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Molecular identification of a *Drosophila* G protein-coupled receptor specific for crustacean cardioactive peptide☆

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Abstract

The *Drosophila* Genome Project website (www.flybase.org) contains the sequence of an annotated gene (CG6111) expected to code for a G protein-coupled receptor. We have cloned this receptor and found that its gene was not correctly predicted, because an annotated neighbouring gene (CG14547) was also part of the receptor gene. DNA corresponding to the corrected gene CG6111 was expressed in Chinese hamster ovary cells, where it was found to code for a receptor that could be activated by low concentrations of crustacean cardioactive peptide, which is a neuropeptide also known to occur in *Drosophila* and other insects (EC_{50}, 5.4×10^{-10} M). Other known *Drosophila* neuropeptides, such as adipokinetic hormone, did not activate the receptor. The receptor is expressed in all developmental stages from *Drosophila*, but only very weakly in larvae. In adult flies, the receptor is mainly expressed in the head. Furthermore, we identified a gene sequence in the genomic database from the malaria mosquito *Anopheles gambiae* that very likely codes for a crustacean cardioactive peptide receptor.

The recent sequencing of the *Drosophila* genome by the *Drosophila* Genome Project consortium [1] represents a milestone in insect research, because it enables researchers to identify all proteins in *Drosophila* and, thereby, to understand an insect at all possible levels (molecular, cellular, organismic, behavioural, etc.). Furthermore, the new knowledge that we will gain from *Drosophila* will certainly also have a strong impact on our understanding of other insects, other arthropods, or even other invertebrates. Our research group is particularly interested in neuropeptide receptors and their ligands (the neuropeptides), because these proteins/peptides occupy a high hierarchic position in the physiology of an insect and steer important processes such as reproduction, development, feeding, and all types of behaviours.

The website of the *Drosophila* Genome Project consortium (www.flybase.org) contains a list of 40–45 annotated (i.e., hypothetical) neuropeptide G protein-coupled receptors. We have earlier used this list as a starting point to clone various *Drosophila* neuropeptide receptors [2–5], but we have often found that the annotations were not correct. Furthermore, the ligands for the annotated receptors are unknown, i.e., they are orphan receptors. Therefore, proper cloning of the annotated receptors, expression in cells, and identification of their cognate ligands are still necessary processes.

In the present paper we have cloned the receptor encoded by the annotated gene CG6111, subsequently corrected its annotation (gene structure), and identified its cognate ligand. We have chosen gene CG6111, because its putative gene product is structurally somewhat related to the *Drosophila* adipokinetic hormone (Drm-AKH) and *Drosophila* corazonin (Drm-corazonin) receptors [3,6]. This would open the possibility for the existence of a second Drm-AKH or -corazonin receptor. We found, however, that the receptor was highly specific

☆ The nucleotide sequence reported in this paper has been submitted to the GenBank Data Bank with Accession No. AY219842.

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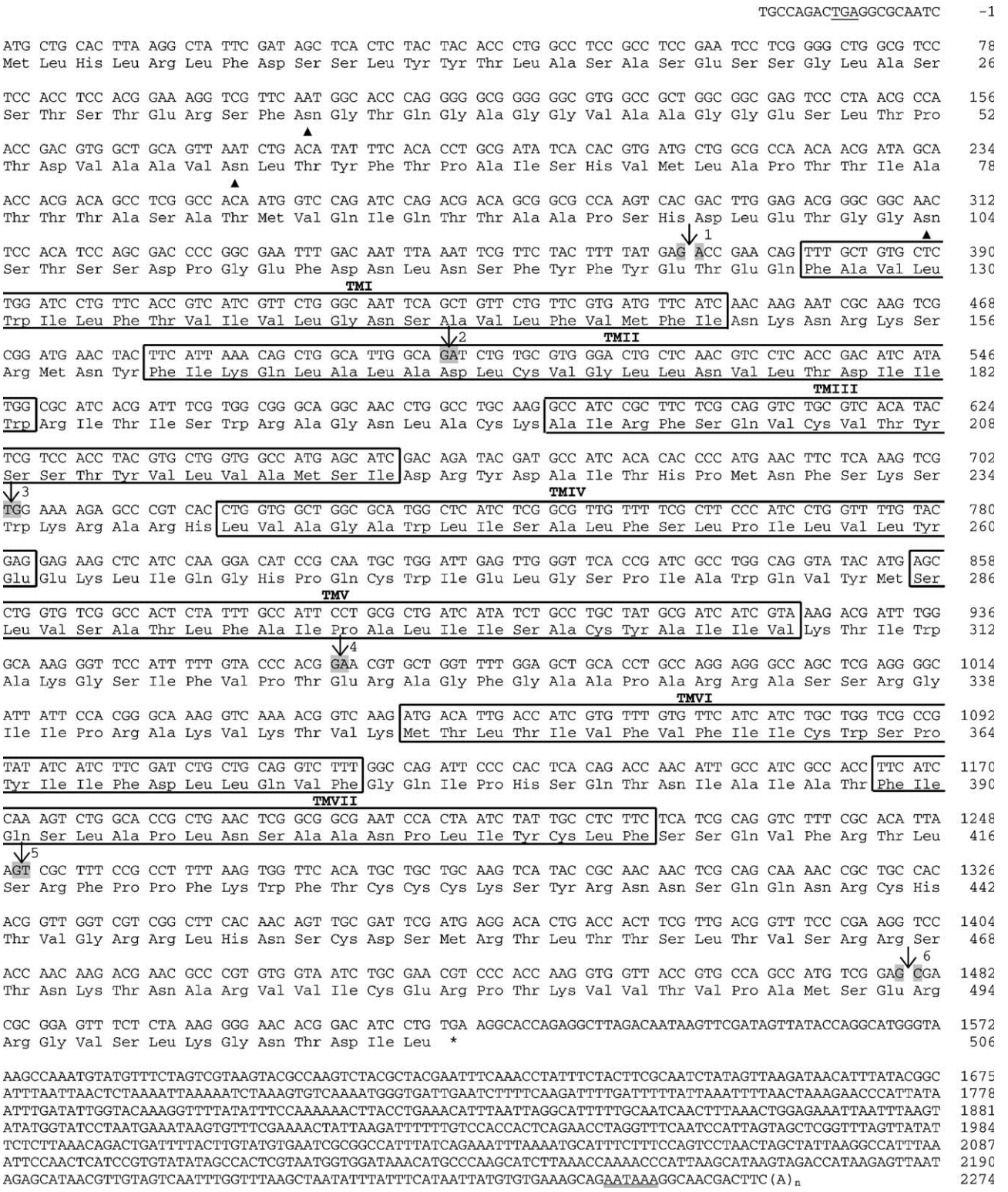

Fig. 1. cDNA and deduced amino acid sequence of the corrected gene CG6111. Nucleotides are numbered from 5′- to 3′-end and the amino acid residues are numbered starting with the first ATG codon in the open reading frame. The six introns are indicated by arrows and the exon nucleotides, bordering these introns, are highlighted in grey. The seven membrane spanning domains are boxed and labelled TMI-VII. The two putative glycosylation sites in the extracellular N terminus of the protein are indicated by filled triangles. The translation termination codon is indicated by an asterisk. The in-frame stop codon in the 5′-noncoding region is underlined. The putative polyadenylation signal in the 3′-noncoding region is underlined twice.

for crustacean cardioactive peptide, which is a neuropeptide that was first discovered in crustaceans, but that also occurs in identical form in insects [7–9].

Materials and methods

Primers were constructed based on the proposed exons of gene CG6111 (www.flybase.org) and used on cDNA from *D. melanogaster* third instar larvae (Canton S) as a template. The sense primer was 5′-ACCGTCATCGTTCTGGGCAATTCA-3′ (corresponding to nucleotide positions 403–426 of Fig. 1) followed by the nested sense primer 5′-GCAAGTCGCGGATGAACTACTTCATTA-3′ (corresponding to nucleotide positions 461–487 of Fig. 1) and the antisense primer was 5′-GATGTCCGTGTTCCCCTTTAGAGA-3′ (corresponding to nucleotide positions 1492–1515 of Fig. 1) followed by the nested antisense primer 5′-GTCCTCATCGAATCGCAACTGTTGTGA-3′ (corresponding to nucleotide positions 1344–1369 of Fig. 1). The PCR program was 1 cycle of 95 °C for 3 min, 58 °C for 1 min, and 72 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 1 min, 72 °C for 2 min, and a final extension step of 72 °C for 10 min. These PCRs were carried out using the Advantage2 PCR enzyme system (Clontech). For the amplification of the 3′-UTR the SMART RACE cDNA kit (Clontech) was used. The 3′-RACE reactions were made with the sense primer 5′-GCTTCACAACAGTTGCGATTCGATGAGGA CACTG ACCA-3′ (corresponding to nucleotide positions 1341–1378 of Fig. 1) followed by the nested sense primers, 5′-GAAGGTCCACCAACAAGACG AACGCCCGTGTGG TA-3′ (corresponding to nucleotide positions 1397–1431 of Fig. 1) and 5′-GCCATGTCGGAGCGACGCGGA GTTTCTCTAA-3′ (corresponding to nucleotide positions 1468–1498 of Fig. 1). The PCR program was: 94 °C for 3 min, then touchdown PCR for 5 cycles, 94 °C for 30 s, 74 °C for 40 s, decreasing 2 °C for 5 cycles, and 72 °C for 3 min, followed by 25 cycles of 94 °C for 30 s, 62 °C for 40 s, 72 °C for 3 min, and a final extension step of 72 °C for 10 min. Due to the presence of CG-rich regions at the 5′-end of the receptor cDNA, instead of performing the 5′-RACE PCR, specific primers were used based on the genomic sequence. The sense primer was 5′-TTGCTAGCGCAATCATGCTGCACTTAAGGCTATTCG-3′ (corresponding to nucleotide positions (−)8–22 of Fig. 1) and the antisense was 5′-GCCCGTGGAATAATGCCCCTCGAGCTGG-3′ (corresponding to nucleotide positions 1001–1028 of Fig. 1). The PCR program was: 96 °C for 3 min followed by 35 cycles of 96 °C for 1 min, 55 °C for 30 s, and 72 °C for 3 min, using the Advantage2 PCR enzyme system (Clontech) and 5% formamide in the PCR mixture. All PCR products were cloned into pCR4-TOPO (Invitrogen), using the TOPO TA Cloning method (Invitrogen).

Northern blots were prepared, using the NorthernMax-Formaldehyde kit (Ambion) and BrightStar-Plus membranes (Ambion). A cDNA probe (corresponding to nucleotide positions 1505–2146 of Fig. 1) was labelled using the Strip-EZ DNA kit (Ambion). The *Drosophila* ribosomal protein 49 probe was generated as described in [10].

Chinese hamster ovary (CHO) cells were grown as described previously [6]. To amplify a full length cDNA coding for the receptor, the following primers were applied: the sense primer 5′-TTGCTAGCGCA ATCATGCTGCACTTAAGGCTATTCG-3′ (corresponding to nucleotide positions 1–22 of Fig. 1) and the antisense primer 5′-TTG AATTCTCACAGGATGTCCGTGTTCCCCTTTAGAG-3′ (corresponding to nucleotide positions 1493–1521 of Fig. 1), using the *Pfu-Turbo* enzyme system (Stratagene). The *Nhe*I and *Eco*RI restriction sites which had been incorporated into the above primers facilitated the direct cloning into the pIRES2-EGFP vector (Invitrogen) after digestion of the PCR product with the above restriction enzymes. The insert was fully sequenced and the plasmid was transfected into CHO cells, as previously described [6]. The bioluminescence assay was performed as in [6,11].

DNA sequence compilation, nucleotide, and amino acid sequence comparisons were performed using the Lasergene DNA Software package (DNASTAR). The secondary structure of the receptor protein was analysed, using the TMHMM (v. 2.0) prediction server from the Center for Biological Sequence Analysis, BioCentrum-DTU (www.cbs.dtu.dk).

Results

The website of the *Drosophila* Genome Project consortium (www.flybase.org) contains the annotated gene CG6111, which is presumed to code for a G protein-coupled receptor. We constructed primers based on the six predicted exons of this gene and performed PCRs, using *Drosophila* cDNA as a template. These PCRs resulted in bands of the expected sizes and sequences, after which we performed 3′- and 5′-RACEs to obtain a more complete cDNA sequence. During this part of the work, it became clear that the gene CG6111 had not been correctly annotated and that there was an extra exon in front (5′) of the annotated six exons (exon 1 in Fig. 1). Furthermore, this additional exon was annotated to be part of a different gene not related to G protein-coupled receptors (CG14547; www.flybase.org).

Table 1
Intron/exon boundaries of the CCAP receptor gene

Intron	5′-Donor	Intron size (bp)	3′-Acceptor	Intron phase
1	GAG gtgggtg...	14352	...cttccag ACC	3
	Glu		Thr	
2	G gtgagtt...	62	...tttccag AT	1
	Asp		Asp	
3	T gtaagat...	190	...cttttag GG	1
	Trp		Trp	
4	G gtaagat...	83	...ttaacag AA	1
	Arg		Leu	
5	AG gtatgtt...	91	...tttccag T	2
	Ser		Ser	
6	GAG gtatgat...	67	...cctttag CGA	3
	Glu		Arg	

Table 2
Nucleotide differences between the cDNA of Fig. 1 and the corresponding genomic sequences from the Berkeley "*Drosophila Genome Project*"

Position of the nucleotide in the cDNA	Type of nucleotide in the gene	Type of nucleotide in the cDNA	Change in amino acid
849	G	A	Val → Val
1134	A	C	Pro → Pro
1721	A	G	—
1731	A	T	—

The cDNA of the corrected gene CG6111 is shown in Fig. 1. It has a length of 2294 bp, a polyadenylation site at its 3′-end, and an in-frame stop codon preceding the first ATG (start) codon in its untranslated 5′-region. The cDNA codes for a protein of 506 amino acid residues, which has seven transmembrane domains, suggesting that it is a G protein-coupled receptor. Furthermore, the protein contains two potential N-glycosylation sites (following the NXS/T consensus sequence) in its extracellular N-terminal region (Fig. 1).

Comparison of the cDNA of Fig. 1 with the genomic sequence of the corrected CG6111 gene revealed that the receptor gene has seven exons and six introns (Fig. 1, Table 1). This comparison also showed that there are a few nucleotide differences between our cloned cDNA and the corresponding genomic sequences from the database (Table 2). These differences, however, did not lead to changes in amino acid residues (Table 2).

We stably transfected CHO cells with DNA corresponding to the coding region of the receptor. These cells did also stably express the promiscuous G protein, G-16 [11]. Two days before the bioassay, we transiently transfected these cells with DNA coding for the protein apoaequorin and 3 h before the assay, we added coelenterazine to the cell culture medium. Addition of receptor ligands and activation of the cloned receptor in these pretreated cells would lead to an IP_3/Ca^{2+}-mediated bioluminescence response that could easily be measured and quantified [2–6,11–13].

We tested a peptide library consisting of 22 *Drosophila* and other invertebrate neuropeptides on the pretreated CHO cells and found that the cloned receptor was activated by low concentrations of crustacean cardioactive peptide (CCAP) (EC_{50}, 5.4×10^{-10} M). No other *Drosophila* peptides, including Drm-AKH and Drm-corazonin, did activate the receptor (Fig. 2).

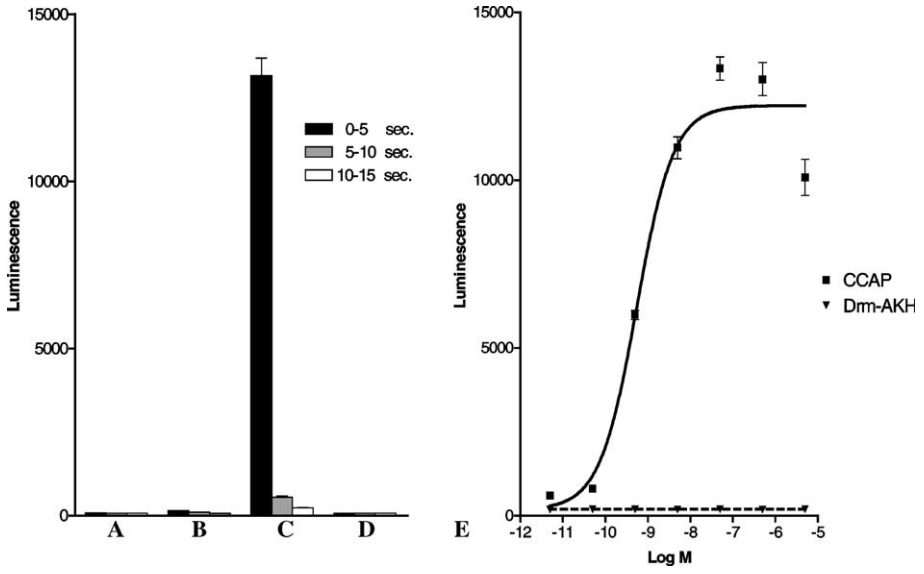

Fig. 2. Bioluminescence responses of nontransfected CHO cells and of a transfected and cloned cell line expressing the receptor of Fig. 1. The SEMs are given as vertical bars, which are sometimes smaller than the symbols. In these cases, only the symbols are given. (A) Responses of nontransfected cells to 10^{-7} M CCAP dissolved in PBS, containing 0.1% BSA. (B) Responses of the transfected cell line to PBS, containing 0.1% BSA. (C) Responses of the transfected cell line to 10^{-7} M CCAP dissolved in PBS, containing 0.1% BSA. (D) Responses of the transfected cell line to 10^{-5} M Drm-AKH. Drm-AKH was also tested on cells expressing the Drm-AKH receptor, where it gave a normal (high) bioluminescence response [6]. (E) Dose–response curve of CCAP for the activation of the receptor (filled squares). Drm-AKH (filled triangles) does not activate the receptor. The following peptides did not activate the receptor either (in concentrations up to 10^{-6} or 10^{-5} M): capa-1 and -2; *Drosophila* corazonin; *Drosophila* ecdysis triggering hormones-1 and -2; *Drosophila* myosuppressin; *Drosophila* myotropin; *Drosophila* pigment dispersing hormone; *Drosophila* pyrokinin-2; *Drosophila* small neuropeptide F-1; *Drosophila* tachykinin-3; Drostatin-A2, -B1, -C; FMRFamide; *Heliothis zea* AKH; hug-γ; Leucokinin III; Leucopyrokinin; and proctolin. For peptide structures see [8,9,20,24–26].

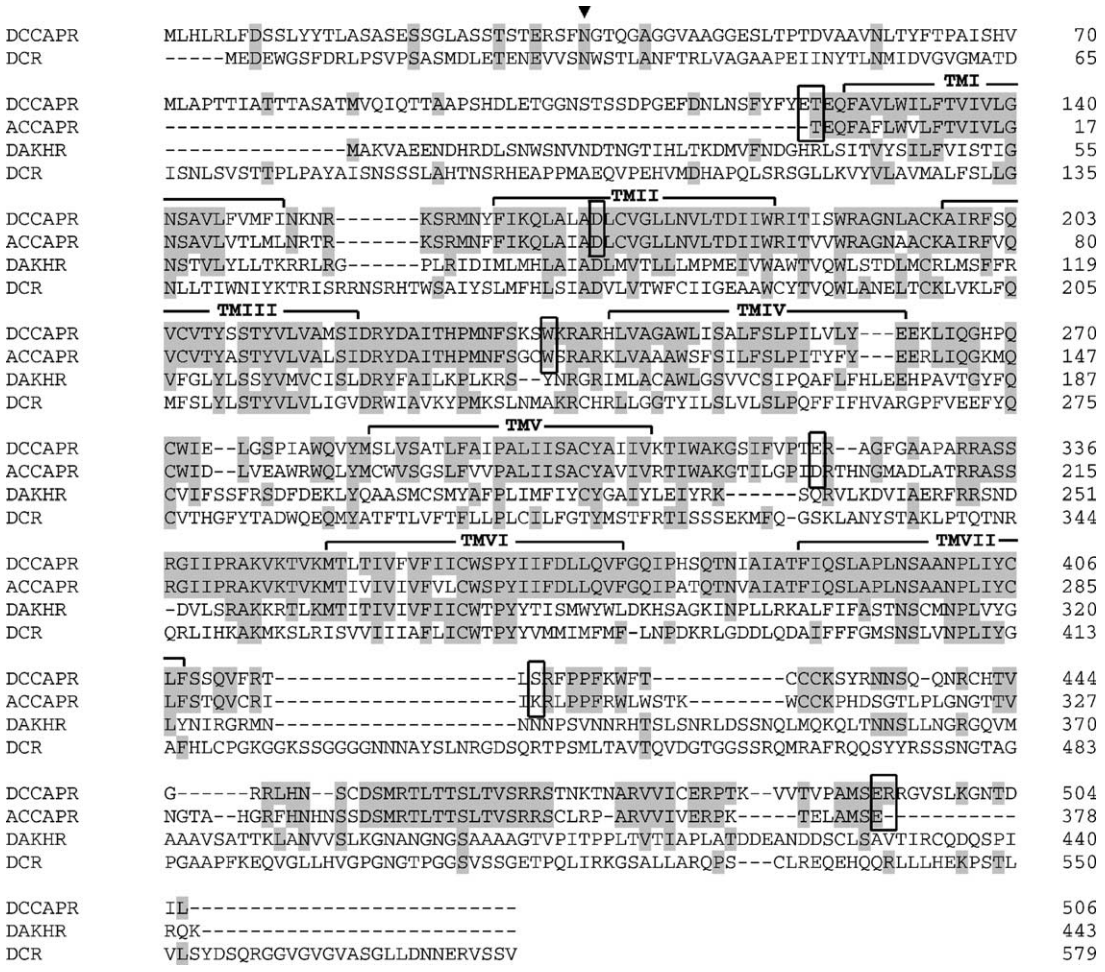

Fig. 3. Amino acid sequence comparison between the *Drosophila* CCAP receptor (DCCAPR), the putative *Anopheles* CCAP receptor (ACCAPR) (corresponding to the annotated gene product with Accession No. EAA01142.1), the *Drosophila* AKH receptor (DAKHR), and the *Drosophila* corazonin receptor (DCR). Spaces are introduced to optimize alignment. Amino acid residues that are identical between DCCAPR and at least one of the other receptors are highlighted in grey. The seven membrane spanning domains (for DCCAPR) are indicated by TMI-VII. Common introns between the DCCAPR gene and one of the other receptor genes are indicated by vertical boxes.

Fig. 2E also shows that high concentrations of CCAP (10^{-5} M) partly inactivates the receptor, which is probably due to receptor desensitization. Furthermore, we noticed that CCAP sticks to the polyethylene tubing and other parts of our luminometer, which made it difficult to wash the peptide away (and get a zero background when new cells were tested) after a CCAP experiment. We solved this problem by dissolving the peptide in PBS + 0.1% bovine serum albumin (BSA).

A comparison of the *Drosophila* CCAP receptor with the other identified or annotated *Drosophila* neuropeptide receptors showed that the receptor was mostly related to the *Drosophila* AKH receptor (23% identical amino acid residues; 32% identities within the seven transmembrane domain; and 51% similar residues), followed by the *Drosophila* corazonin receptor (20% identical residues; 29% identical residues in the seven transmembrane domain; and 50% similar residues) (Fig. 3). However, the *Drosophila* CCAP receptor was somewhat more related to the mammalian vasopressin V1b receptors than to any of the *Drosophila* receptors (26% identical residues; 35% identical residues in the seven transmembrane region; and 55% similar residues). None of the six introns in the CCAP receptor were shared by the Drm-AKH or Drm-corazonin receptors. All this showed that none of the *Drosophila* receptors were closely related to the *Drosophila* CCAP receptor.

A comparison of the *Drosophila* CCAP receptor with the sequences stored in the Genome Project database from the malaria mosquito *Anopheles gambiae* [14] revealed the existence of an *Anopheles* receptor that showed strong sequence similarities with the *Drosophila* CCAP receptor (70% identical amino acid residues; 80% identical residues in the transmembrane domain; and 89% similar residues). Furthermore, all six introns found in the *Drosophila* CCAP receptor gene did also occur (at identical positions and with

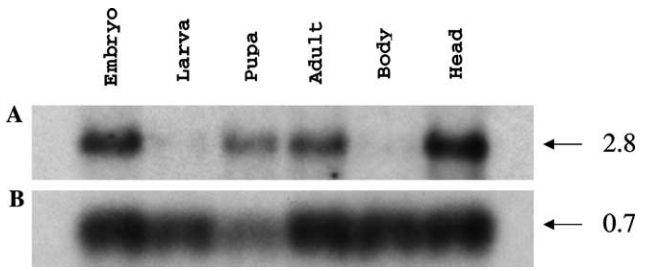

Fig. 4. Northern blots of different developmental stages from *Drosophila*. Each vertical lane contained about 5 μg mRNA from embryos, larvae, pupae, adult flies, and body (thorax/abdomen) and head from adult flies. The numbers at the right give mRNA sizes in kilobase. (A) Hybridization with a cDNA probe specific for the *Drosophila* CCAP receptor mRNA. (B) The blot of (A) was stripped and hybridized with a cDNA probe specific for ribosomal protein-49. This blot gives the loading efficiency.

identical intron phasings) in the *Anopheles* gene, suggesting that this gene is coding for an *Anopheles* CCAP receptor (Fig. 3).

Northern blots showed that the receptor is expressed in all developmental stages from *Drosophila,* but only very weakly in larvae. In adult flies, the CCAP receptor is expressed mainly or exclusively in the head (Fig. 4).

Discussion

Very recently Park et al. [15] have also identified gene CG6111 as being a gene coding for *Drosophila* CCAP. There are, however, major differences between our findings presented here and those of Park et al. First, we find that the CCAP receptor has a much higher affinity for CCAP (EC_{50}, 5.4×10^{-10} M) than that Park et al. reported (EC_{50}, 1.3×10^{-7} M). The reason for this discrepancy might be that Park et al. used a different expression system (frog oocytes) than we did in our present study (CHO cells). Second, we have sequenced the complete coding region and non-coding 3′-end of the CCAP receptor cDNA, whereas Park and co-workers [15] have missed the whole last exon (exon 7, Fig. 1). This means that their expressed CCAP receptor lacks a portion of the C-terminus, which also might explain the low affinity of their receptor for its ligand CCAP. Third, we found that the CCAP receptor is specific for CCAP and that it does not crossreact with AKH or other *Drosophila* neuropeptides (Fig. 2). Park et al., however, find that their receptor is also activated by AKH in concentrations that are comparable to CCAP (EC_{50}, 2.4×10^{-7} M). One possible explanation for this amazing finding (the two peptides do not have similar structures) could be that their equipment (tubing and measuring chamber) still contained adsorbed CCAP after a CCAP measurement, which was then released during subsequent measurements of AKH. As mentioned earlier, we had initially similar problems, which, however, could be overcome when CCAP was dissolved in PBS, containing 0.1% BSA. Therefore, we are convinced that the CCAP receptor is specific for CCAP and that it does not crossreact with AKH.

The *Drosophila* AKH and corazonin receptors are structurally related (30% identical amino acid residues; 56% similar residues) and, moreover, their genes have a common intron with identical intron phasings, showing that the two receptors also are clearly evolutionarily related [3]. As mentioned in the Results part, such a clear evolutionary relationship does not exist between the CCAP receptor and the AKH and corazonin receptors, although the CCAP receptor is structurally somewhat similar to the AKH/corazonin receptor group (Fig. 3). Functionally, however, the three receptors are more clearly related. In locusts it has been shown that CCAP releases AKH from the *corpora cardiaca* [16]. CCAP increases heartbeat in the moth *Manduca sexta* [17,18], as does AKH in the cockroach *Periplaneta americana* [19]. In probably all insects, AKH is involved in the mobilization of lipids from the fat body during energy-requiring activities such as flight and locomotion [20], which are situations where heartbeat is certainly also increased. Corazonin, again, increases heartbeat in cockroaches [21]. Whether all these actions do also occur in a single insect such as *Drosophila* is an open question. However, our Northern blots (Fig. 4) clearly showed that the *Drosophila* CCAP receptor is abundantly present in the head (brain and associated organs such as the corpora cardiaca), but virtually absent in the thorax and abdomen of *Drosophila*. This means that if CCAP would regulate the activity of the heart or other peripheral organs in *Drosophila*, its actions should be indirect, for example by releasing AKH or other hormones from the corpora cardiaca.

CCAP was originally isolated from the shore crab *Carcinus maenas,* because of its prominent cardioactive actions [7]. This cyclic peptide (PFCNAFTGCamide, where the Cs form a disulphide bridge) has later been identified in an identical form from various insects, including *Drosophila* [8,9,16–18]. In addition to its cardioexcitatory and AKH-releasing activities, CCAP also appears to be involved in insect molting, where it might steer the motor behaviour associated with ecdysis [22]. CCAP, therefore, might be a multifunctional peptide involved in various motor behaviours.

CCAP is remarkable in that its structure has remained unchanged during the last 500 million years of evolution (since the divergence of insects and crustaceans [23]). This would suggest that also its receptor has remained relatively invariable (at least in its peptide binding regions) and that it would be easy to identify the CCAP receptors from other arthropods, now that we

have identified the first specific invertebrate CCAP receptor from *Drosophila*. That this is a feasible option is illustrated by our identification of a likely CCAP receptor in the malaria mosquito *Anopheles gambiae* (Fig. 3). This receptor work on other model insects and crustaceans, together with the more genetically orientated work in *Drosophila* (e.g., receptor knock-outs), will certainly advance our knowledge on the actions of CCAP in invertebrates.

Acknowledgments

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