.1(+) constructs was performed using the EndoFree Maxi Kit (Qiagen, Hilden, Germany). Human embryonic kidney cells (HEK 293) were cultivated at 37 °C and 5% CO_2 in minimum essential medium eagle (MEM; Sigma-Aldrich, München) after adding of 10% fetal calf serum (Invitrogen, Carlsbad, USA), 1% L-glutamine (Invitrogen, Carlsbad, USA), 1% Pen/Strep 100× (Invitrogen, Carlsbad, USA) and 1% non-essential amino acids (Invitrogen, Carlsbad, USA). Cells were cultivated on cell culture plates with a diameter of 100 mm (Cellstar, Coppell, USA). For passaging, the medium was removed and cells were washed with Dulbecco's phosphate buffered saline (D-PBS, Invitrogen, Carlsbad, USA) and incubated with trypsine EDTA (Invitrogen, Carlsbad, USA). The cells were resuspended in medium and transferred in a new plate with fresh medium. About 10^6 cells were seeded on a new plate containing 10 ml medium. After 24 h the transfection was performed with PolyFect transfection reagent (Qiagen, Hilden, Germany) for 24 h. Transfected cells were dispensed on a 24-well plate (Nunc, Wiesbaden, Germany) and grown for 24 h at 37 °C and 5% CO_2.

2.5.1. Detection of DopRII activation

Transfected, untransfected and mock transfected cells were washed twice with 100 µM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, München, Germany) D-PBS solution (Invitrogen, Carlsbad, USA) and preincubated for 15 min at 37 °C in the same solution in order to inhibit phosphodiesterase activity. After removing the supernatant, dopamine IBMX solution was added at the appropriate concentration (1 mM–1 nM) and cells were incubated for additional 15 min at 37 °C. This solution was also removed and cAMP levels were determined by cAMP biotrak enzymeimmunoassay system (Amersham Biosciences, Buckinghamshire, UK) according to manufacturers instructions. In contrast to the manual 500 µl lysis buffer were added to the cells.

2.6. Transgenic Drosophila melanogaster

In order to investigate the function of dopamine receptor II in vivo, the model organism *Drosophila melanogaster* was used. A fragment of the fruit fly DopRII (Genbank accession number: NM_079824, FlyBase: CG18741) was cloned into pUASTi vector, kindly provided by Amin Ghabrial (unpublished; Stanford University). This vector is a modification of the pUAST vector designed to stably integrate DNA inserts into the genome, which are regulated by the yeast UAS GAL4 system (Brand and Perrimon, 1993). Upstream and downstream of a spacer (intron of *trachealess*), two identical fragments of the DmDopRII were cloned into this vector. For this purpose a fragment of the sequence AE003770 (Genbank accession number) from basepair 17901 to 18632 according to an

exon of the dopamine receptor II was amplified. The result was a hairpin construct with tail-to-tail orientation. After transcription of this construct the *trachealess* intron will be removed by splicing and the mRNA fragments of DmDopRII will form dsRNA. Transgenic flies were produced by injecting the vector in combination with a helper vector, coding for transposase, into embryos of *white* mutants inducing the integration of the hairpin construct into the genome. Afterwards the flies were crossed with *white* mutants in order to identify transgenic progeny possessing the marker gene *white*. Progeny with the *white* gene had red eyes. Transgenic flies were stabilized with balancer fly lines (Rubin and Spradling, 1982). Expression of the hairpin was induced by crossing homozygous transgenic RNAi flies with the heterozygous actin-GAL4 or tubulin-GAL4 driver lines (Brand and Perrimon, 1993; Duffy, 2002). Therefore, the whole progeny possessed one copy of the RNAi construct, but 50% had the driver gene whereas the other 50% lacked the driver gene (identification by dominant markers). If the knock-down of the gene induces lethality during development, only flies possessing the dominant marker (inherited by the driver line) reach adult life stages. The ratio of flies with and without the dominant marker was determined 5 days after hatching and used for the quantification of lethality (crossings with less than 50 progeny were discarded). Control RNAi fly lines were established in parallel: A fragment of the *white* gene (genomic sequence from FlyBase: CG2759; basepair 10124–10745) and a fragment of the *phosphofructokinase* gene (genomic sequence from FlyBase: CG4001; basepair 7126–7917), respectively were inserted in the pUASTi vector twice. Transgenic *white* and *phosphofructokinase* RNAi fly lines were established as described for the DmDopRII RNAi fly line.

3. Results

3.1. Bioinformatic analysis and cloning of CfDopRII

The identification of new orthologs is based on sequence conservation throughout different species. On the basis of highly conserved regions degenerate primers can be designed, enabling the cloning of expected orthologs. To identify highly conserved insect GPCRs, we performed a bioinformatic sequence similarity analysis, resulting in a selection of GPCRs applicable for identification of orthologs by degenerate PCR.

GPCRs of *Anopheles gambiae* (Hill et al., 2002) and *Drosophila melanogaster* (Brody and Cravchik, 2000; FlyBase) have been described. After the download of GPCRs from *Anopheles gambiae* and genomic sequences from *Anopheles gambiae*, *Drosophila melanogaster*, *Apis mellifera*, *Bombyx mori* and *Aedes aegypti* the bioinformatic software Genlight (Beckstette et al., 2004), comprising all common sequence comparison algorithms, was used for high-throughput sequence analysis. A bidirectional BLAST search was performed between GPCRs from *Anopheles gambiae* and the genomic sequences. The program generates new hit-sets with sequence pairs which can be displayed as alignments. The degree of similarity between two sequences can be quantified by identity and e-value (Selzer et al., 2004). The following criteria were developed to assess the hits: A significant hit had a sequence identity of at least 56% and an e-value of at least 10^{-30}. The identities, e-values and the number of hits for a specific GPCR of each species in different sequence sets were used to determine the conservation level. Moreover, the analysis was repeated using GPCRs from *Drosophila melanogaster*. The results were combined and revealed that the family of dopamine receptors from different species show the highest conservation level. Therefore, these receptors were chosen for the identification of orthologs from the cat flea *Ctenocephalides felis*.

The alignment of dopamine receptors II from *Drosophila melanogaster* (Genbank accession number: NM_170420; Feng et al., 1996), *Apis mellifera* (Genbank accession number: NM_001011567; Humphries et al., 2003; Mustard et al., 2003) and *Anopheles gambiae* (Genbank accession number: XM311193; Hill et al., 2002) showed highly conserved regions. For these sequences degenerate primers were designed, and nested PCRs were performed (Fig. 1).

After discovery of the first fragment of CfDopRII, the 5′- and 3′-ends of the new orthologous gene were identified by RACE-PCR with gene specific primers. The complete sequence was amplified from cDNA of *Ctenocephalides felis*. The CfDopRII open reading frame (Genbank accession number: DQ459405) is 1392 bp in length, coding for a putative 463 amino acid protein. Analysis with the TMHMM server version 2.0 revealed that the protein contains the expected seven transmembrane helices (Fig. 1). Additionally, we found two further variants of CfDopRII by RACE PCRs (Genbank accession numbers: DQ459406, DQ459407) possessing alternative 3′-ends. Because of highest similarity between other insect dopamine receptors II and the longest variant of CfDopRII (DQ459405) all further experiments were performed with this variant. Alignment with DopRII sequences from *Drosophila melanogaster*, *Apis mellifera* and *Anopheles gambiae* revealed a high degree of conservation (identity between CfDopRII and DmDopRII: 58%, identity between CfDopRII and AmDopRII: 66% and between CfDopRII and AgDopRII: 61%). The conservation level of transmembrane helices (TMH) is especially high, whilst greatest divergence can be found in the third intracellular loop, which plays a key role in receptor/G protein coupling selectivity (Hermans, 2003; Gether, 2000; Wess, 1998). The new dopamine receptor contains residues typical for biogenic amine receptors: The aspartate residue (D131) in transmembrane helix III (TMH III) was described to be a relevant amino acid for the binding of the amine group of catecholamines like dopamine. Two serines (S216 and S219) in TMH V are supposed to form hydrogen bonds with the hydroxyl groups of dopamine. CfDopRII also

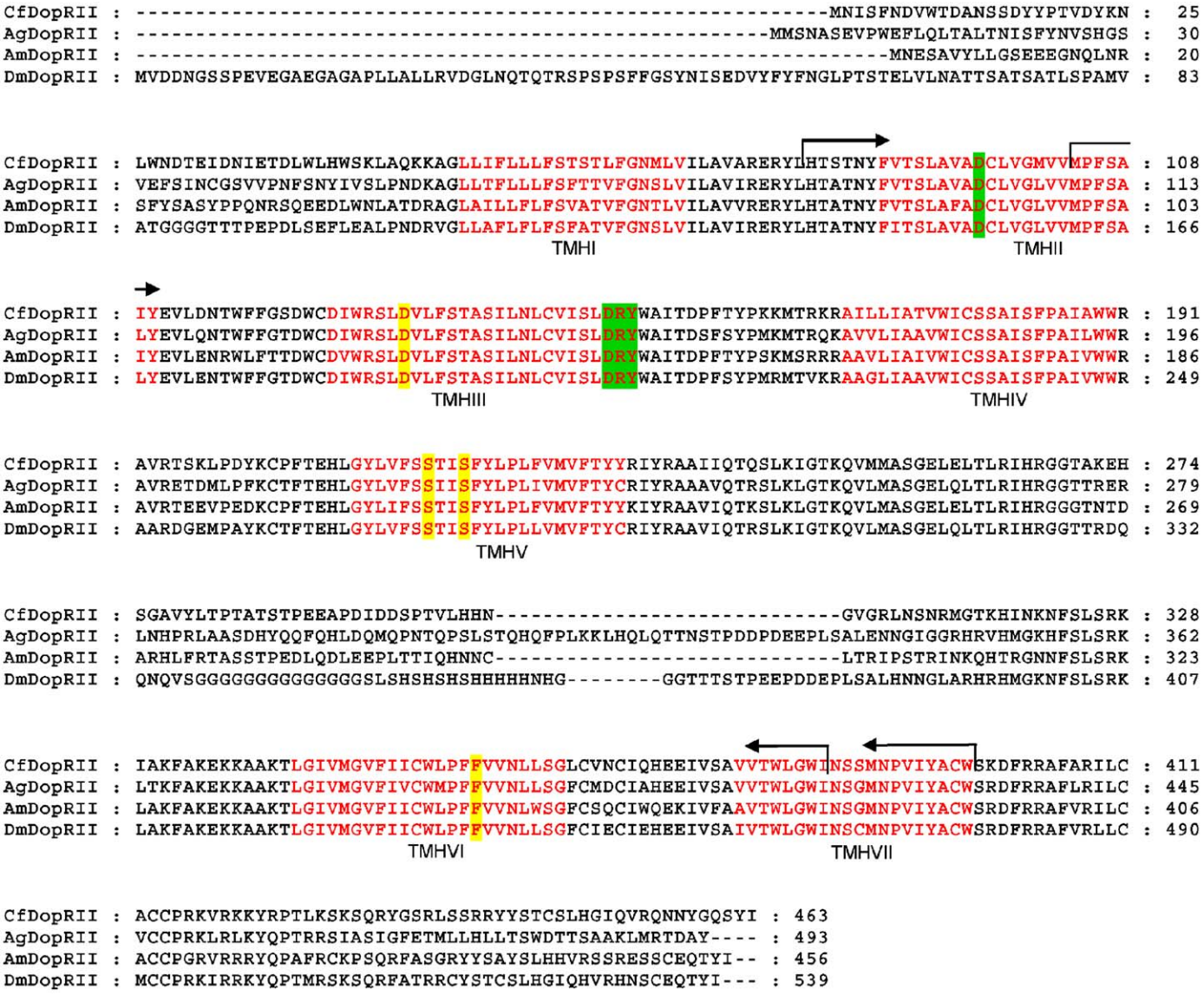

Fig. 1. Multiple sequence alignment of orthologous dopamine receptors II from *Ctenocephalides felis* (CfDopRII), *Apis mellifera* (AmDopRII), *Anopheles gambiae* (AgDopRII) and *Drosophila melanogaster* (DmDopRII). Transmembrane domains are indicated by red amino acids and were predicted with TMHMM server version 2.0. Regions for which degenerate primers were designed are indicated by arrows. Green boxes show amino acids involved in receptor activation. Amino acids with yellow labels are probably involved in ligand binding (Strader et al., 1995; Mustard et al., 2005).

possesses a typical phenylalanine (F356) in TMH VI most likely interacting with the aromatic ring of dopamine. The aspartate residue in TMHII and the DRY sequence, which are described to be involved in receptor activation, are also present (Strader et al., 1995; Mustard et al., 2005).

3.2. Phylogenetic analysis

The phylogenetic analysis illustrates the evolutionary relationship between insect and human biogenic amine receptors. In invertebrates three different dopamine receptors are known (DopRI, II and III) in comparison to five human dopamine receptors classified into D1-like and D2-like receptors (Mustard et al., 2005; Beggs et al., 2005). Human DopR1 and 5 belong to D1-like receptors which increase intracellular cAMP levels. They share the highest degree of conservation with insect dopamine receptors I and II. In contrast D2-like receptors (human DopR2, 3 and 4) inhibit adenylyl cyclase like the insect dopamine receptor III (Missale et al., 1998; Mustard et al., 2005; Beggs et al., 2005). The highest degree of similarity was detected for the CfDopRII and orthologous insect dopamine receptors II, indicating the expected relationship. As an internal control, the histamine receptor from *Drosophila melanogaster* was added. The identity between CfDopRII and the histamine receptor amounts to 7%. This receptor was excluded from the dopamine receptors confirming the phylogenetic tree (Fig. 2). These results indicate that the cloned receptor is an ortholog of the other insect dopamine receptors II. All functionally characterised members of these receptors

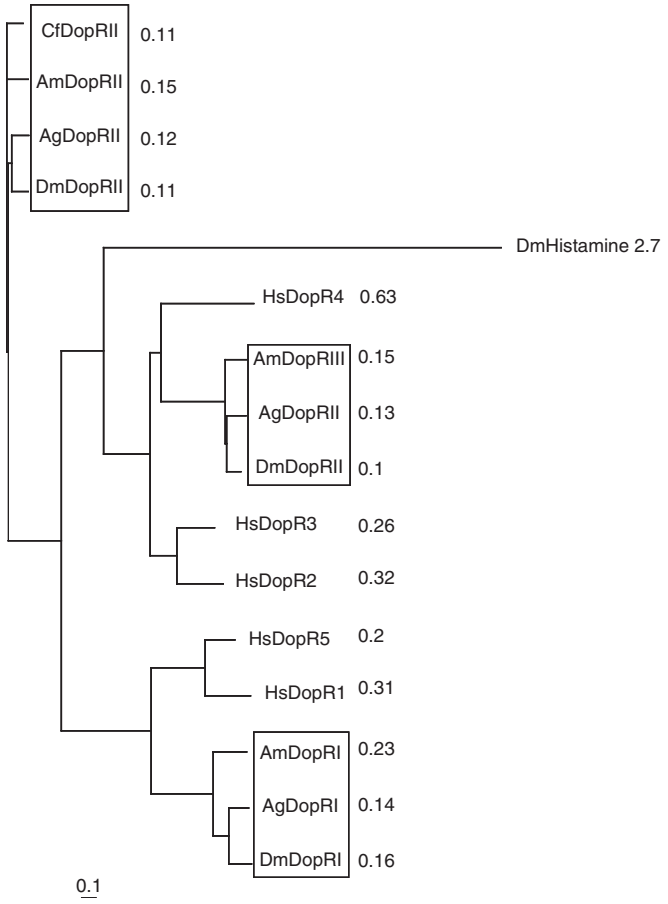

Fig. 2. Phylogenetic tree of biogenic amine receptors. The evolutionary relationship is displayed in the length of the branches. The scale for distances is displayed. The following organisms are listed: *Apis mellifera* (Am), *Drosophila melanogaster* (Dm), *Anopheles gambiae* (Ag), *Homo sapiens* (Hs) and *Ctenocephalides felis* (Cf). Hence CfDopRII clusters with the DopRII group in contrast to the control gene DmHistamine which does not group with any of the dopamine receptors. Genbank accession numbers of used sequences: CfDopRII—DQ459405; AmDopRI—CAA73841; DmDopRI—AAA85716; HsDopR5—NP_000789; HsDopRI—NP_000785; DmDopRII—Q24563; AmDopRII—AAM19330; AmDopRIII—AY921573; DmDopRIII—AAN15955; HsDopR4—NP_000788; HsDopR3—NP_000787; HsDopR2—NP_000786; AgDopRI—EAA10574; AgDopRII—EAA06824; AgDopRIII—EAA09118; DmHistamine—AAF56578.

couple to $G\alpha_s$ proteins, leading to an increase of the cAMP level in cellular expression systems (Feng et al., 1996; Mustard et al., 2003).

3.3. Functional expression of CfDopRII

GPCRs couple to heterotrimeric G-proteins and enable the transmission of signals from the outside of the cell into the cytosol. For functional expression in established assay systems like *Xenopus* oocytes or HEK293 cells the invertebrate dopamine receptor has to couple to vertebrate G-proteins. It cannot be determined in advance to what extent the different vertebrate G-proteins are able to couple to invertebrate receptors. Therefore, we decided to express the receptor in two independent systems, in HEK293 cells and in *Xenopus* oocytes, in order to confirm the results.

First, the CfDopRII was functionally expressed in HEK293 cells. After stimulation with dopamine, a dose dependent increase of the intracellular cAMP levels was detected (Fig. 3A). Because it has been suspected that mock transfection leads to the expression of endogenous receptor populations in HEK293 cells (Beggs et al., 2005), several controls were performed and measured in all independent experiments: Untransfected cells did not respond to stimulation with dopamine. Mock transfected cells without DNA (data not shown) and controls transfected with the vector pcDNA3.1(+), respectively (Fig. 3A), showed a slight increase of cAMP-levels after incubation with dopamine. However, transfection of CfDopRII always resulted in a dopamine-dependent increase of cAMP levels that were significantly higher than the controls, indicating that this receptor is activated by dopamine and couples to $G\alpha_s$. The significant dose-dependent response to dopamine (after subtraction of control values) was used to calculate an EC_{50} of 2.4 μM (Fig. 3B).

CfDopRII and DmDopRII were both functionally expressed in HEK293 cells in order to compare the functionality of these genes in the same expression system. The cells were transfected with one of the genes, respectively, and were stimulated with dopamine. Even with high concentrations of dopamine (1 mM) the increase of cAMP levels was higher in cells transfected with DmDopRII than in cells expressing the CfDopRII (data not shown). Additionally, the comparison of the EC_{50} values (DmDopRII: $EC_{50} = 0.35\,\mu M$ (Han et al., 1996); CfDopRII: $EC_{50} = 2.4\,\mu M$) indicates significant differences.

To prove that CfDopRII is a functional dopamine receptor, HEK293 cells expressing the receptor were incubated with 10 μM of different biogenic amines (Fig. 3C). As expected, norepinephrine led to increased cAMP-levels in CfDopRII expressing cells as well as in cells which were transfected with pcDNA3.1(+). This indicates that HEK293 cells express endogenous adrenergic receptors which respond to a stimulation with norepinephrine (Beggs et al., 2005; Gerhardt et al., 1997; Ohta et al., 2003; Grohmann et al., 2003). The effect of the other biogenic amines on CfDopRII expressing cells was descending in the following order: dopamine > tyramine > octopamine > serotonin. This result confirms that the described receptor is a dopamine receptor.

Oocytes from *Xenopus laevis* were used as an alternative expression system. Injected mRNA is translated and proteins integrate into the cell membrane where they can be activated (Feng et al., 1996; Barnard et al., 1982; Sumikawa et al., 1981). In order to investigate the functionality of CfDopRII, capped mRNA produced in vitro was injected in oocytes. After 5 days of incubation the activation with the natural ligand dopamine was tested. For each value, the cAMP levels of at least five single oocytes were determined. An expected dose dependent

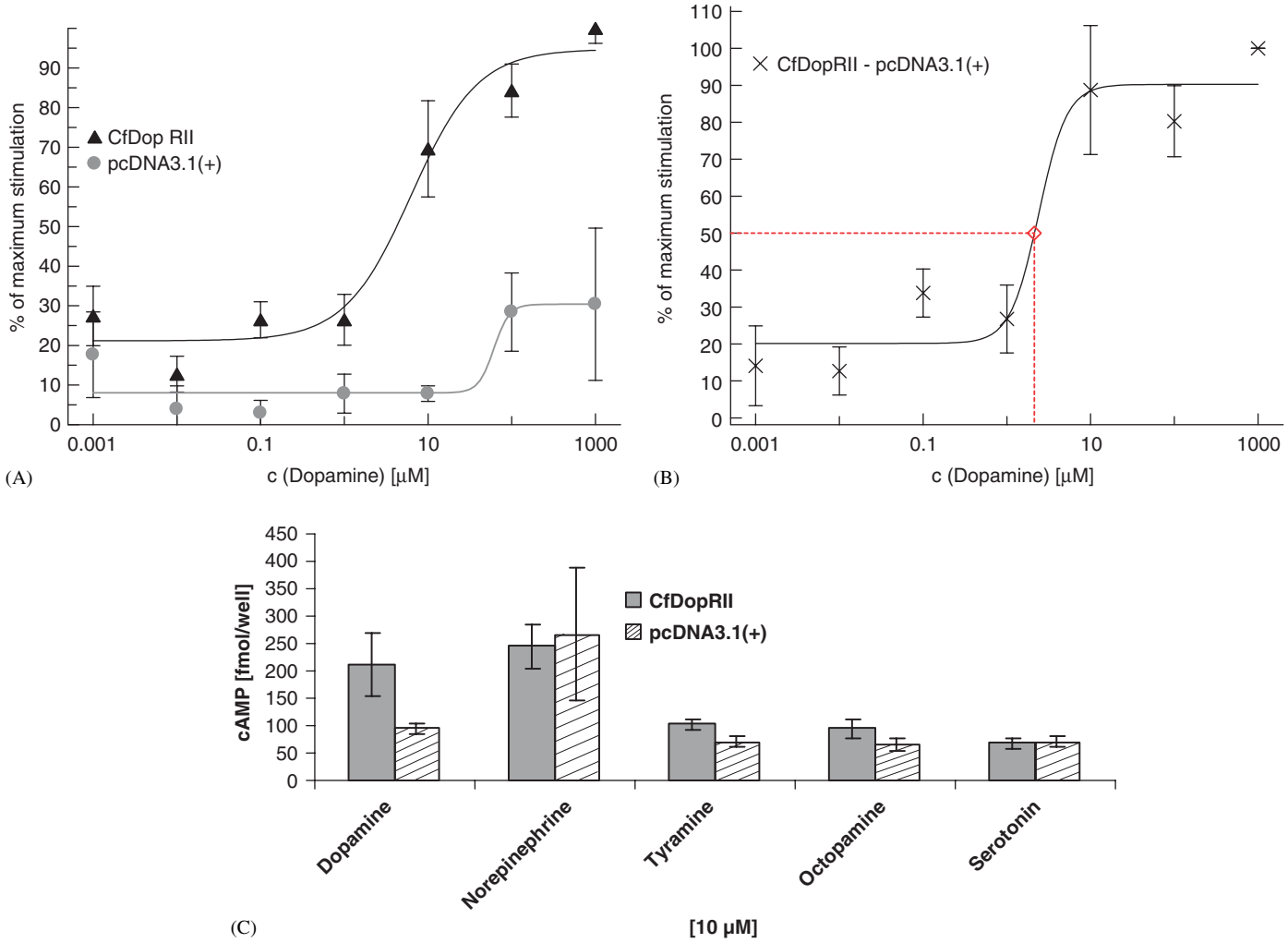

Fig. 3. Functional expression of CfDopRII in HEK293 cells. (A) Detection of the dopamine dose-dependent cAMP increase in CfDopRII expressing HEK293 cells in comparison to cells transfected with pcDNA3.1(+). The intracellular cAMP level of transfected cells without dopamine incubation were measured as controls and subtracted. Subsequent values were normalized. HEK293 cells transiently transfected with CfDopRII or with pcDNA3.1(+) were stimulated with concentrations from 1 mM to 1 nM dopamine. (B) Calculation of the EC50 value. The cAMP values of pcDNA3.1 transfected cells were substracted from the values of activated CfDopRII expressing cells. According to this curve the EC50 value was calculated with the software ExcelFit version 4.1. (C) Effect of biogenous amines (10 µM) on HEK293 cells transfected with CfDopRII or alternatively with pcDNA3.1(+) as control. (A + B + C) In each of the experiments depicted here four samples were measured and the average and standard deviation were determined. Results were confirmed in three independent experiments.

increase of cAMP was detected (Fig. 4). Thus, the functionality of CfDopRII was shown in two independent expression systems.

3.4. Knock-down of DmDopRII in transgenic Drosophila melanogaster

The characterisation of CfDopRII was performed in cellular expression systems (oocytes and HEK293-cells). Additionally, the function of dopamine receptor II for a living organism was investigated in vivo. The genome of *Ctenocephalides felis* has still not been sequenced and genetic transformation protocols have not been established in cat fleas, but they are well established in the insect model organism *Drosophila melanogaster*. Therefore, the dopamine receptor DmDopRII (orthologous to CfDopRII) was chosen for investigation. Because no mutations for DmDopRII are described in the FlyBase or in the literature, we performed a knock-down approach: DmDopRII was down regulated by transgenic RNAi in order to quantify RNAi-mediated lethality during development. Insertion of the RNAi construct into the genome of *Drosophila melanogaster* occurs randomly, so that the transgene could be under control of different gene regulatory elements in independent transgenic fly lines. This might lead to a variation of the expression rate of the RNAi construct. Therefore, four independent transgenic DmDopRII RNAi fly lines were crossed with the ubiquitous driver lines tubulin-GAL4 and actin-GAL4, respectively, in order to induce expression of the RNAi construct (Table 1).

The level of lethality obtained by the two driver lines was comparable.

To confirm the results, the following control experiments were performed: the knock-down of Phosphofructokinase (PFK), a key enzyme of glycolysis with a known lethal phenotype (Spradling et al., 1999; Goldstein et al., 2001) resulted in 100% lethality for six tested independent *pfk*-RNAi fly lines as expected. Moreover, an RNAi approach with the non essential *white* gene mediated no lethality, but revealed the expected *white* eye phenotype (data not shown; compare Lee and Carthew, 2003). Taken together, the positive and negative controls revealed that the RNAi approach is suitable to assess if a particular gene function is essential for the organism. The role of DmDopRII is not as essential as Phosphofructokinase, because the knock-down of the dopamine receptor did not result in 100% lethality. But the knock-down of the gene led to a reduced viability (Table 1). Thus, we conclude that DmDopRII might have an intermediate function during development.

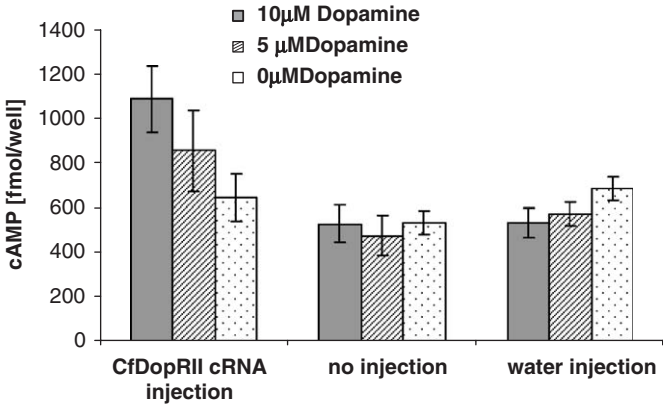

Fig. 4. Effect of dopamine on CfDopRII-expressing and control oocytes from *Xenopus laevis*. For each value the cAMP levels of at least five single oocytes were determined and the average and standard deviation were calculated. Cells expressing CfDopRII were compared with non-injected and water-injected oocytes incubated with 5, 10 µM or without dopamine. Results were confirmed in three independent experiments.

4. Discussion

We identified the first GPCR from the cat flea *Ctenocephalides felis* and characterised it functionally. On the basis of sequence comparisons, we supposed that the gene is orthologous to insect dopamine receptors II. Moreover, by expression in HEK293 cells and in *Xenopus laevis* oocytes, we could demonstrate that CfDopRII is also a functional ortholog; the most potent agonist among biogenic amines was dopamine.

Primarily, we performed a general bioinformatic analysis to identify highly conserved insect GPCRs in order to improve identification of orthologous GPCRs in the relevant parasitic insect *Ctenocephalides felis*. Insect GPCR sequences from genomic sources were analyzed and scored depending on sequence similarity using the software Genlight (Beckstette et al., 2004). The analysis revealed that the dopamine receptor protein family shows the highest conservation among different insect species, comparable to the alignments reported by Mustard et al., 2005. In our work DopRII was selected for the identification of the first GPCR from the cat flea. The gene was identified and cloned successfully. The comparison between CfDopRII and orthologous insect dopamine receptors AmDopRII, AgDopRII and DmDopRII revealed high values of sequence identity (58–66%). The bioinformatic approach was thereby confirmed. Sequence alignments showed that the highest degree of identity was found between CfDopRII and AmDopRII. Lower sequence identities were observed between CfDopRII and the orthologous genes of *Anopheles gambiae* and *Drosophila melanogaster*. Also, the EC_{50} values of CfDopRII ($EC_{50} = 2.4\,\mu M$) and AmDopRII ($EC_{50} = 2.2\,\mu M$; Mustard et al., 2003) were similar and differed from the EC_{50} value of DmDopRII ($EC_{50} = 0.35\,\mu M$; Feng et al.,1996). Thus, the highest degree of similarity was observed for CfDopRII and AmDopRII.

Expression of GPCRs from insects in a vertebrate expression system like *Xenopus* oocytes or HEK293 cells requires coupling between invertebrate receptors and

Table 1
Lethality of DmDopRII knock-down organisms

Target protein	Driver line	Lethality (%)			
		Line A	Line B	Line C	Line D
DmDopRII	Tubulin-Gal4	23	70	34	74
	Actin-Gal4	3	60	48	98
Phosphofructokinase	Tubulin-Gal4	100	100	100	100
	Actin-Gal4	100	100	100	100
White	Tubulin-Gal4	0	18	0	0
	Actin-Gal4	0	0	4	0

Transgenic flies possessing the DmDopRII, *phosphofructokinase* or *white* RNAi construct were crossed with two independent driver lines, tubulin-GAL4 and actin-GAL4, respectively. The lethality of the knock-down organisms was quantified. The knock-down of the *white* gene resulted in the expected *white* eye phenotype of progeny.

vertebrate G proteins which might not work properly. Moreover, the expression of endogenous receptor population may disturb the experiments (Beggs et al., 2005; Mustard et al., 2005). Therefore, both described alternative expression systems were used for functional expression of CfDopRII. Although negative controls (HEK293 cells) were sensitive to dopamine, a significant increase of cAMP mediated by the ligand dopamine was detected in cells transfected with CfDopRII indicating the functionality of CfDopRII. In contrast to the HEK293 system, control oocytes showed no dopamine-dependent increase of cellular cAMP levels, but a dose-dependent increase of cAMP mediated by CfDopRII was detected. The advantage of expression in HEK293 cells is the suitability for high throughput approaches, whereas the *Xenopus* expression system is robust and easy to handle but not suitable for high throughput approaches.

After the characterisation of CfDopRII in two expression systems, we were interested in its in vivo function. Because no genetic transformation experiments have been established for *Ctenocephalides felis*, we used the insect model organism *Drosophila melanogaster* to analyse the effects caused by a DmDopRII knock-down. A model organism has to be extensively studied, should be easy to maintain and shows particular experimental advantages. *Drosophila melanogaster* proved to be perfectly suited as model organism. The fruit fly breeds in large numbers and has a short lifecycle so that several generations can be analyzed in a short period. A variety of mutants is available, the handling of the flies is well established and many genetic and molecular techniques are described (Ashburner et al., 2005).

Although the effect of dopamine on insects has been investigated for *Drosophila melanogaster* (Hearn et al., 2002), and is connected with locomotor activity (Yellman et al., 1997), response to drugs of abuse (Bainton et al., 2000; Li et al., 2000), fertility (Neckameyer, 1996; Pendleton et al., 1996), sexual behavior (Neckameyer, 1998a, b) and memory (Tempel et al., 1984; Neckameyer, 1998a, b), no mutations for DmDopRII have been described. In *Drosophila* many genes (more than 53% of the genome) were mutagenized by random screens with P-element insertions (Bellen et al., 2004; Thibault et al., 2004) or chemicals (Ashburner et al., 2005). The reasons for the absence of DmDopRII mutations could be haplo-insufficiency or lack of essentiality of the gene product. We analyzed the function of DmDopRII in vivo performing a transgenic RNAi approach. Compared to positive and negative controls, an intermediate effect for the knock-down of DmDopRII was found. Therefore, DmDopRII is most likely not as essential for *Drosophila* development as for instance the phosphofructokinase, the key enzyme of glycolysis. Because the model organism *Drosophila melanogaster* allows conclusions on gene functions in other insects by analogy, we propose that CfDopRII has a comparable in vivo function for *Ctenocephalides felis* and other insects.

The bioinformatic analysis revealed that all dopamine receptors are highly conserved in insects. The identification of CfDopRI and CfDopRIII, as well as further characterisation and analysis of coupling, is subject for further studies. Also, a comparison with dopamine receptors from other insects would be very interesting.

For *Anopheles gambiae* three dopamine receptors are predicted by bioinformatic analysis (Hill et al., 2002), but they have not yet been cloned nor characterised. Moreover, the sequence similarity and functional conservation with other non-insect arthropods like ticks would be expedient to investigate. The investigation of the dopaminergic GPCRs from different arthropods would provide information about the degree of sequence similarity and functional conservation of GPCRs in general and of dopamine receptors in particular between different species.

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